Mushroom Biology and Mushroom Products

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Foreword

The World Society for Mushroom Biology and Mushroom Products is an international organization devoted to the enhancement and application of knowledge related to basic and applied aspects of mushroom biology and mushroom products. It was established to provide a platform for mushroom biologists from all over the world, coming from both well-established centers and emerging groups in countries with increasing interests on mushroom biology and mushroom products. A major activity of WSMBMP is its three-yearly Conference organized by the host country under the banner of the Society.

The proceedings document of the seventh conference includes both oral (in volume 1) and poster presentations (in volume 2) submitted at this event by scientists from more than 35 countries. For the first time, the proceedings are provided in electronic format on a memory stick available for the participant at the date of the conference. This saves trees and allows for keyword searching in a document of more than 720 pages. We are grateful to the Scientific Committee members for their assistance in evaluating the papers submitted. The task of error corrections in the manuscripts, technical editing, and compiling the proceedings into an electronic format, was provided by the scientific editors of the proceedings. An open access online publication of the proceedings is going to be proposed by QUAE Edition at the address http://www.symposcience.org. It needs an additive work of edition that will be done after the conference.

The purpose of the 7th International Conference on Mushroom Biology and Mushroom Products (ICMBMP7) was to bring together scientists from all subdivisions of biology with an interest in mushrooms and mushroom products, including those associated with economic, environmental and engineering issues. The main focus of ICMBMP7 is "Towards a Significant Contribution of Mushroom Biology to Sustainable Development" and the following areas are covered: (i) Genomics, Genetics, Molecular Biology and Breeding, (ii) Evolution, Biodiversity and Systematics, (iii) Biochemistry, Physiology, Nutritional and Medicinal Aspects, Innovative Products from Mushrooms, and (iv) Cultivation Technology and Bioconversions.

The development of high-yielding strains of both well-established and more recently cultivated mushroom species using strain improvement and breeding technologies was reported in the 6th International Conference (Bonn, Germany, 2008). Since 2008, the availability of the entire genome sequences of two important cultivated mushrooms, *Pleurotus ostreatus* and *Agaricus bisporus*, has been a key development. This new opportunity for advancing our knowledge and understanding of mushroom genetics and mushroom biology is a major feature of ICMBMP7. The expected progresses in breeding lead to question the ways to disseminate and protect the new strains.

Evolution, systematics, and biodiversity are important topics for the future development of new mushroom-derived products and the cultivation of new mushroom species. They are clearly identified for the first time in the WSMBMP conferences. Research output in these areas should offer a plethora of new opportunities, drive the choice of the species to be studied and influence policy makers in terms of biological resource preservation and management.

Several papers report on medicinal effects observed on cellular models and test animals, and others on mushroom as sources of nutritional and active biomolecules. If there is to be confident in the use of such mushroom extracts and supplements for the treatment of various medical conditions, then the quality and production of the products needs to be standardized and regulated. There is a major lack of such regulation at the moment, and it is essential that progress be made in this area in the future to give consumers confidence and to protect public health from substandard products. The proceedings address this question by contributing to the dissemination of scientifically validated information and proposing discussions between participants from Asia, America and Europe.

Knowledge on the biology of mushrooms (their physiology, their life cycles, their development, their nutrition) is a source of information for the development on new mushroom cultivations or the improvements on conventional cultivation techniques. The presentations on these topics show strong influence of genomics and all the approaches of the molecular biology on the mushroom biology of today.

Technological developments in the mushroom industry in general have resulted in increased production capacities, innovations in cultivation techniques, improvements in the quality of the final products, and the utilization of the natural qualities of mushrooms for environmental benefits. Papers in these Proceedings show the importance of edible and medicinal mushroom cultivation in integrated managements of agricultural wastes products. However, significant challenges remain in maintaining and further improving the current cultivars and cultivation techniques for mushroom production, in reducing mushroom diseases by integrated pest management, and in continuing to seek out new opportunities.

These Proceedings highlight many aspects of mushroom biology and mushroom products and it will our honor if they reach the objective of promoting the contribution of mushroom biology to sustainable development.

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WHOLE-GENOME SEQUENCING OF THE CULTIVATED BUTTON MUSHROOM *AGARICUS BISPORUS*: HISTORY, STATUS, AND APPLICATIONS

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ABSTRACT

The culmination of the first half-century of investigations into the genome of *Agaricus bisporus* was marked by the first whole-genome sequencing for a strain of this species. The genome of a second strain of *A. bisporus* was sequenced the following year. While a structural view of the genome is now effectively complete, functional interpretations of the many gene sequences, supported by accumulating experimental data, are still being developed. A first summary of the physiological capabilities and strategies of this 'humicolous' species is now in preparation; when completed it should illuminate the role of the fungus in its natural ecosystems, and provide the most detailed view to date on the interaction of the fungus with its complex commercial compost substrate. Ultimately all functional and behavioral aspects of the mushroom may potentially be decoded by investigating these and additional genome sequences. The availability of whole-genome sequences to the *Agaricus* research community represents a milestone of unprecedented potential.

Keywords: Genomics; Genetics; Breeding

The genome of the 'Button Mushroom' *Agaricus bisporus*, and the genetic diversity incorporated within every cultivated and wild strain of that species, determines the biological potential and fate of each culture and every crop in whatever environment is present. The list of variable, commercially important traits under genetic control is long and growing:

- Yield: vegetative growth rate, optimum temperature for vegetative growth, substrate utilization efficiency,
- Quality: cap pigmentation, cap smoothness, basidiomatal size, shape, firmness, density, bruising responses, processing characteristics, mushroom flavor, agaritine content,
- Development/Morphogenesis: pinning responses and behavior, developmental scheduling of basidiomata,
- Tolerance to environmental stresses: temperature tolerance,
- Resistance to pathogens: responses to pathogens and antagonists including *Pseudomonas, Trichoderma, Lecanicillium fungicola, Mycogone permiciosa,* and dsRNA viruses,
- Breeding efficiency: strain mating behavior, incompatibility phenotype, and life cycle… among others. (For strain identification and intellectual property rights protections, the molecular fingerprint of each strain also derives directly from the primary DNA structure of the genome.)

Knowledge of the genome of *Agaricus* accumulated slowly at first. Twelve key milestones along the way (while acknowledging many other important contributions) are:

1959: Evans, using light microscopy, established the haploid chromosome number at 13 [1].
1972: Raper et al. and Elliott independently confirmed that a heterothallic life cycle existed in *A. bisporus*, masked by the predominant (pseudo-) homothallic behavior of the species, and that traits could segregate and reassort [2, 3].
1982: May and Royse demonstrated the use of codominant (allozyme) genetic markers to confirm successful mated crosses and to provide genetic fingerprints of, and estimated and deduced relationships among, individual strains [4].
1982: Arthur et al. used reassociation kinetics to estimate the size of the haploid genome at 34 Mbp, and also estimated the G-C content and the proportions of single-copy and repeated DNA present [5].
1988: Hintz et al. used restriction mapping to estimate the size of the mitochondrial genome of *A. bisporus* to be 136 kbp [6].
1992: Royer et al. used CHEF analysis to confirm the chromosome number at 13, and the estimated genome size at 30+ Mbp; using Southern hybridizations and linkage-mapped probes, chromosomal length polymorphisms were also demonstrated [7].
1993: Kerrigan et al. provided a comprehensive genetic linkage map to one hybrid heterokaryotic genome of *A. bisporus*, genetically confirmed all facets of normal meiosis and heterothallism (as well as pseudohomothallism), and provided first estimates of varying recombination rates (including non-recombining segments and asymmetrical segregation ratios) over the genome [8].
1993: Xu et al. used knowledge of molecular marker linkage relationships to locate the mating-type gene on chromosome 1, allowing prediction of mating behavior in crosses from genotype data: the beginning of Marker-Assisted Selection (MAS) in *Agaricus* strain breeding [9].
1996: Imbernon et al., using two different hybrid mapping populations, located the recently discovered BSN locus (which exhibits effectively dominant behavior in determining the number of spores formed on the basidium, and thus the effective 'ploidy' of spores and the life cycle they pursue) on chromosome 1, allowing further marker-assisted selection [10].
1999: Callac et al. used linkage markers to locate the primary pileipellis-pigmentation determining locus PPC1 on chromosome 8 [11].
2002: Moquet et al. determined that a primary Quantitative Trait Locus (QTL) determining resistance to *Pseudomonas*-associated 'blotch disease' is closely linked to PPC1 [12].

After the millennium, the pace of molecular genetic, physiological, and other experimental studies on *A. bisporus* continued to accelerate. The important contributions over the past two decades by the teams at Horst/Wageningen (including Sonnenberg and Baars), Warwick/HRI (including Burton, Challen, and Eastwood), INRA Bordeaux (including Foulongne, Callac, Imbernon, Savoie and Largeteau), and, in the private sector, by the Monterey group (including Loftus and Lodder), CTC (Rodier), and my own group at Sylvan, are too numerous to list here. Much of this work is reported in the public record, including patent filings, and can easily be located via online searching. Some work remains confidential and proprietary within private and contracting quasi-public or academic organizations.

As these studies evolved and expanded, it became increasingly obvious that the lack of available genomic sequence for *A. bisporus* was greatly hampering progress in this field. For example, to look for a marker-linked gene target, or the distribution and behavior of a transposable element, one would have to prepare a cosmid library comprising of several thousand individual clones, blot the DNA from the entire genomic library onto several dozen membranes, prepare labelled probes, Southern-hybridize probes to target membranes and develop and capture hybridization signals via enzymatic, radiographic or chemiluminescent methods, prepare flagged cosmid DNA for subcloning (a second iteration of the above steps) or else use direct sequencing and 'walking' along the chromosomal DNA in 500-nt steps, analyze the segment sequence to identify ORFs, transposons, or other features of interest, attempt to classify gene targets based on limited structure-function databases, and then test, via experimentation, hypotheses based on interpretation of the above results. Having carried out precisely such exercises with the team at Sylvan, I can confirm that it is every bit as cumbersome and tedious, not to mention slow, time-consuming and expensive, as it sounds.

The first fungal genome, that of the yeast *Saccharomyces cerevisiae*, was sequenced in 1996. At 12.2 Mbp, it is about one-third the estimated size of the *A. bisporus* genome. Approximately 6000 genes have been identified on 16 chromomes. The first genome sequence of a filamentous fungus, that of *Neurospora crassa*, was published in 2003. This genome, at 43 Mbp, is larger than that of *A. bisporus*, and is believed to incorporate about 10,000 genes.

In 2002, the first of a series of white papers appeared [13], advocating a concerted effort toward obtaining sequences of more fungal genomes. Fifteen candidate fungi were proposed for whole-genome sequencing, and beginning in 2003 many of these sequencing projects were subsequently completed with the resources of the National Human Genome Research Institute (NHGRI), the Whitehead Institute/MIT Center for Genome Research (WICGR), and the Broad Institute. Of particular interest to this readership is the genomics work on agaric fungi (mushrooms), which in the earliest examples includes work on the coprophillic species *Coprinopsis cinereus* (http://genome.jgi-psf.org/Copci1/Copci1.home.html), the mycorrhizal species *Laccaria bicolor* (http://genome.jgi-psf.org/Lacbi2/Lacbi2.home.html) and the wood-inhabiting species *Pleurotus ostreatus* (http://genome.jgi-psf.org/PleosPC15_2/PleosPC15_2.home.html), a cultivated mushroom. While data from these and several other relevant sequencing projects are now available in public databases, it is too early for all of the supporting publications to have appeared in the literature.

In 2003 a core group of *Agaricus* scientists began to build community and industry support for, and seek opportunities for, whole genome sequencing of *A. bisporus*. This group evolved over several years, with key roles played by Kerry Burton, Mike Challen, Anton Sonnenberg, and myself. After submitting a number of proposals, our plan to sequence and compare two distantly-related haploid genomes of *A. bisporus* was accepted by the USA's Department of Energy, Joint Genome Institute, Community Sequencing Program based in Walnut Creek, California. As sequencing neared completion in 2009-2010, the circumstances of some core PIs changed, and Francis Martin generously agreed to take over coordination responsibilities toward collating interpretive data from participating laboratories, and the preparation of a master paper reporting the results of the project.

The focus of our arguments to justify the value of sequencing genomes of *A. bisporus* conformed to the mission of the DOE program: Energy and the Environment. Emphasis in this program is placed upon organisms (or genes) that may participate in the development of new and sustainable fuel and energy sources in formats that conform to current and near-term infrastructure, and on organisms that play key roles, including that of model systems, in ecosystem processes, particularly those that mediate the flow of carbon through the global biological carbon cycle. Additionally, incorporating phylogenetic diversity has always been a consideration of the agencies choosing fungal genomes for sequencing support.

We stressed that *A. bisporus* would be one of the first species in the order *Agaricales*, and would be *the* first species from the large family *Agaricaceae*, to be sequenced. We particularly emphasized that *Agaricus* and *Agaricaceae* belong to an ecological guild adapted to humus-like substrates such as aged forest litter (and commercial mushroom compost). Very little is known about how forest litter is recycled in nature, in spite of the great quantity of leaf, needle and twig material that accumulates and decomposes in forest ecosystems, biomass that contributes greatly to global atmospheric carbon inputs. We predicted that there was a very good chance of finding novel metabolic strategies that might not be seen in the more 'charismatic' white-rot and brown-rot wood-decomposing fungi, nor in coprophillic fungi such as *Coprinopsis*. Our thesis has been that humicolous species (among the 'detritophiles') may have a distinctly different melange, or deployment, of substrate conversion enzymes.

We additionally pointed out that *Agaricus* was now a well-established laboratory organism, and a good model system for various syndromes including the cellular mechanics of pseudohomothallism. In particular, our strategy allowed for the possibility of identifying the BSN locus controlling the basidial spore number trait. Also of interest is the fact that *Agaricus* is the basis of a multibillion dollar food crop industry that employs a large number of people.

Finally, we specifically proposed that one haploid genome, from the commercial European bisporic (var. *bisporus*) homokaryon H97, be sequenced using the Sanger method. A second, wild North American tetrasporic (var. *burnettii*) homokaryon, JB-137-s8, was proposed for sequencing using newer, less expensive short-read technologies, with assembly guided by the finished assembly of the H97 genome. Completing the picture, tens of thousands of ESTs ('Expressed Sequence Tags' obtained from the sequences of messenger RNAs transcribed from actual functioning genes, obtained at several life-cycle stages) would be used to characterize genes of this species, train gene recognition computer algorithms, and directly confirm activity of specific 'gene models'. The mRNAs used were to be obtained from the heterokaryon U1, a widely cultivated hybrid strain and patriarch of the most widely cultivated commercial strain family today. U1 has two haploid nuclear genomes: one from H97, linking half of the EST data directly to the H97 genome sequence, and the other from the H39 homokaryon, providing many opportunities to compare two different allelic gene sequences (H39 vs H97) drawn from within the same European *A. bisporus* var. *bisporus* population.

Thus two scales of comparison were possible: between alleles within the European population, and between genomes of two different varieties of the species from separate populations on two different continents. Other interesting comparisons, for example between wild and cultivated strains, or between compost-adapted wild strains vs. leaf-litter inhabiting strains [14], cannot be made with strong support based only on these first two genomes, but could well be investigated in the near future using additional strains, with the expansion of lower-cost sequencing techniques.

Our final proposal, drafted by Mike Challen, was accepted in 2007 for the 2008 CSP program, and was carried out essentially as we specified. Both genomes are now effectively completed, although (as is typical for most affordable genome projects) a few small gaps remain, and both are now in the public domain on the JGI's genome portal web site http://genome.jgi-psf.org/.

Key findings from the H97 genome sequence assembly are as follows: The 13 nuclear and one mitochondrial chromosome are represented on 29 assembled 'scaffolds', indicating the presence of 15 substantive gaps in the sequence assembly, these gaps comprising not more than 0.7% of the nuclear genome. Some of the 'missing' genome is likely to consist of tandemly arranged ribosomal RNA cassette repeats which are extremely difficult to reassemble into the original genome sequence. Computer search algorithms have identified 10,438 gene 'models', many of which are supported (as representing 'real' genes) by sequenced mRNA transcripts (ESTs). While many of these genes are sufficiently similar to other known genes that their

identities and functions can be predicted with confidence, a considerable number of genes may be unique (in current databases) to *Agaricus*, or may occur more widely but have functions that remain unknown. Genes known to be involved in nutritional pathways are numerous and diverse, allowing comparisons to the profiles of other fungi with different ecological roles. The mitochondrial chromosome of H97 is comprised of 145, 919 nucleotide pairs. Thousands of individual transposable elements (transposons) belonging to numerous and diverse families also inhabit (or infest) the genome; many of these elements have been observed (by Sylvan and by the Wageningen team) to be actively moving from site to site within the genome, and this provides one possible explanation, with many documented precedents of gene disruption, for the development of anomalies such as sectors or stroma from within otherwise healthy, stable cultures.

Since the release of the genome assemblies, teams of researchers have worked to interpret the sequencing and expression data, understand the structure and function of the metabolic pathways deduced from the identities of the confirmed genes, associate those data with the physiological behaviors of the sequenced strains on defined media, and ultimately determine whether *A. bisporus* has a fundamentally distinct 'ecological guild' signature based either on its repertoire of substrate conversion enzymes or on other aspects of its deduced physiology based on gene expression and other data. Most of those analyses are completed, while some are still underway; at this writing the available and developing information is being collated into a cohesive view of the nature of this mushroom and its role in the environment. The finished report is expected to be submitted shortly to a scientific journal, where it will become a part of the public record in the near future. The specific contributions of the many participating laboratories will be detailed and fully credited at that time.

Meanwhile, two opportunities are being pursued concurrently. First, molecular mycologists working on diverse fungi now have two unusual new genomes to compare with those of other organisms; *Agaricus* is becoming one of the standards for genomic and metabolic comparisons, and each study potentially returns additional information of interest to the *Agaricus* community. Second, the laboratories directly involved in the molecular genetics of *Agaricus* now have access to the most powerful tool ever created for enabling progress in this field. It has become a simple matter to obtain the sequence of a gene, compare diversity of a gene sequence among different strains, develop and evaluate genetic markers linked to genes and QTL regions, locate transposons, and carry out many other related tasks. From 2010 forward, the return on monies invested in molecular genetics research on *Agaricus* will be far greater than at any previous time in history.

Problems that could not be solved a few decades ago, at the beginning of my career, which then became comprehensible but intractable, and next became tractable but infeasible, then feasible but prohibitively complex and costly, have ultimately become straightforward, practical, cost-effective and – gradually – even routine to undertake. Such work is well underway. The release of these *Agaricus bisporus* genome sequences, and the forthcoming master paper that will soon document and interpret the sequencing results, represent what may be the pivotal moment in the development of our field.

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BREEDING AND STRAIN PROTECTION IN THE BUTTON MUSHROOM *AGARICUS BISPORUS*

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ABSTRACT

The button mushroom *Agaricus bisporus* is one of the most widely cultivated edible mushroom species in the world. Being the main species cultivated in the Western hemisphere, its popularity also increases in Eastern Countries such as China and Korea. The world production level for 2009 is estimated at ca. 4 million tons with an economic value of ca. 4.7 $ billion. Despite its economic relevance, it is surprising to see that breeding effort in this species is low. The main reasons for this low effort are the typical life cycle that hampers breeding and the difficulty to protect strains.
The complete life cycle of *A. bisporus* was unravelled in the early 70-ties of the previous century. After an apparently normal meiosis, predominantly bisporic basidia are produced, each containing two non-sister post meiotic nuclei. Upon germination these spores generate heterokaryotic mycelia. Only spores from the rare four spored basidia contain one haploid nucleus and can be used to generate hybrids in breeding programs.

Release of the first commercial hybrid dates back to 1980. Subsequent commercial hybrids were identical or very similar to the first hybrid. It is clear that some "new" varieties were generated by making copies of the first hybrid via tissue cultures of mushrooms. It is, however, unclear how other varieties were generated with apparently identical genotype but nevertheless some clear differences in phenotypes. Recent research indicates that offspring can be generated without recombination between homologous chromosomes. A pairing of non-sister nuclei on bisporic basidia will thus result in a redistribution of homologous chromosomes over the constituent nuclei. This redistribution appears to have phenotypic influence. This phenomenon thus allows for a relatively easy way of generating new varieties.

This paper will present opportunities of this typical meiotic behaviour for breeders but also addresses what consequences it has for strain protection.

Keywords: *Agaricus bisporus*, breeding, meiosis, essentially derived varieties

INTRODUCTION

The button mushroom *Agaricus bisporus* (lange) Imbach is the most widely cultivated mushroom in the USA, Europe and New Zealand/Australia. Its popularity is also increasing in Eastern countries as China and Korea. The world production of button mushrooms in 2009 is almost 4 billion tonnes with an estimated value of 4.7 billion dollars at farm gate (Table 1). Despite the long tradition of cultivation, professionalization of the cultivation system and the economic value it is surprising that efforts in breeding of this species is minimal. The first hybrid varieties for white button mushrooms were released in 1981 [1] and new varieties released afterwards were either identical of very similar to these first hybrids.

Recently, the whole genome of the first hybrid variety (Horst U1) has been sequenced (http://genome.jgi-psf.org/). This allowed the generation of large numbers of markers (SNP's) that have been used recently to study meiosis in the button mushroom and screen the genotypes of

traditional and present-day varieties. Although meiosis of the different subvarieties of the button mushroom has been studied previously [2, 3, 4, 5], the use of SNP markers has generated new data on marker segregation in especially the bisporic subvariety that represents all traditional and present-day commercial varieties and most wild accessions. This subvariety produces mainly two spored basidia and preferentially non-sister nuclei are paired in one spore [6]. Only rarely, four spored basidia are produced in which each spore receives one haploid nucleus. We will illustrate in this article what opportunities the complete absence of recombination offers for breeding. In addition, the SNP analyses will illustrate the likely origin of the present-day white strains and these should be considered as essentially derived varieties.

Life cycle of the button mushroom. Homobasidiomycetes are characterized by the fact that they are haploid during most of their life cycle. Fusion of nuclei only takes place in basidial cells just before spores are produced. Each diploid nucleus produces four haploid nuclei after meiosis and these are distributed to the four spores formed by each basidial cell.

Table 1. World production of button mushrooms in 2009.
The origin of the data are from: 1) Groupement Européen des producteurs de champignons (GEPC); 2) Dedicates estimates; 3) Australian Mushroom Growers Association; 4) New Zealand Growers; 5) American Mushroom Institute; 6) Chinese Chamber of Commerce (CNFA).

Country	Tonnes	Value ($) *1000	Country	Tonnes	Value ($) *1000
Poland[1]	250000	345000	Romania[2]	17000	34000
Netherlands[1]	230000	317400	Serbia[2]	10000	20000
France[1]	102400	141312	Slovakia[2]	3000	6000
Spain[1]	93500	129030	Croatia[2]	5000	10000
Italy[1]	64500	89010	Bosnia[2]	3000	6000
Germany[1]	58000	80040	Macedonia[2]	1500	3000
Ireland[1]	55000	75900	Bulgaria[2]	3000	6000
UK[1]	43000	59340	Russia[2]	9751	19502
Bulgaria[1]	40000	55200	Turkey[2]	30000	60000
Belgium/Luxemb.[1]	34000	46920	Ukraine[2]	30000	60000
Hungary[1]	20000	27600	Australia[3]	61000	310000
Denmark[1]	2300	3174	New Zealand[4]	8500	43197
Austria[1]	700	966	South Africa[2]	20000	40000
Others[1]	24000	33120	USA[5]	356936	924860
			China[6]	2181053	1308632
			South Korea[2]	190000	380000
Total production (tonnes) in 2009: 3,923,000					
Total value (x 1000 $) in 2009 : $ 4,602,100					

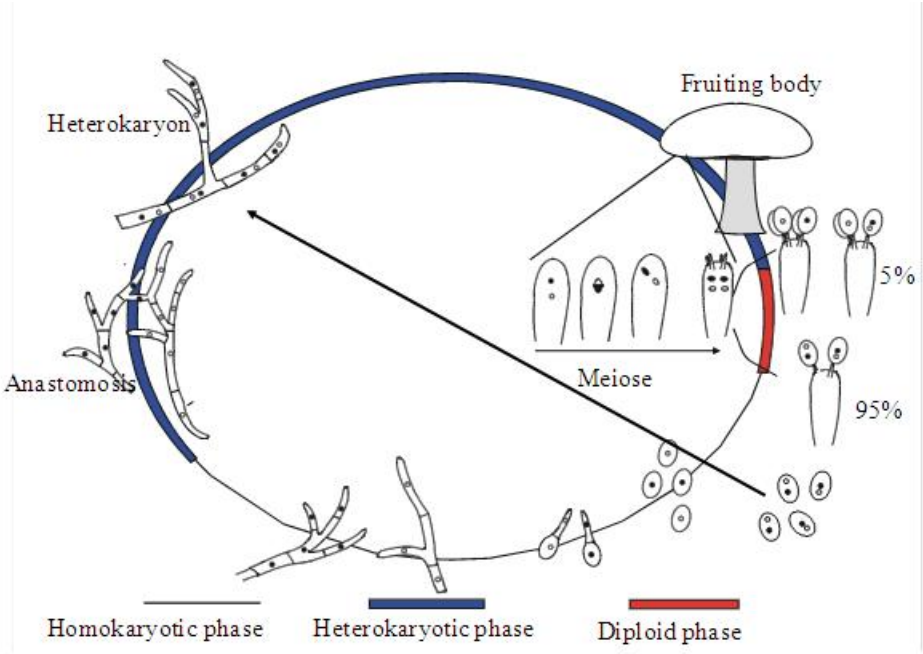

Figure 1. Typical life cycle of *Agaricus bisporus* var. *bisporus*. Most basidia produce 2 spores, each receiving non-sister nuclei. Due to the low recombination frequency between homologous chromosomes, these spores retain (almost) all alleles of the parental nuclei. The homologous chromosomes have an altered distribution over the constituent nuclei.

The spores germinate into haploid mycelium that cannot produce fruiting bodies. These infertile mycelia are designated as homokaryons. Homokaryons with different mating type can anastomose and subsequent exchange of nuclei leads to the formation of heterokaryotic (dikaryotic) mycelium. The presence of both mating types within one mycelial cell triggers a developmental process leading to the formation of fruiting bodies provided environmental conditions are favourable. This non-self compatibility or heterothallism is controlled by one or two unlinked loci. The outbreeding potentials of basidiomycetes are high because they possess numerous distinct mating types [7]. The majority of Homobasidiomycetes show this heterothallic life cycle. The button mushroom *Agaricus bisporus* deviates from this life cycle. Most basidia produce only two spores and the four post-meiotic nuclei are distributed over two spores in such a way that non-sister nuclei are paired in one spore [8, 2] (Fig. 1). This usually leads to mycelia with two different mating types and thus to fertile heterokaryons. This type of life cycle is designated as secondary homothallic. This phenomenon is also referred to as automixis or intra-tetrade mating, a form of selfing where mating occurs among the products of a single meiosis. Only rarely basidia are formed that produce three or four spores. Only on these basidia spores are produced with one haploid nucleus that generate homokaryons and can be used for cross breeding. Two decades ago, a novel variety has been found in de Sonoran desert of California [9]. This variety produces predominantly four spored basidia and each spore germinates into homokaryotic mycelia.

Recombination between homologous chromosomes. Recent breeding programs have shown remarkable differences in recombination frequencies between homologous chromosomes in homokaryotic offspring of the subvarieties *bisporus* and *burnettii*. Whereas in the four-spored subvariety *burnettii* on average eleven recombinations per individual per generation are found [5], a much lower recombination frequency is seen in the two spored *bisporus* variety. Recent analysis of different offspring of the first commercial hybrid Horst U1 showed recombination frequencies

varying from 0.08 to 1.3 per individual per generation, i.e. approximately 100 times lower than offspring of *bisp* x *burnettii*. That indicates that in many individuals most chromosomes are inherited unchanged from either one or the other parent. Present breeding programs indicate that this low recombination frequency might be common in the bisporic variety. Offspring were examined of two hybrids, each having one parent derived from Horst U1. The other parent has been isolated from a wild bisporic variety via protoplasting. These two other parental lines were derived from genetically unrelated wild accessions. Recombination frequencies in these offspring were 0.57 and 0.35 respectively, a similar low frequency as observed in offspring of Horst U1.

Mazheika et al [10] have analysed microscopically meiosis in both the bisporic and tetrasporic variety. They observed incomplete and abnormal axel elements and synaptonemal complexes during meiosis in the bisporic variety whereas in meiosis in the tetrasporic variety all stages can be seen clearly without obvious abnormalities. That could indicate that one or more genes involved in meiosis are mutated. This is obviously a recessive mutation since recombination frequency in a hybrid between the bisporic and tetrasporic variety is considerably higher.

Obstacles and opportunities for breeders. The low recombination frequency between homologous chromosomes in meiosis forms an obstacle for breeding programs since recombination is a prerequisite for mapping QTL's and reducing linkage drag in introgression breeding programs. All traditional and present-day varieties and most of the available wild collected strains are bisporic and it is likely that they all rarely will show recombination between homologous chromosomes in meiosis I. As previously mentioned, alleles responsible for this abnormal behaviour in meiosis are likely recessive. This allows mapping of genes involved and thus offers opportunities to generate advanced breeding stock with normal recombination frequencies. Mapping of these genes, however, is a laborious task since the phenotype. i.e. recombination frequency, has to be analysed for each individual.

Generation of offspring without recombined homologs offers interesting opportunities for breeders. Most steps of meiosis in *A. bisporus* var. *bisporus* seem conventional and a few steps are exceptional. The nuclear numbers per cell are reduced in basidia to two, one for each mating type. After fusion of both nuclei, alignment of homologous chromosomes is rare or incomplete [10] preventing recombination between homologues. In the first reduction division of the meiosis, chromosomes are distributed over two nuclei with an independent distribution of homologues over the two daughter nuclei (although frequently a skewed segregation is seen for some chromosomes). The absence of recombination combined with independent distribution of homologues leads in each basidium to two nuclei with a redistribution of homologues compared to the parental nuclei (Fig. 2). Each nucleus produces subsequently two sister nuclei that are exact copies of each other. The four nuclei can thus be distributed over two, three or four spores. In the latter case, each spore will receive one haploid nucleus in which each chromosome can be of one or the other parental type. This offspring can be used to generate homokaryons in which one chromosome is substituted compared to the parental nuclei. This can be done by crossing these homokaryons with either one or the other parental homokaryon and subsequently selection of chromosome substitution lines in a set of non- or rarely recombining offspring. These chromosome substitution homokaryons can all be mated with one and the same compatible tester homokaryon in order to produce mushrooms. The differences seen in phenotypes will likely be caused by the chromosome that has been substituted since all of these homokaryons have an identical genetic background. In this way, phenotypic effects for each chromosome can be tested. If a trait of interest is located on one chromosome, the introduction of this trait to another variety can be done by only transferring the relevant chromosome to an acceptor line.

In the absence of recombination between homologues, there are two ways in which the four post-meiotic nuclei can be distributed over two spores on bisporic basidia (Fig. 2).

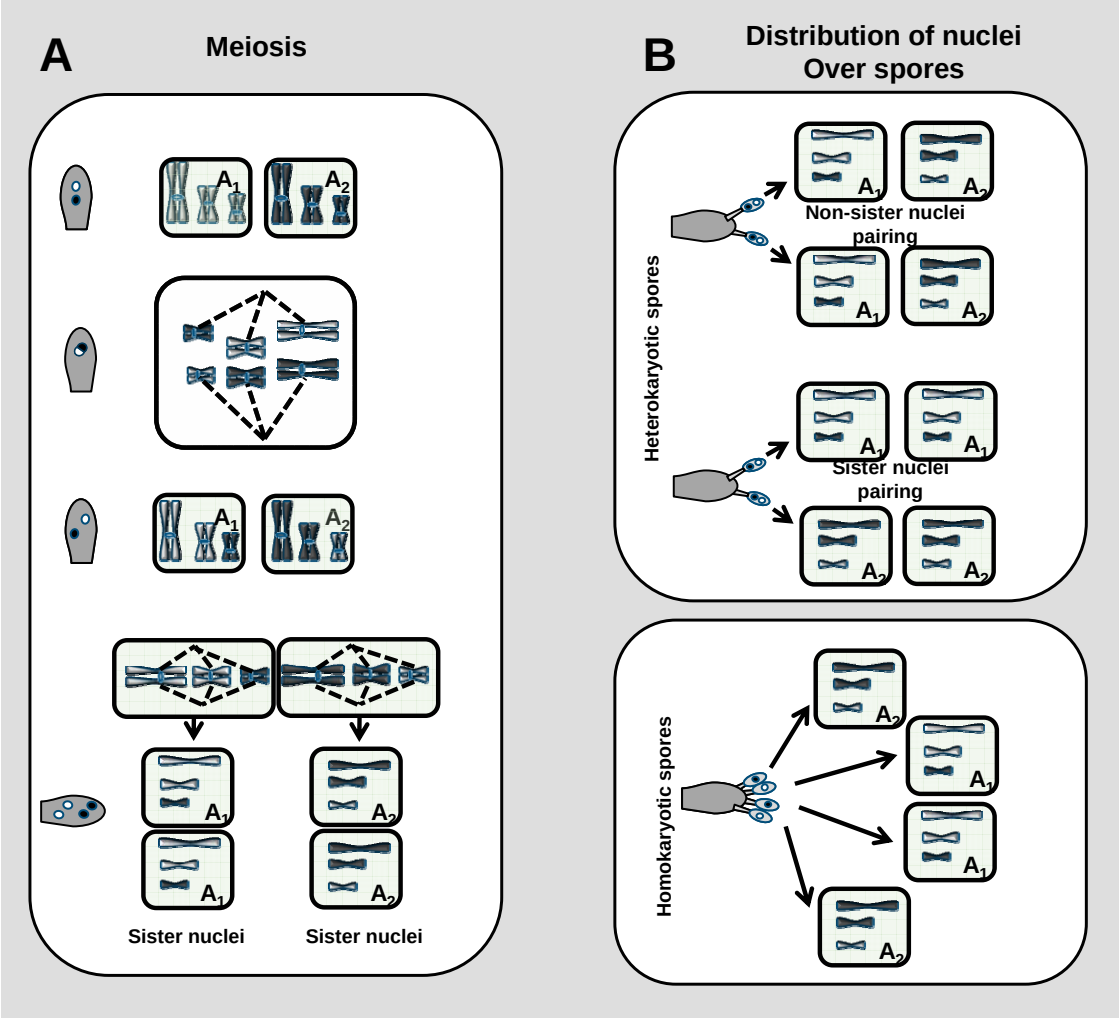

Figure 2. Schematic representation of meiosis (panel A) and distribution of nuclei over spores (panel B) in *A. bisporus* var. *bisporus*. For the simplicity only 3 of the 13 chromosomes are depicted. The different mating types are designated as A_1 and A_2. Without recombination between homologous chromosomes, meiosis leads to four haploid nuclei with 2 sets of sister-nuclei and within each set genetically identical sisters. Previous research indicates that preferentially non-sister nuclei are directed to spores on 2-spored basidia. Without recombination, these two nuclei have the same genetic constitution and show an altered distribution of homologs over the nuclei compared to the parental nuclei. When sister nuclei are distributed over 2 spores, each spore will receive two identical nuclei and will germinate into homokaryons (one nuclear type and one mating type). On 4-spored basidia, each spore receives one haploid nucleus in which chromosomes are substituted.

Either sister or non-sister nuclei are paired in one spore. The pairing of sister nuclei leads to a spore with two identical nuclei and thus one mating type. These will germinate into homokaryons, indistinguishable from homokaryons arisen from four-spored basidia. The pairing of non-sister nuclei leads to spores receiving nuclei that are complementary in chromosome constitution compared to the parental nuclei. All alleles found in the parental heterokaryon are also present in these types of offspring. Compared to the parental nuclei, homologs are redistributed

over the two nuclei. Upon germination, these types of heterokaryons will show the same genotype as the parental heterokaryon. Only by recovering the constituent nuclei via protoplasting, redistribution can be visualized. Analyses of randomly isolated single spore isolates indicate that preferentially non-sister nuclei are paired in one spore in two-spored basidia. How often sister nuclei are paired in one spore is not known but Elliott [8] has shown that this does occur occasionally.

Possible origin of the present-day varieties of white button mushrooms. The genome of the first hybrid Horst U1 has been sequenced recently. One of the parental homokaryons of Horst U1, i.e. H97, has been sequenced by the Joint Genome Institute (http://genome.jgi-psf.org). The other parental homokaryon, i.e. H39, has been sequenced by ServiceXs (http://www.servicexs.com/) using the next sequencing generation techniques of Illumina. The size of the genome and scaffolds generated correlate with the previous observation on genome size (31 Mb) and number of chromosomes (13) in *A. bisporus* variety Horst U1 [11]. Comparison of these two genomes revealed the presence of more than 280,000 single nucleotide differences. This means that on average one out of 110 base pairs differs between these two parental genomes.

Table 2. Strains genotypes with 600 SNP markers developed for alleles in the parental nuclei of the first commercial hybrid Horst U1. In the third column the average percentage of SNP markers is indicated for which both alleles of Horst U1 are present.

Origin	Type	% both alleles identical to Horst U1	# strains tested
Wild accessions	Tetra-sporic	1.3	1
	Bi-sporic	29	16
Traditional varieties	Off-white	50.1	
(used before 1981)	White	45.7	
Post Horst U1 varieties	Present-day white varieties	99	9
(used after 1981)	Brazilian/Chinese commercial	32.4	1

We have used these differences to generate single nucleotide polymorphic markers (SNP's). Six hundred markers were selected evenly distributed over the whole genome. These markers were subsequently used to genotype a number of traditional varieties, present-day commercial varieties and wild accessions [12]. The traditional varieties were used before Horst U1 and Horst U3 were commercially available (1981). Since breeding by hybridisation between homokaryons of different varieties was not common (or not done at all) before 1981, it is expected that these varieties are generated via tissue cultures of superior mushrooms or multi spore cultures derived from one mushroom. The latter is a traditional technique often used to "rejuvenate" strains. The traditional commercial white varieties can be divided in two subvarieties, i.e. white and off-white strains. In these varieties 46 and 50% of both alleles present in Horst U1 were found, respectively (Table 2). Since Horst U1 is a hybrid between a white and an off-white strain, this is an expected outcome. In the bisporic wild accessions on average for 29% of all 600 SNP markers both alleles were found. In the only tetrasporic wild accession tested, only 1.3 % of both alleles of all SNP's were present. This clearly shows that wild accessions are more distantly related to commercial varieties and that germplasm of tetrasporic varieties is not present in the commercial varieties. All tested commercial varieties that were released after Horst U1 was produced show a striking similarity to Horst U1, i.e. for 99% of all 600 SNP markers both alleles are found. Since the SNP marker

scoring has an error of at least 1%, we consider these varieties as identical to Horst U1 and identical to each other. The origin of these varieties is unclear and there are two possibilities, either these varieties are full copies of Horst U1 or they are obtained as fertile single spore isolates of Horst U1. As stated in the previous paragraph, most basidia of the bisporic varieties produce two spores and recombination between homologs is very low. The
pairing of non-sister nuclei in one spore thus results in a preservation of most if not all alleles and an altered distribution of homologs over both nuclei. The latter can be checked by recovering both parental types in these commercial varieties via protoplasting of vegetative mycelium. This procedure was carried out for two commercial varieties. Vegetative mycelium was protoplasted and both nuclear types were recovered as homokaryons. All four homokaryons were genotyped using the SNP markers. The analysis showed clearly that all alleles that grouped in one chromosome in Horst U1 also grouped in one chromosome in nuclei of these commercial varieties, i.e. no recombinations were found between homologs. The distribution of homologs, however, was different from that in Horst U1 and both commercial varieties showed different distributions (Fig. 3). This indicates that it is very likely that these commercial varieties were derived from the first hybrid Horst U1 via selection of heterokaryotic single spore cultures.

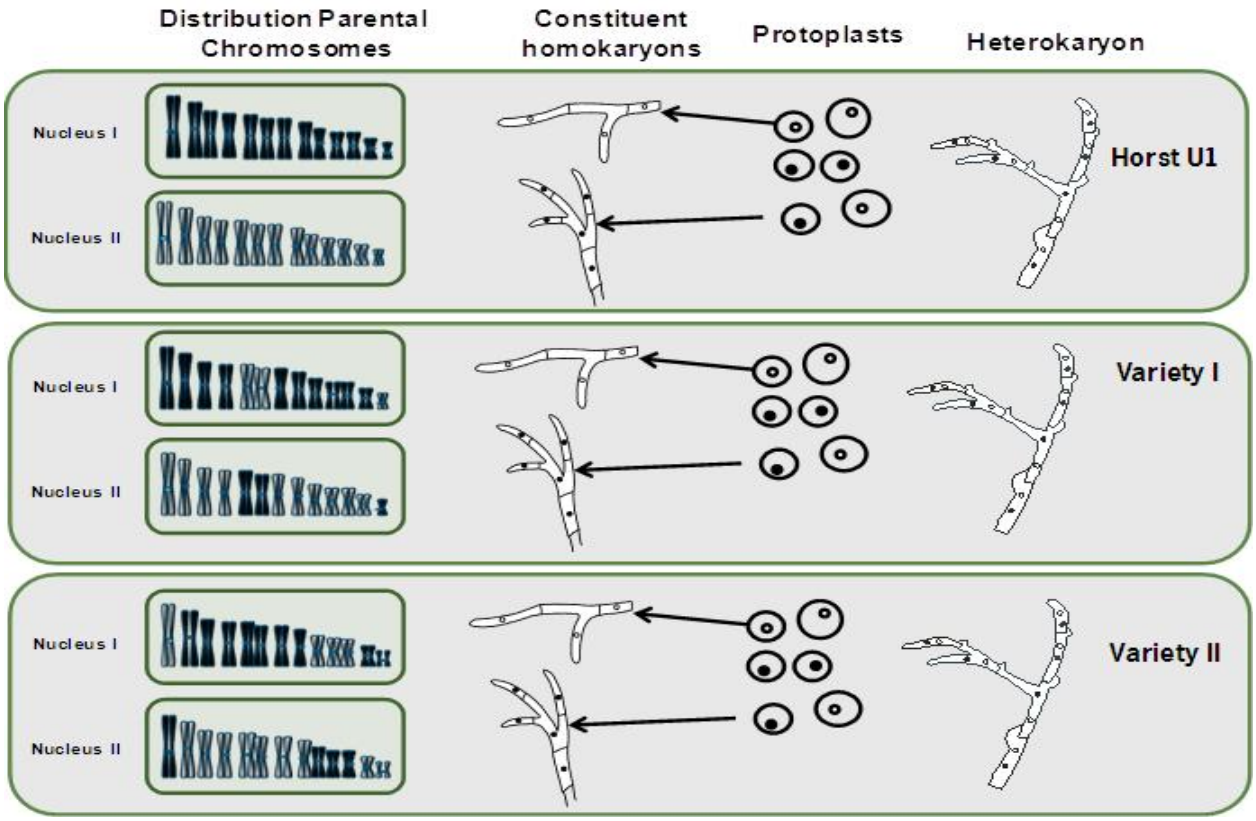

Figure 3. Distribution of homologous chromosomes over the constituent nuclei of the varieties Horst U1 and two other varieties marketed after Horst U1 was released. The constituent nuclei of these varieties were recovered as homokaryons via protoplasting. Six hundred SNP markers, evenly distributed over all chromosomes were generated for the two parental nuclei of Horst U1. These markers were subsequently used to genotype the constituent homokaryons of the two varieties. Alleles that grouped per chromosome in Horst U1, also grouped per chromosome in all homokaryons of the two varieties, indicating that no recombination has occurred. Compared to Horst U1, the homologous chromosomes show an altered distribution over the nuclei. These varieties might have been obtained by isolating fertile single spore cultures from an offspring of Horst U1 that shows no recombination and a normal independent segregation of homologous chromosomes in meiosis I.

It is expected that all other commercial varieties with an identical genotype as Horst U1 have been derived in the same way. It is interesting to see that one strain that is used commercially in Brazil and has an origin in China (Jos Buth, personal communication) has only 32% allele similarity with the other commercial varieties. This variety might have been obtained by true breeding but further genotyping has to be done to exclude the possibility that this strain is identical to one of the traditional commercial varieties.

Despite the striking genetic similarity of the present-day commercial varieties to the first released hybrid Horst U1, phenotypic differences can be seen in scaling, pinning or size of mushrooms. What causes these differences is unknown. It is possible that gene interaction differs between genes within one nucleus compared to genes between different nuclei and that this epistatic effect is the main effector for phenotypic differences between strains with identical alleles. The origin for phenotypic differences could also be epigenetic. One of the epigenetic mechanisms is methylation of nucleotides, usually cytosine. C-methylation does occur in *A. bisporus* and most transposons are methylated (Sonnenberg, unpublished). Whatever the mechanism is, the opportunity to change the distribution over the constituent nuclei and generate minor differences in phenotype is an interesting tool for breeders to "tune" traits of varieties.

Strain protection and definition of essentially derived varieties (EDV's). Although the opportunity for exchange of homologs between nuclei and its effect on phenotype is interesting from a scientific and breeders' point of view, it offers an obstacle to invest in breeding programs. Introducing new traits in varieties by hybridisation of commercial varieties with wild accessions is a laborious task. It involves the assessment of traits which should not be underestimated since many traits are influenced by climate conditions and the quality of substrate. This means that sufficient replications have to be done. Especially efforts have to be put in isolation and analysis of segregating offspring. The latter is a laborious task for several reasons. All present-day hybrids and most wild accessions have mainly bisporic basidia. Large numbers of single spore cultures have to be isolated and analysed to find sufficient homokaryons, since only homokaryons can be used for matings. In addition, due to the low recombination frequency, even more homokaryons have to be isolated and analysed to find sufficient individuals with at least one recombination. Including the repeated backcrossing needed to introduce the trait without too much linkage drag we estimate that the length of a breeding program can vary from four up to ten years. Once released, these bisporic varieties can be used to generate heterokaryotic offspring. These offspring will have all (or almost all) alleles of the original variety and will differ in distribution of homologs between the constituent nuclei. From the phenotypic differences found, an interesting one can be selected and used to market a new strain. Since these varieties have been through meiosis, they are considered to be generated via breeding. This way of breeding, however, will take months or at the most two years. Such new varieties will compete with the original variety as we have seen with "new" varieties marketed after the release of the first hybrid Horst U1. That means that there will be no or insufficient return of investment in large breeding programs. This, together with the typical life cycle of the button mushroom, is the main reason why no new varieties were released for the white button mushroom in last three decades. A solution to this problem would be to define the heterokaryotic offspring of A. *bisporus* var. *bisporus* as essentially derived varieties as defined by the UPOV convention. This does not mean that generating varieties via heterokaryons should be prohibited. They can be useful for growers and processors but a breeder of such derivatives should be tributary to the breeder of the original varieties and negotiate for a licence fee. A good definition and a general acceptance of essentially derived varieties for button mushrooms will certainly help to get investments in breeding program and thus offer the opportunity to generate new varieties that contribute to a much-needed innovation in the mushroom industry worldwide.

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PLANT POLYSACCHARIDE DEGRADATION BY FUNGI

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ABSTRACT

Plant biomass is the major carbon source for many fungal species. Due to its complex polymeric nature, degradation of this biomass to digestible monomers requires a large range of enzyme activities. With the availability of an increasing number of fungal genomes, new insights into the diversity of fungi with respect to plant biomass degradation have been obtained. Recent progress in this area will be reviewed in this paper.

Keywords: fungi, plant biomass, polysaccharide degradation, CAZy, growth profiling

PLANT BIOMASS COMPOSITION AND STRUCTURE

Plant biomass is the most abundant organic matter on earth and the major substrate for the majority of fungal species. It consists mainly of polysaccharides, but also contains proteins and the aromatic polymer lignin. Plant polysaccharides can be divided into plant cell wall polysaccharides (cellulose, hemicelluloses, pectin) and storage polysaccharides (e.g. starch, inulin, gums) [1]. They consist of many different monomeric components that are attached to each other by a variety of linkages.

Cellulose is the most abundant plant polysaccharide. It consists of a linear chain of β-1,4-linked D-glucose residues (Fig. 1). These chains are organised in bundles called microfibrils [2] that provide the main strength and structure for the plant cell wall.

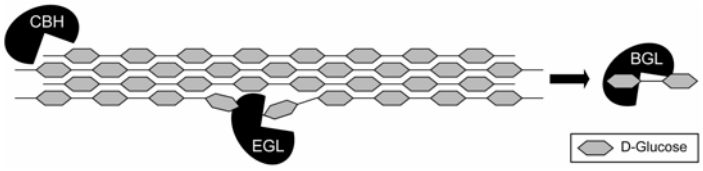

Figure 1: Schematic presentation of cellulose, its degradation and release of D-glucose. Reprinted from [3] with permission from the publisher (Copyright remains with the original publisher).

Hemicelluloses are more diverse in nature and three main types are present in plant cell walls: xylan, xyloglucan and mannan (Fig. 2). Xylan consists of a backbone of β-1,4-linked D-xylose residues [4, 5]. Other residues can be attached to this backbone, such as α-linked L-arabinose or (4-O-methyl-) D-glucuronic acid residues, α- or β-linked D-galactose residues and acetyl residues [5-8]. The L-arabinose residues can be further decorated with feruloyl residues [9-11]. Xyloglucan has a backbone that consists of β-1,4-linked D-glucose residues that are decorated with α-1,6-linked D-xylose residues [12]. Attached to the D-xylose residues can be L-arabinose, D-galactose and L-fucose residues. Mannan is often also referred to as galactomannan as this

hemicelluloses consists of a of β-1,4-linked D-mannose backbone with α-1,4-linked D-galactose residues and acetyl residues as decorations [13]. Depending on the plant species, the D-mannose backbone can also be interrupted with single D-glucose residues.

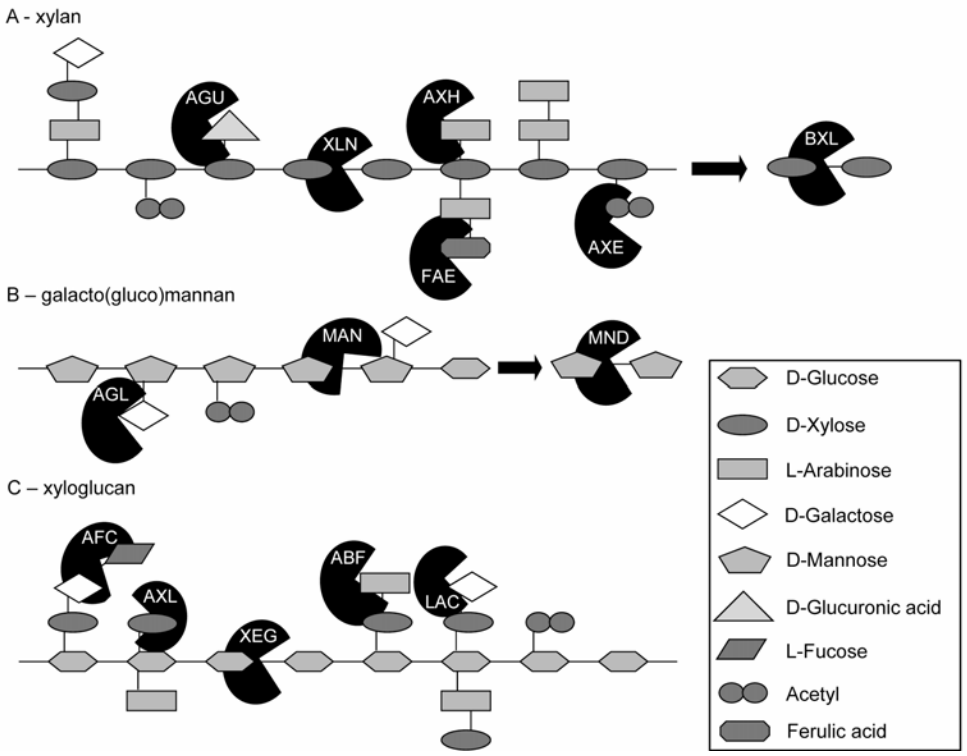

Figure 2: Schematic presentation of hemicellulose components (xylan, galacto(gluco)mannan, xyloglucan) and their degradation. Reprinted from [3] with permission from the publisher (Copyright remains with the original publisher).

Pectin is the third plant cell wall polysaccharide and it consists of several sub-structures (Fig. 3) [14, 15]. Homogalacturonan is a linear polymer of α-1,4-lined D-galacturonic acid residues that has various degrees of acetylation and/or methylation. Xylogalacturonan is a modified homogalacturonan with β-1,3-linked D-xylose residues attached to D-gaalcturonic acid. Rhamnogalacturonan 1 consists of a backbone of α-1,4-linked D-galacturonic acid and α-1,2-linked L-rhamnose residues. Side chains consisting of L-arabinose and/or D-galactose residues are attached to many of the L-rhamnose residues creating a structure often referred to as the 'hairy' region of pectin. Terminal feruloyl and *p*-coumaroyl residues can be attached to these side chains.

Starch is one of the main storage polysaccharides. It consists of an α-1,4-linked polymer of D-glucose residues that contain α-1,4-linked branching points [16]. Another major storage polysaccharide is inulin, that consist of a β-2,1-linked chain of D-fructose with a terminal d-glucose residue [17]. Another varied group of storage polysaccharides are the gums that contain many different structures [18]. Some gums (e.g. locust bean gum, guar gum) are highly similar in structure to cell wall galactomannan.

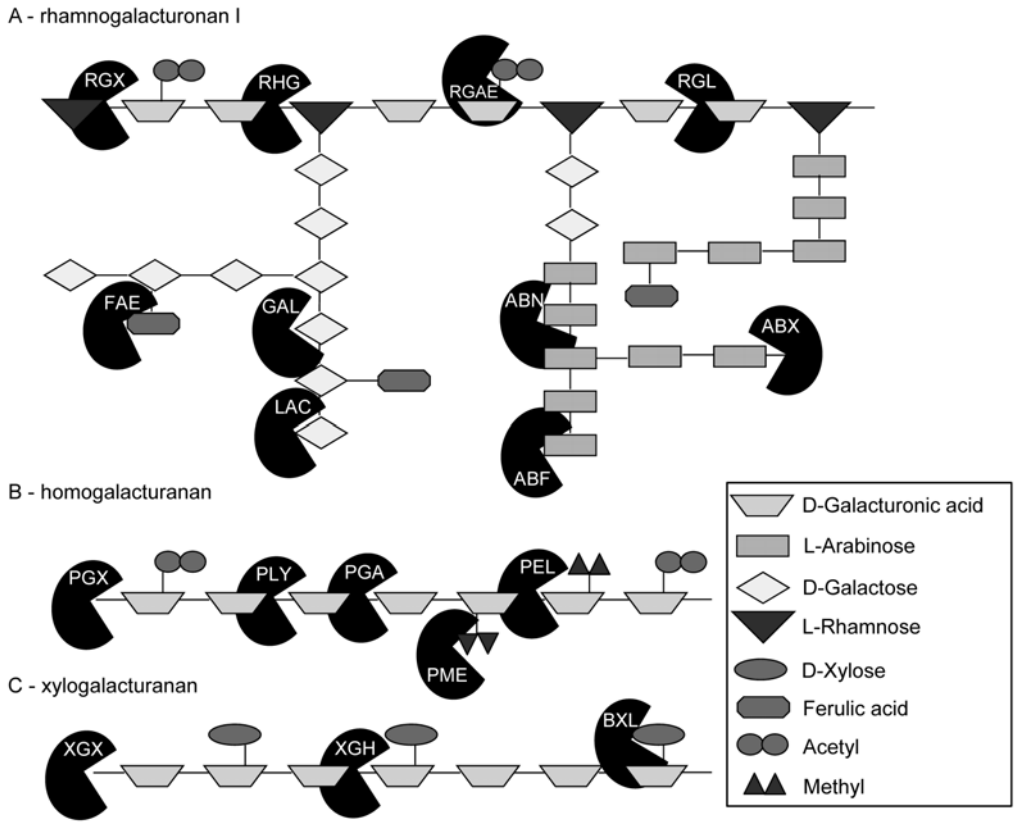

Figure 3: Schematic presentation of pectin components and their degradation. Reprinted from [3] with permission from the publisher (Copyright remains with the original publisher).

BIODEGRADATION OF PLANT POLYSACCHARIDES

Due to the diverse nature of plant polysaccharides, a large range of enzyme activities is required to degrade them to their monomeric components (Table 1) [1, 19]. These enzymes can be divided into families based on modules in their amino acid sequence resulting in a classification system called Carbohydrate Active enZymes (CAZy, www.cazy.org) [20]. Using the CAZy annotation pipeline for fungal genomes has been shown to be a highly powerful tool to obtain insight in the carbohydrate potential of a fungus and has been shown to be superior to manually annotated carbohydrate related genes of fungal genomes. The pipeline has been used for several genome annotations including several basidiomycetes [19, 21-35]. This demonstrated that significant differences exist in the plant polysaccharides degrading potential of *Schizophyllum commune*, *Coprinopsis cinerea*, *Laccaria bicolor*, *Postia placenta*, *Phanerochaete chrysosporium*, *Cryptococcus neoformans* and *Ustilago maydis* [25]. Overall, the genome of *C. neoformans* contained the lowest number of genes encoding putative plant cell wall (PCW) degrading enzymes, followed by the genomes of *U. maydis* and *L. bicolor*, which can be linked to their lifestyle as a animal/human pathogen, a biotrophic plant pathogen and a mycorrhizae, respectively. The saprobic basidiomycetes all contain significantly higher numbers of genes encoding putative PCW enzymes.

Table 1. Fungal enzymatic activities involved in plant polysaccharide degradation. The enzymes are sorted alphabetically on the enzyme code. Modified from [19].

Enzyme class	Enzyme code	Substrate	CAZy families
α-L-arabinofuranosidase	ABF	Xyloglucan, xylan, pectin	GH51,54
endoarabinanase	ABN	Pectin	GH43
exoarabinanase	ABX	Pectin	GH93
α-L-fucosidase	AFC	Xyloglucan	GH29,95
α-1,4-D-glucosidase	AGD	Starch	GH31
α-1,4-D-galactosidase	AGL	Xyloglucan, xylan, galactomannan	GH27,36
α-glucuronidase	AGU	Xylan	GH67,115
α-amylase	AMY	Starch	GH13
acetyl xylan esterase	AXE	Xylan	CE1
arabinoxylan arabinofuranohydrolase	AXH	Xylan	GH62
α-D-xylosidase	AXL	Xyloglucan	GH31
β-1,4-D-glucosidase	BGL	Cellulose	GH1,3
β-1,4-D-xylosidase	BXL	Xylan, pectin	GH3,39,43
cellobiohydrolase	CBH	Cellulose	GH6,7
β-1,4-D-endoglucanase	EGL	Cellulose	GH5,7,12,61
feruloyl esterase	FAE	Xylan, pectin	CE1
β-1,4-endogalactanase	GAL	Pectin	GH53
Glucuronyl esterase	GE	Xylan	CE15
Glucoamylase	GLA	Starch	GH15
β-1,6-endogalactanase	GLN	Pectin	
galactomannan acetyl esterase	GMAE	Galactomannan	
Endo-inulinase	INU	Inulin	GH32
Exo-inulinase	INX	Inulin	GH32
β-1,4-D-galactosidase	LAC	Xyloglucan, xylan, pectin, galactomannan	GH2,35
β-1,4-D-endomannanase	MAN	Galactomannan	GH5,26
β-1,4-D-mannosidase	MND	Galactomannan	GH2
pectin acetyl esterase	PAE	Pectin	
pectin lyase	PEL	Pectin	PL1
rhamnogalacturonan lyase	RGL	Pectin	PL4,11
endopolygalacturonase	PGA	Pectin	GH28
exopolygalacturonase	PGX	Pectin	GH28
pectate lyase	PLY	Pectin	PL1,3,9
pectin methyl esterase	PME	Pectin	CE8
rhamnogalacturonan acetyl esterase	RGAE	Pectin	CE12
rhamnogalacturonan galaturonohydrolase / exorhamnogalacturonase	RGX	Pectin	GH28
α-rhamnosidase / rhamnogalacturonan rhamnohydrolase	RHA	Pectin	GH78
rhamnogalacturonan hydrolase / endorhamnogalacturonase	RHG	Pectin	GH28
Invertase / fructofuranosidase	SUC	Inulin	GH32
d-4,5 unsaturated -glucuronyl hydrolase	UGH	Pectin	GH88
unsaturated rhamnogalacturonan hydrolase	URH	Pectin	GH105
β-1,4-exogalactanase	XFG	Pectin	
xyloglucan-active β-1,4-D-endoglucanase	XEG	Xyloglucan	GH12,74
xyloglucan acetylesterase	XGAE	Xyloglucan	
β-1,4-D-endoxylanase	XLN	Xylan	GH10,11
β-1,6-exogalactanase	XSG	Pectin	
β-1,3-exogalactanase	XTG	Pectin	

EXPANSION OF BASIDIOMYCETE GENOMES

Basidiomycete species form a relative small section among the fungi for which genome annotations have been published as could be seen from the comparison mentioned above. This prevents in depth comparisons as have been published for ascomycete species as the coverage of the basidiomycete tree of line is insufficient to draw conclusions on evolutionary changes in these species related to plant polysaccharide degradation. However, a much larger set of basidiomycete genomes is currently in progress which will soon enable such detailed studies (Table 2). With the rapidly reducing costs of genome sequencing, this number of genomes is likely to grown exponentially over the next years.

Table 2. Basidiomycete genomes that are finished or in progress.

Species	Order	Sequencing Institute
Agaricus bisporus var bisporus	Agaricomycotina	JGI
Agaricus bisporus var. burnettii	Agaricomycotina	JGI
Auricularia delicata	Agaricomycotina	JGI
Ceriporiopsis subvermispora	Agaricomycotina	JGI
Coniophora puteana	Agaricomycotina	JGI
Coprinopsis cinerea	Agaricomycotina	Broad
Cryptococcus neoformans	Agaricomycotina	Broad
Dacryopinax sp.	Agaricomycotina	JGI
Dichomitus squalens	Agaricomycotina	JGI
Fomitiporia mediterranea	Agaricomycotina	JGI
Fomitopsis pinicola	Agaricomycotina	JGI
Ganoderma sp.	Agaricomycotina	JGI
Gloeophyllum trabeum	Agaricomycotina	JGI
Hemileia vastatrix	Pucciniomycotina	Genoscope
Heterobasidion annosum	Agaricomycotina	JGI
Laccaria bicolor	Agaricomycotina	JGI
Malassezia globosa	Ustilaginomycotina	JGI
Melampsora laricis-populina	Pucciniomycotina	JGI
Microbotryum violaceum	Pucciniomycotina	Genoscope
Phanerochaete carnosa	Agaricomycotina	JGI
Phanerochaete chrysosporium	Agaricomycotina	JGI
Phlebia brevispora	Agaricomycotina	JGI
Pleurotus ostreatus	Agaricomycotina	JGI
Postia placenta	Agaricomycotina	JGI
Puccinia graminis	Pucciniomycotina	Broad
Punctularia strigosozonata	Agaricomycotina	JGI
Rhodotorula graminis	Pucciniomycotina	JGI
Schizophyllum commune	Agaricomycotina	JGI
Serpula lacrymans	Agaricomycotina	JGI
Sporobolomyces roseus	Pucciniomycotina	JGI
Stereum hirsutum	Agaricomycotina	JGI
Trametes versicolor	Agaricomycotina	JGI
Tremella mesenterica Fries	Agaricomycotina	JGI
Ustilago maydis	Ustilaginomycotina	Broad
Wolfiporia cocos	Agaricomycotina	JGI

GROWTH PROFILING OF FUNGI VALIDATES DIFFERENCES IN FUNGAL GENOME SEQUENCES RELATED TO PLANT POLYSACCHARIDE DEGRADATION

Recently a novel fungal database has been initiated (www.fung-growth.org) to host growth profiles on a set of 35 carbon sources related to plant biomass. These growth profiles have already been used to provide biological support for differences observed in ascomycete genomes with respect to plant polysaccharide degradation [19, 22-24, 27]. The database currently contains growth profiles for more than 100 fungal species with genome sequences that are finished or in progress and has already shown significant differences in the ability of basidiomycete fungi to use various plant polysaccharides. These comparisons are part of several basidiomycete genome papers that are currently submitted or in progress and will provide a better understanding of the evolutionary changes in basidiomycete fungi.

CONCLUDING REMARKS

Considering the importance of plant biomass as a carbon source for many fungi, a better understanding of the diversity of fungi with respect to carbon utilisation will provide a significant increase in our understanding of fungal evolution. The expansion of basidiomycete genome sequences and other datasets (e.g. transcriptomics, proteomics) will enable studies into these fungi to much higher level and is likely to result in new strategies for improving mushroom cultivation.

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GENOME SEQUENCE, FUNCTIONAL GENOMICS OF SHIITAKE MUSHROOM *LENTINULA EDODES*

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ABSTRACT

Lentinula edodes (Shiitake/Xianggu) is a popular cultivated mushroom species. Understanding the genomics and functional genomics of *L. edodes* is essential to improve its cultivation and quality. Genome sequencing of *L. edodes* provides numerous molecular genetic markers for breeding and genetic manipulation. We sequenced the genome of *L. edodes* monokaryon L54A using Roche 454 and ABI SOLiD. Sequencing reads of about 1011 Mb were *de novo* assembled into a 39.8 Mb genome. We compiled the genome sequences into a searchable database with which we have been annotating the genes and analyzing the metabolic pathways. Over 13,000 gene models were predicted from the genome sequence. The gene models were annotated by BLASTX and categorized according to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). For functional genomics, we have been using many molecular techniques including RNA arbitrarily primed-PCR, SAGE, LongSAGE, EST sequencing and cDNA microarray to analyze genes differentially expressed during development. Protein families of *L. edodes* genome sequence compared across genomes of several fungi identified protein families conserved to mushroom-forming fungi. We are learning more about the molecular biology and genetics of this economically important mushroom.

Keywords: Shiitake Mushroom, genome sequence, transcriptome, fruiting body development

INTRODUCTION

Lentinula edodes (Berk.) Pegler, or the shiitake mushroom, is the second most cultivated mushroom worldwide, especially in China and Japan. Understanding the genomics and functional genomics of *L. edodes* is essential to improve its cultivation and quality. *Lentinula edodes* follows a typical basidiomycete life cycle. Two monokaryotic mycelia with compatible mating types fuse to form a dikaryon. Under appropriate environmental conditions, dikaryotic mycelia aggregate to form a primordium and then mature into a fruiting body. The molecular biology of Agaricales fruiting body initiation and development remains to be elucidated.

Since 2008, reports of genome sequences from mushroom-forming basidiomycete fungi (Agaricales) has been emerging, including *Laccaria bicolor* [1], *Coprinopsis cinerea* [2], *Schizophyllum commune* [3]. Comparison of the mushroom genome sequences will help distinguish genes shared by different mushroom species from those that are specific to individual species. Further characterization of the conserved genes may provide insights into the complex developmental process.

We sequenced the genome of the monokaryotic *L. edodes* strain L54A. This provides resources for the study of growth and development of this mushroom. We compiled the genome sequences, annotations, genetic variations and mushroom genome comparisons into a genome database and analysis platform.

MATERIALS AND METHODS

Genomic DNA was obtained from *L. edodes* monokaryon L54A and subject to shotgun and paired-end sequencing using Roche 454 GS-FLX/Titanium system. The pool of 454 sequencing reads was *de novo* assembled using Newbler version 2.3 (Roche). The intrinsic sequence error of 454 sequencing associated with homopolymers was fixed by additional shotgun sequencing by ABI SOLiD 3 system.

Protein-coding genes were predicted using AUGUSTUS *L. edodes* model [4] (trained with *Schizophyllum commune* proteome, assisted with *L. edodes* ESTs). The reference gene set was subject to automated annotation based on the amino acid sequences. Protein domains and functional sites were predicted by InterProScan [5]. KEGG orthology identifiers were assigned and biological pathways were associated by KEGG Automatic Annotation Server (KAAS) [6]. Gene Ontology (GO) terms were assigned to genes by inferring GO terms of assigned Pfam protein domains [7].

Genome sequences and protein sequences of 14 other fungi, including 3 Agaricales, 4 other basidiomycetes and 7 ascomycetes (Table 1), were collected from public sequence databases. Protein sequences of all 15 fungal genomes were filtered, pooled together and subject to all-versus-all comparison by BLASTP [8]. The software OrthoMCL [9] was used to cluster the proteins into families based on the comparison results.

Table 1: List of 15 fungal genomes compared

Phylum	Species	Reference
Basidiomycota	**Agaricales**	
	Lentinula edodes	This study
	Laccaria bicolor	[1]
	Coprinopsis cinerea	[2]
	Schizophyllum commune	[3]
	Others	
	Cryptococcus neoformans	[10]
	Phanerochaete chrysosporium	[11]
	Postia placenta	[12]
	Ustilago maydis	[13]
Ascomycota	*Apergillus fumigates*	[14]
	Apergillus nidulans	[15]
	Apergillus oryzae	[16]
	Neurospora crassa	[17]
	Saccharomyces cerevisiae	[18]
	Trichoderma reesei	[19]
	Tuber melanosporum	[20]

RESULTS AND DISCUSSION

We have sequenced the genome of *L. edodes* L54A, a parental monokaryon of L54, by Roche 454 GS-FLX/Titanium and ABI SOLiD sequencing at over 11-fold coverage. The 39.8Mb draft genome sequence consists of 767 scaffolds with N50 sequence size of 111.9kb. Over 13,000 gene models were predicted from the genome sequence. Using the genome sequence of *L. edodes* L54A and 14 other fungal genomes, we have built a searchable genome database and analysis platform for bioinformatics and genome analysis.

To identify proteins conserved in all 4 Agaricales, predicted proteomes from *L. edodes* and 14 other fungal genomes were pooled and clustered to identify protein families. Over 1000 families are conserved among Agaricales but absent in ascomycetes (may present in at least one non-Agaricale basidiomycetes). To determine any enriched biological processes or molecular functions among the Agaricales-conserved protein families, Gene ontology (GO) terms of the corresponding *L. edodes* proteins were compared with that of all *L. edodes* proteins. A total of 54 GO Biological Process terms (Table 2) and 46 Molecular Function terms (Table 3) are significantly enriched ($P < 0.05$). Agaricale-conserved protein families were identified to be rich in putative regulators of biological processes, including gene expression (GO:0010468; P=1E-26), transcription (GO:0006355; P=3E-24), signaling (GO:0023051; P=9E-07). These regulators may represent a complex regulatory network in orchestrating fruiting body development of Agaricales. As there are Agaricales-conserved proteins enriched for nucleic acid binding transcription factor activity (GO:0001071; P=1E-15), these putative transcription factors could represent the switches in different stages of fruiting body development.

Table 2: Top 20 Gene Ontology (GO) Biological Process terms enriched among *L. edodes* proteins that are conserved among Agaricales but absent in ascomycetes

Accession	Term	Bonferroni corrected *P*-value	No. of *L. edodes* proteins
GO:0050789	regulation of biological process	2E-38	69
GO:0065007	biological regulation	3E-38	69
GO:0050794	regulation of cellular process	3E-37	67
GO:0019222	regulation of metabolic process	4E-27	47
GO:0010468	regulation of gene expression	1E-26	44
GO:0009987	cellular process	4E-26	157
GO:0060255	regulation of macromolecule metabolic process	4E-26	44
GO:0031323	regulation of cellular metabolic process	6E-26	45
GO:0080090	regulation of primary metabolic process	6E-26	45
GO:0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	2E-25	44
GO:0051171	regulation of nitrogen compound metabolic process	2E-25	44
GO:0009889	regulation of biosynthetic process	7E-25	42
GO:0010556	regulation of macromolecule biosynthetic process	7E-25	42
GO:0031326	regulation of cellular biosynthetic process	7E-25	42
GO:2000112	regulation of cellular macromolecule biosynthetic process	7E-25	42
GO:0006355	regulation of transcription, DNA-dependent	3E-24	41
GO:0051252	regulation of RNA metabolic process	3E-24	41
GO:0044238	primary metabolic process	2E-16	120
GO:0043170	macromolecule metabolic process	4E-16	99
GO:0019538	protein metabolic process	7E-14	64

Table 3: Top 20 Gene Ontology (GO) Molecular Function terms enriched among *L. edodes* proteins that are conserved among Agaricales but absent in ascomycetes.

Accession	Term	Bonferroni corrected *P*-value	No. of *L. edodes* proteins
GO:0005488	binding	3E-59	226
GO:0005515	protein binding	1E-32	76
GO:0001071	nucleic acid binding transcription factor activity	1E-15	25
GO:0003700	sequence-specific DNA binding transcription factor activity	1E-15	25
GO:0046914	transition metal ion binding	5E-15	57
GO:0008270	zinc ion binding	2E-14	42
GO:0003824	catalytic activity	4E-14	156
GO:0046872	metal ion binding	6E-14	59
GO:0043167	ion binding	1E-13	59
GO:0043169	cation binding	1E-13	59
GO:0003676	nucleic acid binding	1E-13	74
GO:0003677	DNA binding	4E-09	44
GO:0030695	GTPase regulator activity	3E-08	14
GO:0004672	protein kinase activity	4E-08	26
GO:0060589	nucleoside-triphosphatase regulator activity	5E-08	14
GO:0005085	guanyl-nucleotide exchange factor activity	7E-08	11
GO:0016301	kinase activity	9E-08	28
GO:0030234	enzyme regulator activity	2E-07	15
GO:0016773	phosphotransferase activity, alcohol group as acceptor	3E-07	27
GO:0016787	hydrolase activity	7E-07	61

We have been using a battery of molecular techniques, including RNA arbitrarily primed-PCR, serial analysis of gene expression (SAGE), LongSAGE, cDNA sequencing and cDNA microarray, to analyze genes differentially expressed along the developmental stages [21, 22]. From mycelium to sporulating fruiting bodies, many physiological and biochemical changes occurs as revealed by analysis of the transcriptome. Through the transition from mycelial to primordial stages, different hydrophobins were expressed abundantly in the two stages, fewer structural genes were expressed, transcription and translation became active, and different genes involved in intracellular trafficking and stress responses were expressed. Massive cDNA pyrosequencing of mature fruiting bodies indicated that the mushroom (1) senses the external environment, (2) transmits signals to express genes through regulatory systems, (3) produces many proteins, (4) degrades unwanted proteins, (5) performs extensive biosynthesis, (6) generates energy, (7) regulates the internal environment, (8) transports molecules, (9) carries out cell division, and (10) differentiates and develops.

CONCLUSION

The genome sequence of *L. edodes* enriched genomic resources for mushroom research. Our works provided a holistic understanding of the molecular basis of the growth and development of mushrooms. Having a genome analysis platform, the mushroom community should also benefit from the access to the datasets in an organized manner.

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USE OF ISSR MARKERS FOR STRAIN IDENTIFICATION IN THE BUTTON MUSHROOM, *AGARICUS BISPORUS*

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ABSTRACT

The white button mushroom *Agaricus bisporus* is the most widely cultivated specious of edible mushrooms all over the world. Originating from a limited heritage line, commercial strains of *A. bisporus* are supposed to be genetically very similar. Many highly polymorphic molecular markers have been exploiting for strain identification in wild and commercial strains of this mushroom. ISSR marker with the whole-genome coverage, accuracy and reproducibility as well as robustness has proved to be a promising marker for genetic diversity analysis of many crops. The objective of this work was to evaluate the potential of ISSR markers for genotype identification in common button mushroom, *A.bisporus*. For this purpose, 18 *A.bisporus* genotypes, including four cultivars, 13 hybrid strains and their single spore progenies along with an indigenous wild strain, were assessed for their similarity using 20 ISSR primers. Out of 20 primers, 10 proved to be discriminative in *A.bisporus*, producing 110 scorable and 76 polymorphic bands. The similarity degree was calculated for each couple of genotypes according to the Jaccard coefficient and grouping was carried out by the UPGMA clustering analysis. ISSR primers successfully identified every single individual; however, high similarity was detected among genotypes. As we expected, the wild genotype Dezful exhibited little relatedness with other genotypes and placed in a separate individual group. Genotypes IM0037 and Dezful with similarity coefficient of 0.44 and genotypes 737 and IM00Ca12 with similarity coefficient of 0.937, were accordingly the least and the most similar genotypes. Our result demonstrates that ISSR markers are powerful enough for detection of polymorphism among closely related genotypes of *A. bisporus*.

Keywords: *Agaricus bisporus*; Genetic Diversity; Molecular marker; Strain identification

INTRODUCTION

Button mushroom (*Agaricus bisporus*), belongs to the genus *Agaricus*, family *Agaricaceae*, Order *Agaricales* and phylum *Basidomycota*. *Agaricus* is one of the biggest genera of Mushrooms which includes about 300 edible and poisonous species.

One of the most important species of this genus is the white button mushroom which is widely cultivated in most parts of the world. Commercial strains of white button mushroom are classified based on the morphological characters, which are easily affected by the environmental factors. Environmental factors along with the close genetic relation between the isolates, make their isolation and identification difficult and sometimes impossible. In the past two decades, different molecular markers based on nucleic acid polymorphisms, have been exploited in genetic studies of the edible mushrooms.

Castle et al.[1] used RFLP technique for genotyping of *A. brunnescens*, Moore et al. [2] used RAPD for separating cultivars of the button mushroom, Ma and Luo [3] used ITS-RFLP in

genotype identification in the genus *Pleurotus*, Chillali et al. [4] used analysis of ITS and IGS regions for evaluation of genetic diversity in the fungus *Armillaria*, Zhang et al. [5] and Guan et al.[6] used ISSR markers for strain identification in *Lentinula edodes* and isolation of different strains in *A. bisporus*, respectively. Using AFLP, Ghorbani Faal et al. [7] succeeded in partly differentiating closely related hybrid strains of white button mushroom and also obtained some unique bands for a few strains. Variable nature of microsatellite regions, minimum requirements and easy application as well as the reasonable cost, has made ISSR marker a very useful tool for most systematic and ecological evaluations [8]. The aim of this study was to evaluate the ability of ISSR marker, for genetic detection and strain identification of white button mushroom strains, being cultivated in Iran.

MATERIALS AND METHODS

Strains. Eighteen genotypes of *A. bisporus* were used in this study (Table 1).

DNA Extraction. DNA was extracted from 18-20 day-old cultures on compost extract (CE\CYM) liquid medium. Extraction was based on Soltis laboratory protocol [9] with little modifications.

ISSR Analysis. 10 ISSR primers (Table 2) were screened from 20 initial ISSR primers. The amplification reactions was carried out in 20 µl volume containing 30 ng DNA template, 0.5 mM primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1 U Taq DNA polymerase (Genet Bio) and 1x PCR buffer.

Table 1: The 18 *A. bisporus* genotypes used in this study

Strain no.	Strain type	Origin	Strain no.	Strain type	Origin
1	IM002	hybrid strain	10	IM00H15	single spore isolate from commercial strain 737
2	IM003	hybrid strain	11	IM00Ca10	single spore isolate from an unknown commercial strain
3	IM005	hybrid strain	12	IM00Ca12	single spore isolate from an unknown commercial strain
4	IM006	hybrid strain	13	IM00Ca14	single spore isolate from an unknown commercial strain
5	IM008	hybrid strain	14	Dezful	An Iranian wild genotype
6	IM0037	hybrid strain	15	737	commercial strain
7	IM00H2	single spore isolate from commercial strain 737	16	A15	commercial strain
8	IM00H12	single spore isolate from commercial strain 737	17	F60	commercial strain
9	IM00H14	single spore isolate from commercial strain 737	18	2200	commercial strain

The reactions were performed as following: a per- denaturation at 94°C for 3 min, 30 cycles of amplification: 45 sec at 94°C, 1 min at 46-51°C (Table 2), 1 min at 72°C and a final extension at 72°C for 5 min. Amplified products were separated using 1.5% agarose gel and stained by ethidium bromide. DNA ruler 100 bp plus was used as size marker. Band scoring was performed using Total lab software. The similarity degree was calculated for each pair of

genotypes according to the Jaccard coefficient and grouping was carried out by the UPGMA clustering analysis using NTSYS.2 software.

Table 2: Ten selected ISSR primers and the polymorphic bands generated by each

Primer name	Sequence (5'-3')	T_a (^oC)	Total bands	Polymorphic bands	Primer name	Sequence (5'-3')	T_a (^oC)	Total bands	Polymorphic bands
807	$(AG)_8T$	46	11	6	834	$(AG)_8YT$	49	11	6
808	$(AG)_8C$	49	13	9	835	$(AG)_8YC$	51	8	6
809	$(AG)_8G$	49	10	3	836	$(AG)_8YA$	49	7	6
810	$(GA)_8T$	47	7	5	841	$(GA)_8YC$	50	18	15
811	$(GA)_8C$	48	10	5	842	$(GA)_8YG$	51	15	15

RESULTS AND DISCUSSION

Ten primers (listed in Table 2) created clear bands suitable for analysis, whereas other primers (805, 812, 840, 843, 844, 845, 858, P8, P38 and P39) either produced no band or non scorable ones. These 10 primers totally produced 110 scorable bands, among which 76 were polymorphic. The ability of primers to detect polymorphism among different genotypes was different. Primer 842 with 15 polymorphic bands out of 15 (Figure 1) and primer 841 with 15 polymorphic bands out of 18 comprised the most discriminative primers.

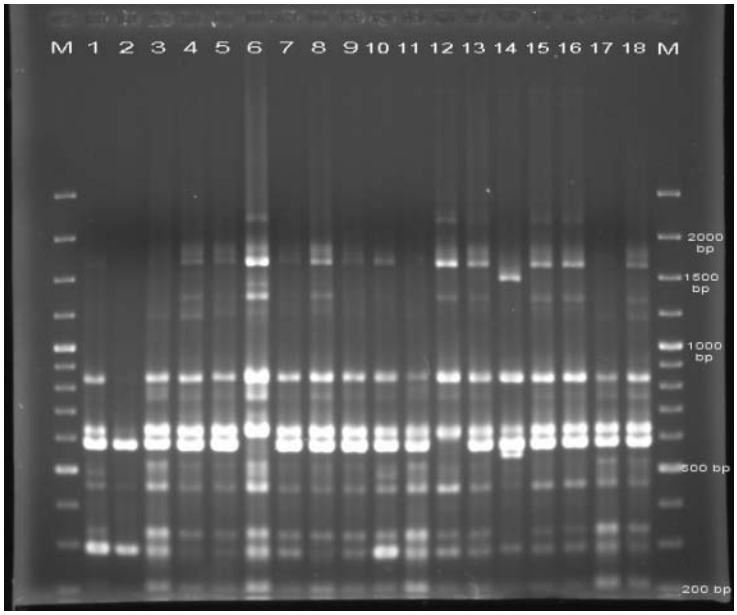

Figure 1: ISSR amplified pattern by primer 842, M: size marker 100 bp plus, Lanes 1-18: Genotypes IM002, IM003, IM005, IM006, IM008, IM0037, IM00H2, IM00H12, IM00H14, IM00H15, IM00Ca10, IM00Ca12, IM00Ca14, Dezful, 737, A15, F60 and 2200.

Based on the sequence of these primers, it seems that GA and AG motives have higher proportion and better distribution in the genome of *A. bisporus*. This is consistent with the results of Guan et al., too [6].

According to the similarity matrix, genetic similarity of sample pairs varied between 0.448 and 0.938. Genotypes 737 and IM00Ca12 with similarity coefficient of 0.937, and genotypes IM0037 and Dezful with similarity coefficient of 0.44 were the most and the least

similar genotypes, respectively (Figure 2). Genotype Dezful which is a wild genotype revealed a completely different banding profile with all 10 selected primers and stood quite distinct from other genotypes in the final cluster.

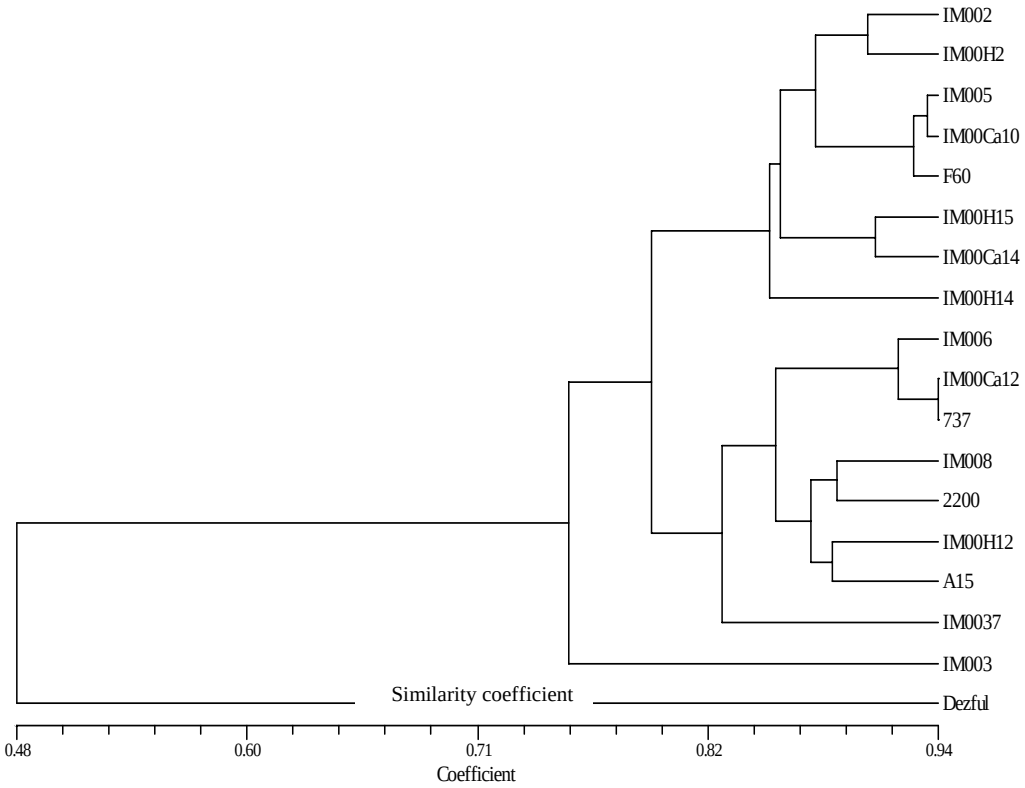

Figure 2: Dendrogram based on ISSR fingerprints for 18 *A. bisporus* strains

Based on the similarity coefficient and the relating dendrogram, strains IM00Ca10, IM00Ca12 and IM00Ca14, all three single spores of an unknown Canadian strain, proved to be very similar to each other and the commercial strain 737. This result led us to this hypothesis that these might have been originated from a single strain, 737. Moore et al. [2] had also described that commercial *A. bisporus* cultivars from different companies reflected a lack of genetic diversities.

In a work by Guan et al.[6], 12 main strains of *A. bisporus* were collected from different provinces in China and tested by six ISSR primers. Although all the strains were successfully differentiated, the results showed a high similarity coefficient between the strains, implying that they might originate from a single maternal strain, U1.

The results of our study also showed high similarity between the commercial strains of *A. bisporus* which are currently cultivated in Iran. At the mean time, ISSR markers provided a good resolution ability to detect small differences.

CONCLUSIONS

Overall, it can be concluded that ISSR markers are useful tools to assess genetic variations among closely related strains of *A. bisporus*. Furthermore, primers containing repetitive sequences of AG or GA will be more favorable for genetic identification and strain detection in this species.

ACKNOWLEDGEMENTS

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CONVENTIONAL AND MOLECULAR APPROACHES FOR BREEDING BUTTON MUSHROOM

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ABSTRACT

A total of 294 single spores were isolated from eleven-button mushroom strains and 132 were evaluated for their fertility of which 24 were found non-fruiting whereas eleven single spore isolates started fruiting after second flush of cropping and remaining were fertile. There were significant variations in the morphological traits like stipe length, gill size, average fruit body weight, etc. The colour of gills was also highly variable and it ranged from dark brown to off white and in some cases gills were almost absent. There was segregation for fruit body colour and SSIs of some of the varieties produced fruit bodies of white, off white, light brown and brown colour. The variability has been analyzed and selected SSIs were subjected to RAPD and gene sequencing of 5.8S rDNA for identifying markers related to fertility. The non-fruiting isolates also produced fruit bodies towards the end of the crop possibly due to natural hybridization in the cropping rooms. DNA from such natural hybrids and original SSIs have also been subjected to RAPD and ITS sequencing for confirmation of natural hybridization. The promising natural hybrids have also been cultured for comparing their yield performance and quality with selected SSIs.

Keywords: *Agaricus bisporus*, single spore isolates, homokaryon, RAPD, ITS sequences

INTRODUCTION

The life cycle and the sexuality pattern of only a few species of basidiomycetes are known, yet there seem to be the predominance of heterothallic sexual pattern in majority. Similarly the compatibility system governing the sexual behavior of these fungi are mostly bifactorial (tetra-polar) in nature, with only a few species showing uni-factorial control.

The breeding of *A. bisporus* is complicated proposition because of its unusual secondary homothallic sexual behavior where majority of the basidia produce two spores each containing two nuclei of opposite mating-type and only a few basidia are tri- or tetrasporic yielding homokaryotic spores. There is no way to identify the latter as *A. bisporus* mycelium unlike other basidiomycetes lacks clamp connections and is multinucleate. The only method to identify non-fertile isolates is the fruiting trial.

The diversity of gene pool is one of the most important prerequisite for any breeding program. During the present study, 294 number of single spore were isolated from eleven different strains (existing and wild), 132 of these were screened for their fertility and some of them were evaluated for morphological traits to analyze the diversity as well as the DNA profiling using RAPD and ITS 5.8S gene sequencing to identify a marker for the fertility so that cumbersome fruiting trial of a large SSI population may be avoided.

MATERIAL AND METHODS

Germplasm used. A total of 294 single spores were isolated from eleven strains, namely, S-11, U-3, A-4, A-6, A-16, A-2, A-15, A-94, S-465, S-130, and a wild strain W.I.-1. All the strains used in the study were obtained from culture collection of Directorate of Mushroom Research, Solan, and Indian Agriculture Research Institute, New Delhi.

Single spore isolation. All the strains were cultivated on pasteurized compost using standard cultivation practice to see the morphological variation and also to collect the spore prints for single spore isolation. SSIs were isolated using serial dilution technique on the standardized media containing dextrose – 5.0 g; $MgSO_4$ – 0.25 g; KH_2PO_4 – 0.95 g; succenic acid – 1.25g; Agar – 15.0 g; water – 500 ml and pH- 5.0. Placing *Agaricus* mycelium on the lid triggered the spore germination where the plates were placed in inverted position. The single germinating spores were marked under an inverted microscope and transferred to slants.

Cultivation of Single spore isolates. The spawn of a total of 132 isolated single spores was prepared on wheat grains following standard practice and was cultivated on pasteurized compost in 10 kg bags. Each SSI was replicated three times. The yield of the each bag was taken up to four weeks and then average for each SSI was calculated and subjected to statistical analysis using anova. The fruit bodies of selected SSIs were analyzed for stipe length, gill size & colour, average fruit body weight and colour of pileus.

DNA Isolation. Genomic DNA was extracted from liquid nitrogen dried mycelium grown on malt extract broth medium (malt extract 10 g and dextrose 5 g) according to the modified method described by Punja & Sun [1]. The DNA concentration was estimated by electrophoresis in 1% Agarose gel containing 0.05% ethidium bromide. The DNA concentration was further estimated spectrophotometrically by taking optical density of the DNA solution at 260 and 280nm. The final working concentration of DNA was standardized to 5ng μl^{-1}.

RAPD analysis. Multilocus genotyping by RAPD was performed using 30 primers supplied by Operon Technologies namely, OPA-01-10 and OPP-01-20. Amplification was performed in a total reaction mixture of 25µl following Singh *et al.* [2]. PCR amplification products were electrophoretically separated on 1.6% agarose gel (Sigma) prepared in 1x TAE. The gel was run for 3 h at 45 V. The staining was done with ethidium bromide and visualized under 300 nm UV light and photographed. The gel photographs were scored for presence and absence of scorable bands with the assumption of positional homology. To establish the genetic relationship among the isolates, similarity coefficients were calculated between isolates and dendrogarm drawn using UPGMA Algorithm (Unweighted Pair Group Method using Arithmetic Averages) of the NTSYS-pc, Version 2.02h programme [3,4].

PCR amplification of ITS regions of 5.8S ribosomal DNA gene. The PCR primer ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'- TCC TCC GCT TAT TGA TAT GC-3') developed by White et al [5] were used to amplify the ITS of ribosomal DNA, which encompasses the 5.8S gene and both ITS-1 and ITS-2 regions. Amplifications were done following Singh *et al.* [2]. The PCR products were visualized on 1% agarose gel in Tris-Acetic acid-EDTA (1x TAE) buffer at 80 V for 60 min. Agarose gels were stained with ethidium bromide and photographed under UV light for amplified ITS products.

Sequencing and sequence analysis of ITS region. PCR products were directly sequenced using automated sequencer of Applied Biosystems (3730 analyser) using ITS-1 and ITS-4 primers. Nucleotide sequence comparisons were performed by using the Basic Local Alignment Search Tool (BLAST) network services against the National Centre for Biotechnology Information databases. The multiple sequence alignment of ITS region of twenty samples (10 each of fertile and sterile monospore) was performed using the CLUSTAL X (1.8) and unrooted neighbour joining tree was plotted to determine grouping amongst the monospores.

RESULTS

A total of 294 single spores were isolated from eleven button mushroom strains and 132 were evaluated for their fertility. Out of 132 single spore isolates some of them were discarded due to very low yields or poor quality fruiting bodies and a total of 64 SSIs were selected for further studies. Twenty-four SSIs were found non-fruiting whereas eleven single spore isolates started fruiting after second flush of cropping that may be due to natural hybridization and remaining were fertile (Table 1). There were significant variations in the morphological traits like stipe length ranging between 1.2 to 4.6 cm, gill size between 0.5 to 4.0 mm, average fruit body weight between 6.86 to 14.9 g, and pileus dia between 1.8 to 4.7cm. The colour of gills was also highly variable and it ranged from dark brown to off white. During the evaluation of the single spore isolates, some of the isolates were found to have negligible gill and also the colour of the gills were very light. It has been noticed that the colour of the SSIs of white strains also varied from white to light brown, which may be due to segregation of traits for fruit body colour (Fig 1). The stipe and pileus size of the SSIs were found to be highly variable character, however, the pileus:stipe ratio was found to be quite constant ranging from 1.6 to 2.5.

The fertile isolates were used for selection of the high yielding and good quality mushroom fruit bodies whereas non-fertile isolates were subjected to hybridization experiments. In the hybridization experiments the isolates were mated in inter and intra-strainal combination so as to determine mating types and develop inter-strainal hybrids.

The variability amongst 18 randomly selected SSIs comprising 13 non-fertile and 5 fertile isolates has been analysed using 30 RAPD primers. The analysis exhibited a very low percent of polymorphism (9.0%) in single spore isolates of *A. bisporus* tested. The clustering and similarity analysis of the single spore isolates was done using NTSys PC version 2.02. The dendrogram generated is presented in Figure 2. The dendrogram shows that the fertile and the non-fertile isolates could be clearly separated through the RAPD primers and are grouped in separate clades. Besides, the some of the RAPD primers was successful in identifying a marker amongst fertile single spore isolates and are marked in the Figure 3. Different primers generated fragments of different sizes in the fertile isolates whereas these fragments were not found in the non-fertile isolates. OPP-1 has generated a fragment of size 2100 bp in the fertile isolate whereas OPP-3 generated a fragment of 1500 bp size. OPP-4 and OPA-10 has generated fragments of size 1250 and 1350 bp, respectively. These bands are of quite large size hence primers against these fragments can be developed and tested to avoid one of the major demerit of the Random Amplified Polymorphic DNA technique i.e. the reproducibility of the fragments under varied conditions, which has always been a major issue while using RAPD markers in any genetic analysis.

Table 1: Number of fertile, non-fertile and natural hybrids from 6 strains of *A. bisporus*

Strain	Fertile SSI	Non-fertile SSIs	Natural Hybrid
A-94	20	2	--
A-6	9	--	--
A-4	5	--	--
U-3	36	22	11
A-15	20	--	--
WI-1	7	--	--

Figure 1. Variation fruit body colour, shape, gill size amongst Single Spore Isolates

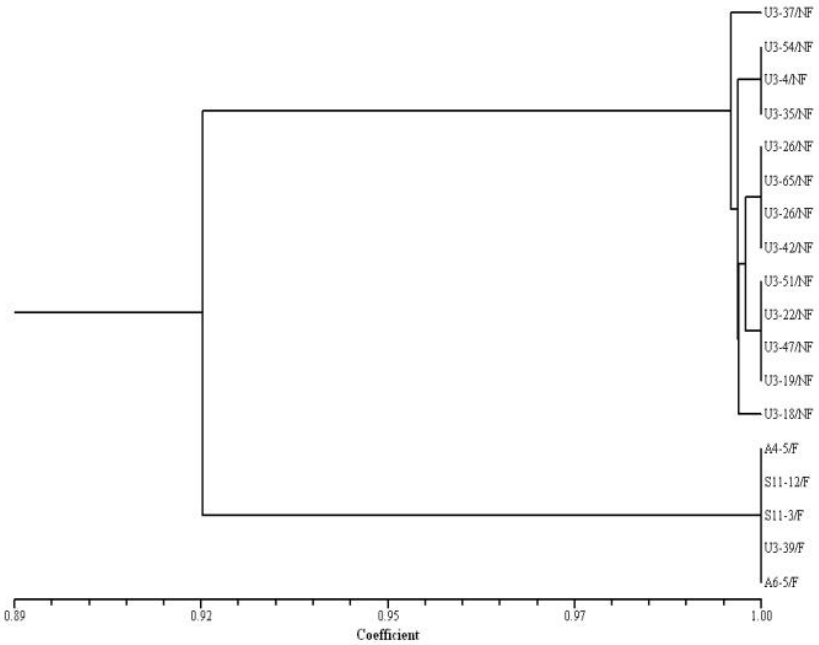

Figure 2. Dendrogram of 18 SSIs generated by NTSys pc based on the thirty primers

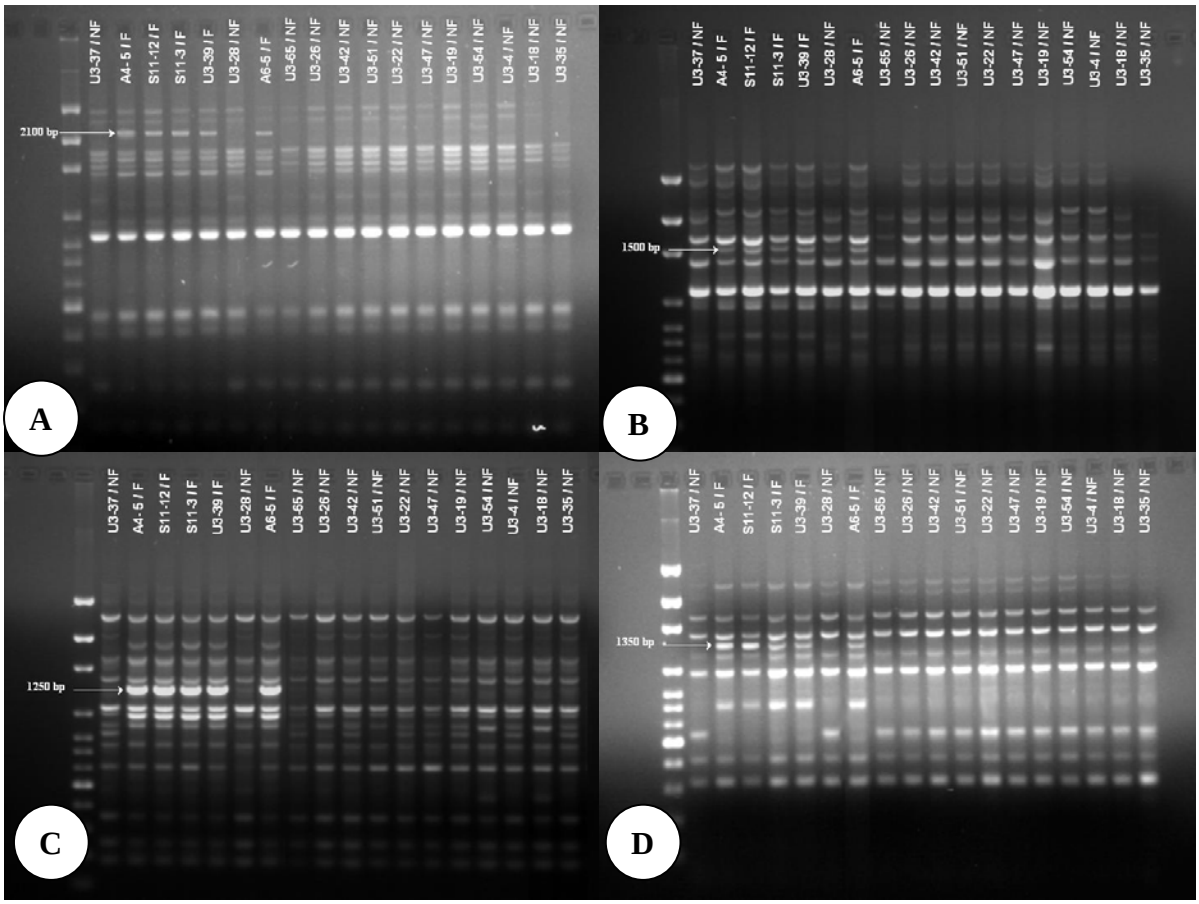

Figure 3. RAPD gel photographs showing marker band in fertile isolates of button mushroom (A = OPP-1; B = OPP-3; C = OPP-4; D = OPA-10).

For ITS sequencing a total of 18 SSIs were taken for the analysis and 10 of them were fertile isolates while the other 8 were non-fertile (table 2). The Electrophoretic separation of amplified ITS 5.8s rDNA region has shown the amplification of a fragment near about 750 bp. The PCR products have been sequenced for diversity analysis as well as to identify a marker for monokaryon identification. The multiple sequence alignment of SSIs exhibited a low level of polymorphism but the generated tree shown in Figure 4 clearly indicated three groups (i) the group of non-fruiting SSIs, (ii) Group of fruiting SSIs, and (iii) a group in which both types of SSIs are included.

Table 2: List of ITS sequenced Single Spore Isolates

Non-Fruiting isolates		**Fruiting Isolates**	
U-3-32/NF	U-3-4/NF	U-3-54/F	U-3-59/F
U-3-42/NF	U-3-18/NF	U-3-39/F	U-3-50/F
U-3-47/NF	U-3-23/NF	U-3-5/F	U-3-43/F
U-3-51/NF	A-94-10/NF	U-3-45/F	A-94-4/F
		U-3-31/F	A-94-9/F

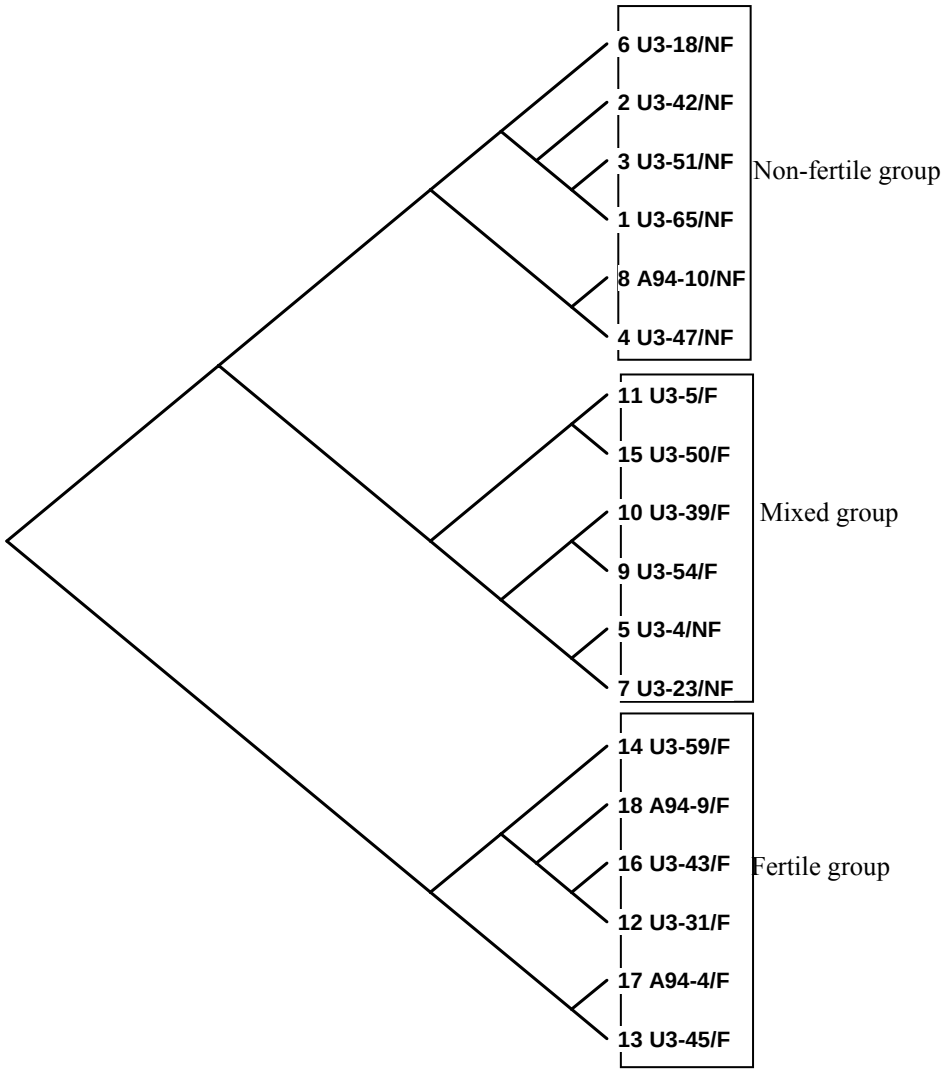

Figure 4. Sequence cladogram showing grouping of SSIs

DISCUSSION

Although button mushroom is an economically important crop, the breeding activity was based mainly only on selection until the late 1970s [6]. Fritsche in The Netherlands took the first systematic breeding approach, and this led to the introduction of the "hybrid" strains Horst U1 and Horst U3 [7]. By the late 1980s most of the cultivated strains in the world were identical to or derived from those strains [8], leaving the industry with a crop that was genetically very limited and had a high risk of sensitivity to disease.

The availability of uninucleate self-sterile homokaryons is a pre-requisite for producing hybrids in the conventional manner. However, homokaryons are difficult to obtain by conventional basidiospore isolation from *A. bisporus* strains because of secondary homothallic life cycle [6]. Similarly the lack of any morphological distinguishing features, such as a clamp connection between homokaryons and heterokaryons, remains a problem, as heterokaryosis can only be confirmed by time-consuming fruiting trials. Hence a molecular-based method for distinguishing homokaryons from heterokaryons is required. Efforts to breed new strains of the cultivated mushroom *A. bisporus* have been hampered by the rarity of the genetic markers that are necessary for a controlled breeding program. The genetic diversity in the crop is very limited and also, the haploid, monokaryotic propagules are rare. DNA restriction fragment length polymorphisms (RFLPs) have been used as genetic markers in a wide variety of organisms,

including humans [9], plants [10, 11, 12], fungi [13, 14, 15], and protozoa [16]. Nuclear ribosomal DNA (rDNA) is frequently used for taxonomic and phylogenetic studies of different species of edible fungi. The RAPD technique has been used both for studies on wild and cultivated strains of *A. bisporus* [1] or in delineation of its homokaryons [18].

In the present study, SSIs of eleven different strains of *A. bisporus* have been taken to identify marker for distinguishing between homo- and heterokaryon without going for a lengthy fruiting trials. Out of 294 a total of 132 SSIs were evaluated for their fertility first and then the molecular analysis was done using RAPDs and ITS sequences on some of them. Twenty-four non-fertile isolates could be identified during the present study and out of which 13 randomly selected SSIs along with five fertile isolates were tested for RAPD profiles for identification of markers. These studies could identify marker band of various molecular weight for fertile isolates by random primers OPA-10, OPP-1, OPP-3, OPP-4 and OPP15. These fragments could only be observed in the fertile isolates and not in the non-fertile ones. Hence this can be used in screening of a large number of populations of SSIs to restrict the number of SSIs to be taken in to the crossing for hybrid development. As the sample size in the study is not so large hence further analysis is under progress to confirm the findings in larger populations and across different strains of button mushroom.

On the other hand, the monokaryon and the heterokaryon differ in their ploidy level, the ITS 5.8S rDNA sequences may differ in the two. To take advantage of the differences in ploidy levels of the fertile and non-fertile isolates, the 5.8S rDNA was sequenced and analysed. However, the analysis of the sequences showed three groups of fertile, non-fertile and mixed group. The non-fruiting isolates, which are grouped completely separate may be the monokaryons and group of fertile SSIs are true heterokaryons but the mixed group contains the fruiting isolates along with the non-fruiting isolates. This may possibly due to four reasons (i) the non-fruiting isolates may be the bi-nucleate homokaryons (ii) isolates are heterozygous but non-fruiting is due to some genetic aberration (iii) wrongly identified as non-fruiting or (iv) error in sequencing. Further analysis of the sequences is under progress to specify the base pair changes, which may serve as a marker for fertility in button mushroom. The finding of the analysis is of immense importance in *Agaricus* breeding program but needs to be validated with larger number of populations across different strains.

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INHERITANCE PATTERN OF BRUISING SENSITIVITY TRAIT IN *AGARICUS BISPORUS*

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ABSTRACT

Bruising during mechanical harvesting causes discoloration of button mushrooms (*Agaricus bisporus*), which leads to a shorter shelf life and lower quality. A full automation of harvesting is technologically feasible but requires the availability of strains that are less susceptible to mechanical bruising. Investigating the inheritance of traits and identification of the QTLs are prerequisites for gene discovery and trait introgression breeding. Here, we studied the inheritance of the bruising sensitivity of *A. bisporus* through inter-crossing between homokaryons with extreme insensitivity and sensitivity. We screened a large number of genetically unrelated strains for discoloration of cap tissue after mechanical damage, and phenotyping of bruising sensitivity trait was processed by computer image analysis. From a number of these lines, the parental nuclear haplotypes were recovered via a protoplasting technique. These so called homokaryons were inter-crossed in all possible combinations, and hybrids were tested for bruising sensitivity. Performance of each homokaryon was thus tested in different genetic background revealing differences in expression of the trait. According to the expression of the trait, homokaryons of three different heterokaryotic strains were found to have obvious different inheritance patterns. The differences in inheritance of bruising sensitivity point to the possible involvement of different mechanisms.

Keywords: *Agaricus bisporus*, Button Mushrooms, Bruising sensitivity, Inheritance

INTRODUCTION

Agaricus bisporus is one of the most widely cultivated fungi. The Netherlands are one of the largest button mushroom producers in the world (255.000 tons in 2008) [1]. Mechanical harvesting is widely used in the mushroom industry in order to reduce the high labour cost. Mushroom bruising and discoloration caused during mechanical harvest lead to a lower mushroom quality and a shorter shelf life. Fully automated harvesting of button mushroom requires superior strains that are less sensitive to mechanical damage. Mushroom discoloration caused by mechanical damage is a consequence of the enzyme-catalyzed oxidation of phenols into quinones. These slightly colored quinones undergo further reactions forming dark melanins [2]. The enzymes and substrates are assumed to be physically separated in different cellular compartments. The study of Esquerre [3] using visible-near-infrared spectroscopy confirms that water is released from internal structures as a result of the physical damage by cytoplasm breakdown, releasing vacuolar and vesicular contents and thereby increasing the free water. This causes further activation of tyrosinase which catalyzes the oxidation of phenolics to quinones. It can be

concluded that several factors are involved in discoloration after bruising; substrates, enzymes and intracellular membranes.

A. bisporus is a secondarily homothallic basidiomycete with a life cycle that deviates from typical basidiomycetes, as described by Sonnenberg [4]. In most basidiomycetes, two types of haploid nuclei exist side by side in the mycelium cells. Fusion of nuclei only occurs just before meiosis. This is the reason that the two constituent homokaryotic parental lines (homokaryons) can be easily separated. Homokaryons can be used for breeding purposes by hybridizing compatible ones from different origins. A collection of wild strains and traditional cultivars was screened for bruising sensitivity. The results indicated that some wild strains showed less bruising sensitivity than commercial lines [5]. A very common strategy for breeding is introducing a new trait derived from a wild line (donor strain) into an existing commercial line (recipient strain) [4]. Knowing dominance or recessiveness of the trait is a prerequisite for identifying genes and trait introgression, which can be examined through crossing different lines. The two constituent homokaryotic parental lines of *A. bisporus* can be recovered through protoplasting [6]. Compatible homokaryotic lines are mated into hybrids and fruited. The sensitivity of mushrooms produced by those hybrids can be screened and analyzed, and the dominant or recessive inheritance pattern can be investigated. The aim of this study was to investigate the inheritance pattern of bruising sensitivity in *A. bisporus.*

MATERIALS AND METHODS

Selection for heterokaryotic parental strains. A broad selection of wild strains and traditional and present-day cultivars was made in previous studies [5]. Strains showed a large variation in bruising sensitivity. Seventeen representative bruising insensitive strains and sensitive strains have been selected and used to recover the constituent homokaryotic parental lines by protoplasting the vegetative mycelium (Table 1).

Table 1: Selected bruising insensitive and sensitive strain

No.	Name	sensitivity	colour	No.	Name	sensitivity	colour
1	TW8	insensitive	white	9	WW7	sensitive	white
2	TW1	insensitive	white	10	WW8	sensitive	white
3	CH1	insensitive	white	11	TW7	sensitive	white
4	TO7	insensitive	white	12	TW6	sensitive	white
5	WW1	insensitive	offwhite	13	TW5	sensitive	brown
6	WB2	insensitive	brown	14	WB17	sensitive	brown
7	WB4	insensitive	brown	15	WB16	sensitive	brown
8	WB5	insensitive	brown	16	CH2	insensitive	white
9	WB18	insensitive	brown	17	WW2	unknown	white

* TW=traditional white; CH= commercial hybrid; TO= traditional offwhite; WW= wild white; WB=wild brown;

Protoplasting and protoclone isolation. Vegetative mycelium of selected strains was protoplasted according to the method that was developed by Sonnenberg [6]. Suspensions containing protoplasts were diluted by a series of steps and plated onto the medium plates (MMP

plate (1% malt extract, 0.5% mycological peptone, 10mM MOPS, and 1.75% agar, PH7.0)+0.6M Sucrose) for regeneration,. After incubation at 24 °C for 5-7 days **r**egenerated single protoplasts (protoclones) were isolated under a stereo microscope and transferred to a new MMP plate. Protoclones were subcultured onto MMP plates covered with cellophane. After 10-14 days incubation at 24 °C, the mycelium was removed from the cellophane and harvested into an microcentrifuge tube for DNA extraction.

Identification of Homokaryons. Homokaryons were identified using PCR. Primers were designed based on known sequences, and were tested for their ability to produce polymorphism between heterokaryons and homokaryons. The absence of one or more bands was taken as an indication that a particular protoclone was homokaryotic. Alternatively, ISSR –PCR was used.

Table 2: Molecular markers used for identification of homokaryons

Marker		Sequence (5'-3')	Ta (°C)	Strains identified	Reference
G-6-PD	Forward	GTAATGTACACGGAGAC	58	TW8, WB2,TW7, WW2	This article
	Reverse	ACTCTGAAGGAACTTGG			
39Tr 2/5-2/4	Forward	CCTCGCGCAAGCAGATACAA	58	TW1, WB5, TW5	This article
	Reverse	TTGTCCGAGACTTACTCACG			
PIN 150	Forward	AGGTGACATGTCAGAAGCGC	58	CH1, CH2, TW6	7
	Reverse	CAATCTCAAGCTTGCCTGG			
P33N10	Forward	ACTATAAAGCGTGAGCTATACG	58	WW7	7
	Reverse	TATCTTCTGCGCTGTGTTGCT			
ISSR A2		HVV(GTT)5	55	TO7, WW1, WB18	This article
ISSR A7		VVH(TTG)5	55	WB4	This article
ISSR B		NDV(CT)8	55	WW8, WB16	This article
ISSR A		NDB(CA)7-C	55	WB17	This article

Crossing between homokaryons. Homokaryons obtained through protoplasting were inter-crossed on compost agar plates (75 g milled phase 2 compost of 0,1 mm particle size was suspended in 1 L of tap water together with 17.5 g of agar. The medium was sterilised for 1 hr at 121^{o}C and poured into petri dishes), and crossings were identified based on the morphology of mycelium interactions and confirmed using PCR methods. Mycelium of the interaction zone was isolated and transferred onto a new MMP medium plate to confirm that it was homogenous, after which the cross was confirmed with the same PCR primers that were used for homokaryon identification.

Fruiting test. Crossings that were identified as hybrids were put in a climate controlled fruiting test. Strains were grown in trays (56 x 36 x 20 cm) filled with 16 kilo commercial phase II compost. Each treatment had two replicates (trays). Cultivation conditions were described by Weijn [5]. Trays were distributed randomly on five layers in the growing room.

Quantification of the bruising sensitivity. The mushrooms of each replicate (tray) at the peak of the first and second flush were picked for screening of bruising sensitivity. Bruising sensitivity

tests were performed with the protocol used in the previous study [5]. In short, mushrooms were damaged mechanically and pictures were taken of the mushrooms after 60 minutes incubation at room temperature in a humid chamber. Mean whiteness difference (WI_DIFF) was quantified from the pictures using computer image software. For each strain 20 mushrooms from two replicates (ten per tray) were analyzed. Brown hybrids and white hybrids were analyzed separately, due to the differences in background color. In addition, the pictures were ranked by eye in order to check the correlation between the data of computer and human perception.

RESULTS AND DISCUSSION

Recovery of homokaryons. All the 16 strains selected had been used to recover homokaryons. Initial selection of putative homokaryons was based on colony morphology and a growth rate that differed from the original heterokaryon. The presence of only one nuclear type in putative homokaryotic protoclones was confirmed by using PCR primers that generate DNA fragments of different sizes in heterokaryons (Table 2). Not all constituent nuclei were recovered as homokaryons from the selected strains. Of seven strains (TW1, CH1, WW1, WW7, TW7, CH2, and WW2) both homokaryotic parental types were recovered and of six strains (TW8, WB2, WB4, WB5, TW6 and TW5) only one homokaryotic parental type was recovered (Table 3).

Crossing between homokaryons. All recovered homokaryons were crossed in all possible combinations. Eighty nine crosses were selected based on interaction seen in the contact zone. PCR primers described above (Table 2) were used to confirm heterokaryon formation. Sixty six crosses were confirmed as being heterokaryons.

Table 3: List of strains which have recovered homokaryons

No.	Name	sensitivity	colour	Homokaryon I	Homokaryon II
1	TW8	insensitive	white	M31	
2	TW1	insensitive	white	K2	K20
3	CH1	insensitive	white	O1	O13
4	TO7	insensitive	off white	MES 09199	MES 09200
5	WW1	insensitive	brown	MES 09143	
6	WB4	insensitive	brown	01557-8	
7	WB5	insensitive	brown	S3	
8	WW7	sensitive	white	Z6	Z8
9	TW7	sensitive	white	Q1	Q26
10	TW6	sensitive	white	N8	
11	TW5	sensitive	brown	MES 09119	
12	CH2	insensitive	white	CH2A	CH2B
13	WW2	unknown	white	MES09206	MES09208

Fruiting test. Fifty hybrids were selected for fruiting and assessment of bruising sensitivity (Table 4). One of the present-day commercial hybrids was used as a control. Forty eight out of 50 treatments produced mushrooms. Treatments 41 (O13 × N8) and 43 (Q26 × N8) failed to produce mushrooms. Some trays had a very low productivity and did not produce enough mushrooms at the desired developmental stage for analysis of bruising sensitivity. For some treatments, the two

replicates (trays) showed large differences with respect to time of pinning and first day of harvest. Replicates of four brown hybrids and one white hybrid showed significant differences in cap color between replicates. Such unexpected behavior may have been caused by cultivation problems and this may influence bruising sensitivity. Therefore analysis of these strains was omitted.

Table 4: Treatments in fruiting test

Treat-ment	Cross	Sensitivity pairing	Parent 1	Parent 2	Treat-ment	Cross	Sensitivity pairing	Parent 1	Parent 2
1	Cr 02	IS	CH2A	Z8	26	Cr 41	IS	mes09143	Q26
2	Cr 03	IS	CH2A	N8	27	Cr 42	IS	mes09143	MES09119
3	Cr 07	II	CH2A	mes09143	28	Cr 43	IU	mes09143	MES 09206
4	Cr 08	IS	CH2A	Q26	29	Cr 44	IU	mes09143	MES 09208
5	Cr 10	IS	CH2B	Z8	30	Cr 49	II	O1	S3
6	Cr 11	II	CH2B	O1	31	Cr 50	IS	O1	Z8
7	Cr 12	II	CH2B	mes09143	32	Cr 51	II	O1	mes09143
8	Cr 13	IS	CH2B	Q1	33	Cr 52	IS	O1	Q26
9	Cr 14	IS	CH2B	Z6	34	Cr 53	IS	O1	N8
10	Cr 15	IS	CH2B	Q26	35	Cr 54	IU	O1	MES 09206
11	Cr 16	IS	CH2B	MES09119	36	Cr 57	II	O1	mes 09199
12	Cr 18	II	CH2B	S3	37	Cr 58	IS	O13	Q1
13	Cr 19	IS	K2	Z6	38	Cr 59	IS	O13	Z6
14	Cr 20	II	K2	mes 09199	39	Cr 60	IS	O13	Z8
15	Cr 21	II	M31	O13	40	Cr 61	IS	O13	Q26
16	Cr 23	IS	M31	Z8	41	Cr 62	IS	O13	N8
17	Cr 24	II	M31	mes09143	42	Cr 66	II	O13	mes09143
18	Cr 25	IS	M31	Q26	43	Cr 68	SS	Q26	N8
19	Cr 26	IU	M31	MES 09206	44	Cr 70	SU	Z6	MES 09208
20	Cr 30	IS	mes 09199	Z8	45	Cr 76	SS	Z6	Q26
21	Cr 32	IS	mes 09199	Z6	46	Cr 78	SS	Z6	N8
22	Cr 34	II	mes 09200	mes09143	47	Cr 84	IS	M31	N8
23	Cr 35	IS	mes 09200	Z6	48	Cr 85	IS	Mes 09143	N8
24	Cr 39	IS	mes09143	Z6	49	Cr 86	II	01557-8	Mes09143
25	Cr 40	IS	mes09143	Z8	50	Cr 87	IS	01557-8	Z6
					51	CH2			

Screening of the bruising sensitivity. Figure 1 shows the bruising sensitivity of white hybrids (First flush). The values for WI_DIFF range from 15.6 to 57.0. A good correlation was found between data of computer software and ranking by eye (Fig 2). A good correlation between computed data and ranking was seen as well in the brown hybrids. Mean value of WI_DIFF among brown hybrids ranges from 2.1 to 26.7. Mushroom of hybrids kept a similar correlation in the second flush. The mean value of WI_DIFF in the second flush ranges from 12.3 to 46.6 for white hybrids, and ranges from 1.4 to 16.9 for brown hybrids (Figures not shown).

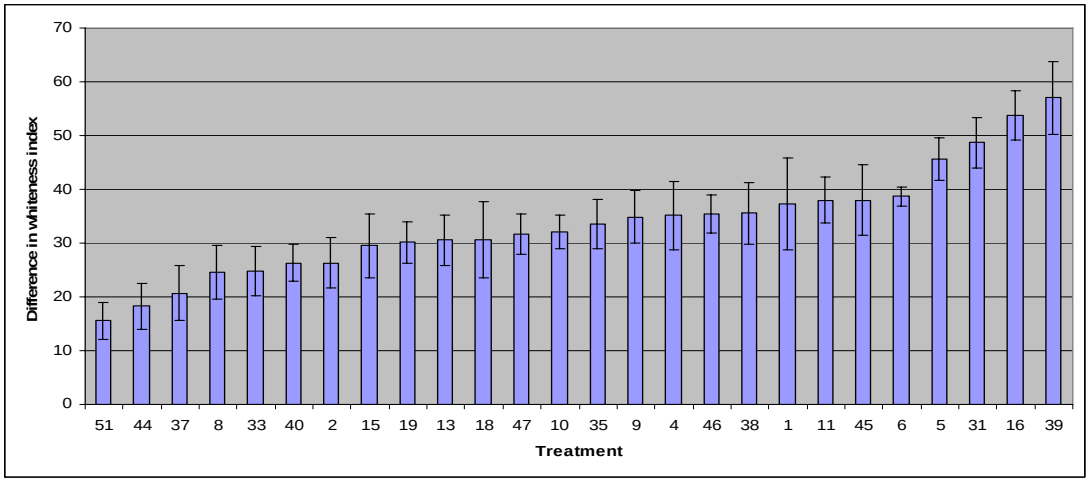

Figure 1: Bruising sensitivity of white hybrids expressed as the difference in whiteness between the bruised area and the non bruised area (1st flush). .

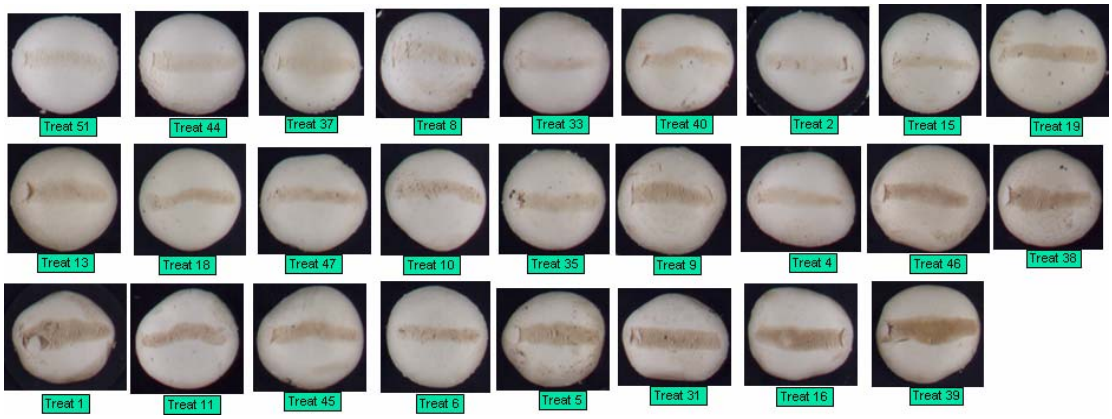

Figure 2: Ranking of bruised mushrooms of white hybrids (1st flush).

With respect to overall cap color, hybrids between homokaryons derived from white strains produced white mushrooms with limited variation (Whiteness Index (WI) ranging from 52 to 70, not shown here). Five homokaryons were derived from wild accessions with a brown cap color. All crosses containing one of these homokaryons produced mushrooms with cap colors varying from light-brown to brown (WI ranging from 11 (brown) to 45 (light brown), not shown here). All brown hybrids are lighter than the present-day commercial brown strains. Since a very light discoloration might be masked by a darker background color, the discoloration is measured relative to the non-bruised area, and thereby corrected for the darker background in brown strains.

Inheritance of bruising sensitivity. Performance of each homokaryon was tested in different genetic backgrounds, thereby revealing differences in expression of the trait (Table 5). According to the expression of the trait, homokaryons of three different heterokaryotic strains were found to have markedly different inheritance patterns.

Table 5. Bruising sensitivity of homokaryons in crosses

Cap color of original variety	Parental strain	Homokaryon	Sensitivity of original hybrid	Average whiteness Score in all crosses	S.D.	n	Range
Brown	WB2	mes09143	I	6.3	3.0	14	1.4-11.2
Brown	WB5	S3	I	9.0	0.6	2	8.6-9.4
Brown	WB4	01557-8	I	10.4	4.5	2	7.2-13.6
Brown	WW1	mes 09200	I	11.7	7.3	2	6.6-16.9
Brown	WW1	mes 09199	I	15.5	4.4	4	12.4-21.9
White	TW1	K2	I	24.0	13.2	2	14.6-33.4
White	CH2	CH2B	I	25.6	13.3	7	7.4-37.0
White	CH2	CH2A	I	20.4	8.5	7	8.7-32.6
White	TW8	M31	I	30.2	13.2	6	6.1-45.8
White	CH1	O13	I	19.3	11.3	6	2.6-32.7
White	CH1	O1	I	20.4	10.2	4	8.6-34.0
White	TW7	Q1	S	19.1	9.7	2	12.3-25.9
White	TW7	Q26	S	22.1	9.7	7	3.4-32.6
White	TW5	MES09119	S	21.4	22.0	2	5.8-36.9
White	WW7	Z8	S	28.5	16.0	7	1.4-45.8
White	WW7	Z6	S	21.8	7.8	10	11.2-33.4
White	TW6	N8	S	19.2	9.2	5	4.9-27.9
White	WW2	MES 09208	U	13.1	14.0	2	3.2-23.6
White	WW2	MES 09206	U	22.2	13.5	3	8.7-35.7
		Average of all crosses		19.0			

In all crosses made, MES 09143, a homokaryon of a wild insensitive brown strain, produced hybrids that are relatively insensitive to bruising (Fig 3). The mean WI_DIFF for MES 09143 hybrids after bruising was 6.3, and varied between 1.4 and 11.2. This indicates that MES 09143 has a dominant inheritance pattern for bruising insensitivity.

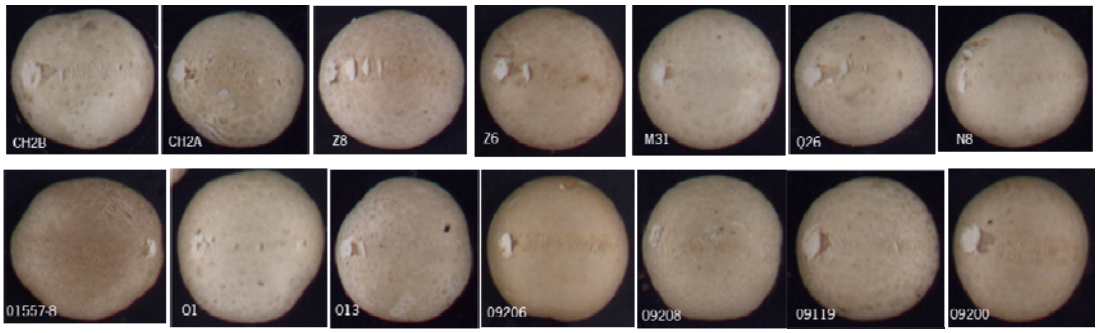

Figure 3. Bruised mushrooms of hybrids with MES 09143 as one of the parental lines.

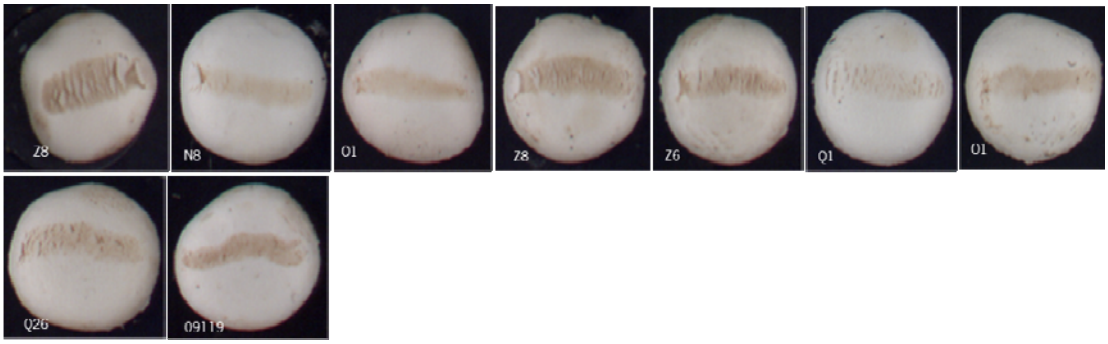

Figure 4. Bruised mushrooms of hybrids with CH2A or CH2B as a parental line

CH2A and CH2B are the two constituent homokaryons of a white insensitive commercial strain (CH2). Hybrids with either CH2A or CH2B as a parental line varied in sensitivity, according to the partner in the cross. Some hybrids with CH2A or CH2B as a parental line were bruising sensitive, while others were bruising insensitive (Fig 4). The mean score for bruising sensitivity of CH2A in crosses was 20.4, and varied between 8.7 and 32.6; the mean score for bruising sensitivity of CH2B in crosses was 25.6, and varied between 7.4 and 37.0. The heterokaryotic parental strain (CH2) showed a lower bruising sensitivity compared those white hybrids (Fig. 1, WI_DIFF of about 15). This suggests that the parental homokaryons of this strain complement each other for the genetic components contributing to insensitivity for bruising but are not dominant. This indicated that CH2A & CH2B showed a recessive inheritance pattern for bruising insensitivity.

Z6 and Z8 are the two constituent homokaryons of a wild sensitive white strain (WW7). All hybrids with Z6 or Z8 as a parental line were bruising sensitive except the one with MES 09143 (the strain that showed dominant inheritance pattern for bruising insensitivity) (Fig 5). The mean score for bruising sensitivity of Z6 in crosses was 21.8, and varied between 11.2 and 33.4; the mean score for bruising sensitivity of Z8 in crosses was 28.5, and varied between 1.4 and 37.0.

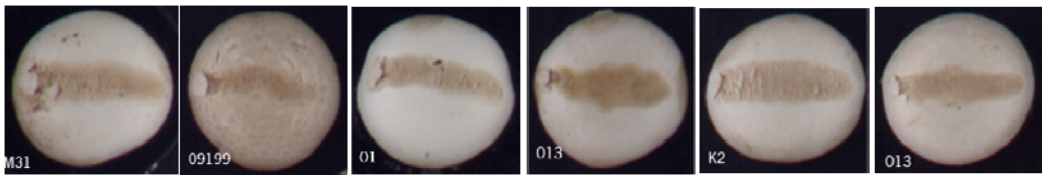

Figure 5. Bruised mushrooms of hybrids with Z6 or Z8 as a parental line

CONCLUSIONS

The differences in inheritance pattern of bruising sensitivity suggest the involvement of at least 3 mechanisms of sensitivity towards bruising and discoloration among the *Agaricus bisporus* strains tested. Hybrids of these homokaryons were selected to generate offspring segregating for these different mechanisms. These offspring will be used to map QTLs for bruising sensitivity. Subsequently advanced breeding stock will be generated by stacking beneficial genome regions involved in bruising insensitivity.

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MOLECULAR IDENTIFICATION OF MATING TYPE GENES IN ASEXUAL SPORES OF *CORDYCEPS MILITARIS*

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ABSTRACT

Cordyceps species are important medicinal mushrooms widely used in traditional Chinese medicine for maintaining health and vitality. *C. sinensis* is the most highly sought-after species, but production of fruit bodies in artificial culture has yet to be achieved. However, strains of *C. militaris*, a closely related species, will fruit on artificial media although degeneration into non-fruiting strains often occurs following continued sub-culture. In order to better understand the molecular basis of fruiting in this species, we have examined the nature of *C. militaris* mating-type genes and their distribution in fruiting and non-fruiting strains. Two parental strains, CM-23B and CM-H07, which produce fruit bodies and abundant asexual spores in artificial culture, were selected for this purpose and 100 single spore isolates were obtained from each strain. Three specific primer pairs, designed according to the reference mating type genes of *C. militaris* deposited in the NCBI database, were used for PCR amplification of the mating type genes present in all the single spore isolates and their parental strains. Both parental strains contained two kinds of mating-type loci, MAT-HMG (MAT1-2-1 gene) and MAT-alpha (MAT1-1-1 and MAT1-1-2 genes). Of the 100 isolates derived from CM-23B, 40%, contained only MAT-alpha, 25% only MAT-HMG and 35% both MAT-alpha and MAT-HMG. Of the 100 isolates derived from CM-H07, 33%, contained only MAT-alpha, 30% only MAT-HMG and 37% both MAT-alpha and MAT-HMG. We infer from these data that, during the formation of asexual spores, some spores (heterokaryons) contain nuclei carrying both mating-type loci (MAT-HMG and MAT-alpha) while others (homokaryons) contain nuclei carrying only one type of mating-type locus, either MAT-HMG or MAT-alpha. Since both mating-type loci are essential for fruiting, it is important to confirm that both MAT-HMG and MAT-alpha are present in strains selected for artificial cultivation.

Keywords: *Cordyceps militaris*; asexual spore; mating type gene

INTRODUCTION

Cordyceps militaris (L.) Link, as a species similar to *Cordyceps sinensis* [1], has the same or similar medicinal value with *Cordyceps sinensis*. Since *C. militaris* is often used as a substitute for *Cordyceps sinensis*, the market of *C. militaris* has a good development prospect; in scientific research *Cordyceps* is also used as the type species of the genus *Cordyceps* [2].

Asexual reproduction is an important part of the life cycle of *C. militaris*. In the production of *C. militaris*, it is mainly through asexual reproduction to inoculation, propagation, conservation. According to our observation, *C. militaris* species produced large amounts of asexual spores in the process of asexual reproduction. These asexual spores were easy to germinate, and the mycelium after germination grew fast. Then we were interested in whether these mycelium germinated from asexual spores had the same genetic background with parent mycelium, which might be related to the phenomenon that *C. militaris* is prone to degradation.

There are two different sources mating type locus of *Cordyceps militaris*. One is mating type loci MAT-alpha, contains two kinds of mating type gene sequence, named MAT1-1-1 and MAT1-1-2, while the other mating type loci MAT-HMG contains only one mating type gene sequence, named MAT1-2-1 [3]. In the process of sexual reproduction in *C. militaris*, homocaryotic mycelium carrying distinct mating type genes are compatible with each other. After plasmogamy, these compatible homocaryotic mycelia form pairs, heterocaryotic mycelium, initiating the dikaryophase of the sexual cycle. Then after karyogamy and meiosis and the formation of ascospores, heterocaryons complete sexual life cycle. According to the life cycle of *Cordyceps militaris*, homocaryotic mycelia have one type of mating type genes, while heterocaryotic mycelia have two types of mating type genes. So we can identify the nuclear phase, homocaryon or heterocaryon, by indentifying the type of mating type genes of *Cordyceps militaris*.

This study isolated 200 strains germinated from asexual spores of two *Cordyceps militaris* strains. We indentified mating type genes of these 200 isolates by PCR.

MATERIALS AND METHODS

Strains. *Cordyceps militaris* strains, CM-23B and CM-H07, were obtained from Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences (SAAS). *Cordyceps militaris* mycelia cultured on potato dextrose agar medium (PDA).

Preparation of asexual spore suspension and single spore isolates. Strains were inoculated on PDA plates, 25 ℃ for 10 days dark culture. Then PDA plates covered with mycelium were washed by 1mL of sterile water to collect mycelium. Asexual spores were isolated by G-2 glass filter and suspended by sterile water and diluted to 10^5 ml^{-1}. 100μl spore suspension was poured onto a PDA plant. The plate was incubated at 25 ℃ in a dark place. When visible colonies appeared, these colonies were subcultured individually onto fresh PDA plates, and incubated at 25 ℃ in a dark place until they were covered with mycelium completely. Then these mycelium were scraped down and freeze-dried until use.

Genomic DNA extraction method of *Cordyceps militaris*. The genomic DNA was extracted from *Cordyceps militaris* with the improved CTAB method [4]. Methods briefly described as follows: freeze-dried *Cordyceps militaris* hyphae ground to powder was added to 65℃ preheated 2 x CTAB extraction liquid (2% CTAB; 1.4 M NaCl; 100mM Tris-HCl; 20mM EDTA, pH8.0), and was incubated at 65°C at least 30 min, then centrifuged at room temperature, 12000 rpm for 10 min. The supernatant was transferred into another fresh 1.5 ml tube，then equivalent volume of phenol-chloroform(1:1) was added, mixed gently but thoroughly about 1 min. This mixture centrifuged (12000 rpm) for 10min. The supernatant was transferred into another fresh 1.5 ml tube and equivalent volume of chloroform-isoamyl alcohol (24:1) was added, well mixed, then centrifuged (12000 rpm) for 10min. The supernatant was transferred into another fresh tube, and 1/10 volume 3M NaAc was added, then 2/3 volume -20°C precooled isopropanol was added, and then incubated at -20°C for 20 minutes, centrifuged at 4℃, 12 000 rpm for 10 min. The precipitation was washed with 75%ethanol for twice, air dried and resuspended in 30μl TE buffer. 1μl RNase(10mg/ml) was added to the DNA solution for 1 hour at 37°C to remove RNA. The final DNA extracts were stored at -20°C until use.

Specific primers amplification for identifying mating type genes of *Cordyceps militaris*. Three specific primer pairs, shown in Table 1, designed according to the reference mating type genes of *C. militaris* deposited in the NCBI database(registration number AB084257 and AB194982), were used for PCR amplification of the mating type genes MAT1-1-2, MAT1-1-1 and MAT 1-2-1.

Using single spore isolates' or their parental strains' mycelia total DNA as templates, mating type genes fragment was amplified under the following conditions: PCR reaction mixture(100 μl) contained 100 ng DNA, 1 x PCR buffer, 2 mmol/L Mg^{2+}, 0.2 mmol/L dNTPs, 0.25 μmol/L forward and reverse primer, and 3 U Ex *Taq* polymerase (Takara Bio Co., Dalian). Amplification conditions were: 1 cycle of 94 °C for 2min; 30 cycles of 94°C for 30 s, 50-55 °C (according to TM of primers) for 60 s and 72 °C for 40-60 s (according to the length of fragment), then a final extension at 72 °C for 5 min. All amplifications were carried out using a TP-600 Thermal Cycler Dice (Takara). PCR products were separated on 1% (w/v) agarose gels.

Table 1: Specific primers used for amplification of mating type genes of *Cordyceps militaris*

Types of mating type genes		Primers	Sequences
MAT-alpha	MAT1-1-2	MAT112F	5'- ATGGAACACAGATCGAGCGACAC -3'
		MAT112R	5'- ATATACCTTCGCGATCATTGCCCAG - 3'
	MAT1-1-1	MAT111F	5'- TTCAGCTTCAGTCCGTTCTGGACA -3'
		MAT111R	5'- GGCAGACATCGTACCTGGTCAAAT - 3'
MAT-HMG	MAT1-2-1	MAT121F	5'- ATGGATCTGCAACTGGATCGGACCA- 3'
		MAT121R	5'- CTACATGATTGACTCCGGGCTCATTG- 3'

RESULTS AND ANALYSIS

Asexual spores of *Cordyceps militaris* on PDA medium initiated germination easily. Generally spore germination could be observed in three days after plated, and in 5 days visible small colonies could be found. The speed of spore germination and hypha growth in each plate are about the same. We didn't find colonies formed from spores germinating slowly (Fig. 1). In this study, 100 single spore isolates were obtained from each strain, CM-23B and CM-H07. After subcultured individually onto fresh PDA plates, the hyphae grew about the same speed. We did not find the strains grew quickly or slowly obviously.

After DNA extraction from 200 asexual single spore isolates and their parent strains, their mating type genes were identified by PCR amplification. Fig. 2 shows the partial results of identification. The length of the mating type gene MAT 1-1-1 was 457bp, while the length of the mating type gene MAT1-1-2 and MAT1-2-1 were 1063bp and 839bp, respectively.

Statistical results of PCR identification showed that the parent strains were contained two kinds of mating-type loci, both MAT-alpha (MAT1-1-1 and 1-1-2) and MAT-HMG (MAT1-2-1). Of the 100 isolates derived from CM-23B, 40% , contained only MAT-alpha (MAT1-1-1 and 1-1-2), 25% only MAT-HMG (MAT1-2-1) and 35% both MAT-alpha(MAT1-1-1 and 1-1-2) and MAT-HMG (MAT1-2-1) as their parent strains. Of the 100 isolates derived from CM-H07, 33%, contained only MAT-alpha, 30% only MAT-HMG and 37% both MAT-alpha and MAT-HMG. The results are shown in Table 2.

The parent strains are heterokaryons; each hypha cell contains two kinds of the nucleus; each nucleus contains one type of mating type genes. So parent strains contain both two types of mating-type genes. For single spore isolates, 62 strains were the same as their parent strains

containing two types of mating-type genes. So these strains were believed to be heterokaryons containing two kinds of nucleus. The other 138 single spore isolate strains only contained one type of mating-type loci, which showed that their hypha cell just contain one kind of nucleus and they were homokaryons.

Table 2: The statistical results of the kind of mating type genes of asexual spore strains, isolated from *Cordyceps militaris*

Types of asexual spore strains	Kinds of mating type genes	strains derived from CM-23B	strains derived from CM-H07
Parental type	MAT1-1-1, MAT1-1-2, MAT1-2-1	35	37
Alpha type	MAT1-1-1, MAT1-1-2	40	33
HMG type	MAT1-2-1	25	30
Total		100	100

Figure 1. The germination status of asexual spore (The seventh day)

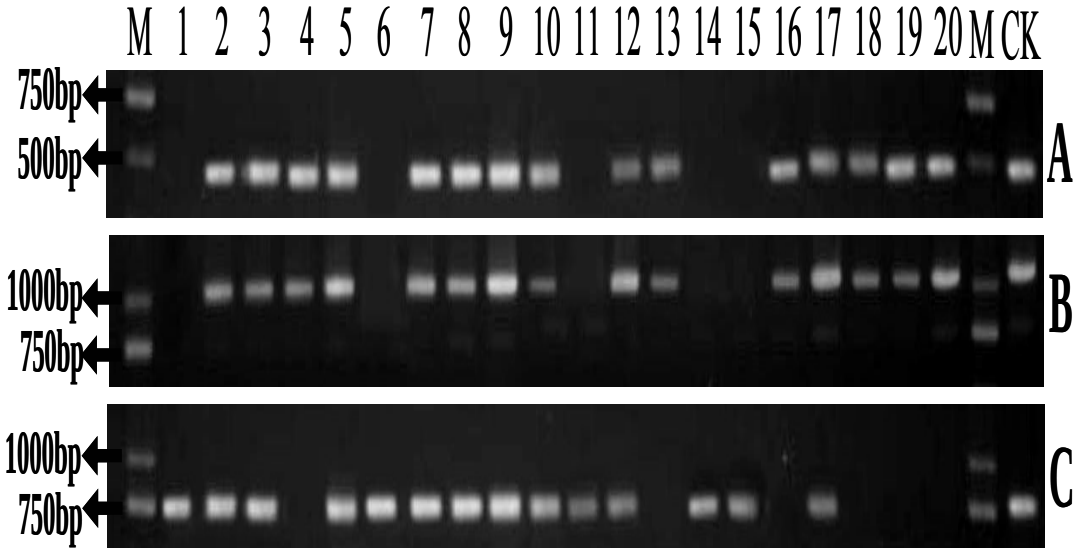

Figure 2: The partial amplification results of mating type genes fragment in single-spore isolates (M: D2000 DNA marker; 1-20: isolates No. 1-20 from CM-23B; CK：CM-23B；A：MAT1-1-1; B:MAT1-1-2; C:MAT1-2-1)

DISCUSSION

The study found that *Cordyceps militaris* strains, CM-23B and CM-H07, could produce a lot of asexual spores. We separated 200 single spore isolates and indentified their mating type genes. We found that a lot of single asexual spore isolates only contain MAT-alpha or MAT-HMG, while their parent strains contained both. Mating type genes regulate the genetic basis of sexual compatibility and sexual reproduction. *Cordyceps militaris* is a typical bipolar heterothallism mushroom [5]. It must grow from two sexual compatible pairs, which are germinated from two mono-ascospores, and then dicaryons grow to the fruiting bodies. The different degree absence of mating type genes of the mycelium grown from asexual spores may be the genetics reason why degeneration of Cordyceps militaris into non-fruiting strains often occurs following continued sub-culture. The question is worth further research. Since both mating-type loci are essential for fruiting, it is important to confirm that both MAT-HMG and MAT-alpha are present in strains selected for artificial cultivation.

According to the proportion of isolates which contained different kinds of mating type genes in this study, we supposed that the two nucleuses of *Cordyceps militaris* heterocaryons, containing MAT-alpha and MAT-HMG respectively, would be packed by plasma membrane with same probability when they formed the asexual spores. Then three kinds of asexual spores would appear, containing MAT-alpha genes only and containing MAT-HMG only and containing them both. Asexual spores only containing MAT-alpha or MAT-HMG would grow to homocaryotic mycelia, while asexual spores containing both would grow to heterocaryotic mycelia. The speculation should be further affirmed by morphological observation.

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DISTINGUISHING LEVEL OF PLOIDY IN *TRICHOLOMA MATSUTAKE*

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ABSTRACT

In order to analyse the ploidy level of the *Tricholoma matsutake* strain NBRC30773 which was obtained by fruit body tissue method, a molecular marker based on the sequence of the region (about 1760bp) upstream of homeodomain protein gene (Tmhox1) was developed. Genomic DNA was prepared from *T. matsutake* NBRC30773 strain and was used as template to amplify fragment with primers (Tm-dis1 and Tm-dis2). We obtained two different nucleotide sequence (Tm-1 and Tm-2) with 92% of similarity between each other. These results indicated that the *T. matsutake* strain NBRC30773 was heterokaryotic. On the other hand, in Tm-1and Tm-2, different cutting sites of restriction enzymes (PstI, BamHI, Bg1II and EcoT22I) were also investigated. Base on these results, the technique of restriction fragment length polymorphism (RFLP) can be used to distinguish the level of ploidy in *T. matsutake*. In the future, the *T. matsutake* strains which were obtained by spore germination and protoplast regeneration will be distinguished.

Keywords: *Tricholoma matsutake*; level of ploidy; heterokaryon; homokaryon; strain

INTRODUCTION

An ectomycorrhizal fungus, *Tricholoma matsutake* (S. Ito & Imai) Sing. is the most prized and the most expensive mushroom in Japan. The annual harvest of *T. matsutake* was 12,000 tons in 1941, but the harvest in the past decade has reduced to between 30 to 100 tons per year and it became 24 tons in 2009 [1]. Most progress towards understanding the sexuality of some ectomycorrhizal fungi has been made with the germination of the basidiospores and the establishment of homokaryotic cultures [2]. Moreover, genetic analysis and selective breeding of cultivated mushroons require the isolation of homokaryons from heterokaryotic stocks [3]. It means that level of ploidy of strain is important information for study of fungus. Obtain of homokarytic strain is important for the study of genetics and breeding application of *T. matsutake*.

In the study of *Tuber melanosporum*, Bonfante Fasolo and Brunel showed that only heterkaryotic mycelium resulting from plasmogamy is able to form mycorrhizas [4], but for ascomycetes, microscopic determination of homokaryotic or heterokaryotic mycelium is difficult. One major reason was that unlike the basidiomycetes, no clamp connections can be found in vegetative hyphae in

ascomycetes [5]. Similarly, less clamp connections can be found in hyphae of *T. matsutake* [6, 11]. So determination of homokaryotic or heterokaryotic mycelium by microscope is nearly infeasible.
In most heterothallic mushroom species, inbreeding is avoided by a sexual incompatibility system determined by two loci each with multiple alleles (the A and B mating-type loci) [12]. In tetrapolar mushroom, the A locus comprises multigenes encoding homeodomain proteins. On the basis of the homeodomain sequence, the mating-type proteins of the A locus are divided into two subgroups: HD1 and HD2 [13], [14]. The N-terminal region of the homeodomain (HD1) protein displays higher level of sequence variation between alleles than C-terminal [15].The high levels of sequence variation in N-terminal region of the HD1 makes this region a candidate for restriction fragment length polymorphism (RFLP) analysis between different level of ploidy.
On the other hand, however, in *T. matsutake*, little is known about the level of ploidy in *T. matsutake* strain which were isolated by either fruit body tissue method, spore germination or protoplast regeneration. So study of the level of ploidy in *T. matsutake* will provide the basic knowledge for *T. matsutake* genetics and breeding application. In this study, we have developed a molecular marker based on the sequence near the homoedomain gene that could be used to resolve this question of level of ploidy in *T. matsutake* strain.

MATERIALS AND METHODS

Fungal strains and culture conditions. *T. matsutake* NBRC 30773 was used in this study. *T. mastutake* NBRC 30773 was obtained from the National Institute of Technology and Evaluation of Japan and isolated by fruit body tissue method. The mycelium of *T. matsutake* NBRC 30773 were routinely cultured on modified Hamada's agar medium (0.5% KH2PO4, 0.2% yeast extract, 2% glucose, and 1.5% agar, pH 5.1) prepared with tap water [7]. For the preparation of genomic DNA, 3 square agar blocks (5 x 5 x 5 mm) of fungal mycelium culture were cut and transferred into 20 ml modified Hamada's liquid medium in a 100-ml Erlenmeyer flask and were then incubated at 25 °C for 30 days. Mycelia were then collected, lyophilized, and used for genomic DNA extraction.

Preparation of DNA. Genomic DNA was prepared from lyophilized mycelia of *T. matsutake* strain NBRC 30773 using a GENEALL Plant SV Mini kit (Toyobo Co., Osaka, Japan) according to the manufacturer's instructions.

Amplification of the *T. matsutake* homeodomain protein gene (Tmhox1). Initially, fragments of genomic DNA encoding the mitochondrial intermediate peptidase protein (Tmmip) were amplified by PCR with the degenerate oligonucleotide primer pair MIP2F and MIP2R. The MIP2F and MIP2R primers were designed based on the amino acid sequences GLGEPKF and FDLWEEI, respectively, which are conserved in the mitochondrial intermediate peptidase protein of *Coprinopsis cinerea* and *Schizophyllum commune* [16], [17]. PCR was performed in a 50-µl reaction containing 1 x Ex Taq buffer (Takara Bio Co.), 50 ng genomic DNA, 50 pmol each primer, 0.2 mM each dNTP, and 1.25 U Ex Taq polymerase (Takara Bio Co.). The PCR reaction was performed with a Takara PCR Thermal Cycler Personal (Takara Bio Co.) and consisted of an initial denaturation for 4 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 2 min at 50 °C, and 45 s at 72 °C, and a final elongation step at 72 °C for 10 min. The amplified PCR fragment (approximately 250 bp) was directly cloned into the pT7Blue (R) T-vector (Novagen, Madison, WI, USA), generating pTMGLU1. To amplify three segments of genomic DNA covering complete sequence of the Tmhox1 gene and its upstream region, 3 oligonucleotide primer pairs (Tmmip1nd1/ Tmmip1nd2, Tmmip2nd1/ Tmmip2nd2, Tmmip3nd1/

Tmmip3nd2) were designed based on the partial Tmmip sequence acquired and the nucleotide sequences of the DNA fragments amplified by the cassette PCR method. Template DNA for cassette PCR was prepared with a Takara LA PCRTM In vitro Cloning Kit (Takara Bio) according to the manufacturer's instructions. Genomic DNA from *T. mastutake* was digested with restriction endonuclease, ligated with nucleotide linker and used as templates for PCR.

Amplification of the *T. matsutake* fragments Tm-1and Tm-2. Nucleotide sequencing of fragments Tm-1 and Tm-2 were conducted using cassette amplification by PCR with one primer sets (Tm-hox-up1: 5'-ATCAACCATCTGGAGACACT-3' and Tm-hox-up2: 5'-TTTGACTGCTCAACATAGACCT-3'). The primer sets were designed based on the nucleotide sequences of upstream of Tmhox. Template DNAs for cassette PCR were prepared with a Takara LA PCR In Vitro Cloning kit (Takara Bio Co.) according to the manufacturer's instructions [18]. Genomic DNA from *T. matsutake* was digested with XbaI and the fragments were ligated with nucleotide linkers and used as templates for PCR. For fragments Tm-1and Tm-2, an approximately 2.0-kbp PCR product from Tm-hox-up2 to a XbaI site were cloned into the pT7Blue (R) T-vector (Novagen), generating pTMGLU1. The sequences for Tm-1 and Tm-2 were obtained. To amplify the entire genomic sequence of Tm-1 and Tm-2 respectively, oligonucleotide primers Tm-dis1/Tm-disF and Tm-dis2/Tm-disF were designed based on the nucleotide sequence of DNA fragment amplified by the cassette PCR method. PCR reactions were performed in a 100-µl mixture containing 1 ´ Ex Taq buffer, 100 ng extracted genomic DNA, 100 pmol each primer, 0.2 mM of each dNTP, and 2.5 U Ex Taq polymerase. PCR reactions were conducted using an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 30 s at 94 °C and 2 min at 55 °C. DNA sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) using the chain-termination procedure with a BigDye Terminator Cycle Sequencing version 3.1 kit (Applied Biosystems) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

To investigate the genomic structure of the region of upstream of Tmhox1 in *T. matsutake*, two nucleotide region containing fragment Tm-1and Tm-2 were amplified and sequenced. The characterization data for fragments are listed in Table1.

In process of isolation of *T. matsutake* strain, the *T. matsutake* hyphae was described as "slow-growth, sterile, clamp-less and dikaryotic hyphae" [6]. In this present study, we obtained two different nucleotide sequences that showed 92% of identity between each other. These results indicated that the *T. matsutake* strain NBRC30773 was heterokaryotic. Based on the sequence of the two fragments Tm-1and Tm-2, the different cutting sites of restriction enzymes (PstI, BamHI, Bg1II and EcoT22I) were also investigated in them. The difference of cutting sites of restriction suggested that in distinguishing the level of ploidy in *T. matsutake*, the technique of either restriction fragment length polymorphism (RFLP) or PCR-RFLP was feasible.

On the other hand, the two fragments sequence Tm-1and Tm-2 with different cutting sites of restriction were investigated in *T. matsutake* strain NBRC30773, but the existence of this character in most of *T. matsutake* strain need to be investigated. At the same time, the feasibility of RFLP or PCR-RFLP with restriction enzyme in distinguishing level of ploidy in most of *T. matsutake* strain will also be checked. Then, based on these results, in the future, the *T. matsutake* strains which were obtained by spore germination and protoplast regeneration will be distinguished.

Table 1: Character of fragments Tm-1 and Tm-2 in *T. matsutake*

Fragment	Length (bp)	cutting sites of restriction enzymes	identity
Tm-1	1760	*Pst*I, *Bam*HI, *EcoT22*I and *Bg1*II	92%
Tm-2	1763	*Pst*I, *Bam*HI and *Eco*T22I	

CONCLUSIONS

In the present study, two fragments sequence Tm-1 and Tm-2 with different cutting sites of restriction were investigated in *T. matsutake* strain NBRC30773. Thus, the conclusion of that level of ploidy in *T. matsutake* strain NBRC30773 was heterokaryon was indicated. Base on these results, the technique of restriction fragment length polymorphism (RFLP) or PCR-RFLP can be used to distinguish the level of ploidy in *T. matsutake*. These results will provide the basic knowledge of genetics and breeding application for *T. matsutake*.

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STRAIN IMPROVEMENT OF EDIBLE FUNGI WITH *PLEUROTUS ERYNGII* NEOHAPLONTS

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ABSTRACT

Pleurotus eryngii is an edible fungus with increasing interest for the local market as a gourmet product, due to its nice sensory characteristics and its thick and fleshy stipe. *Lentinula edodes*, on the other hand, is a fungus with interesting therapeutic properties whilst *Pleurotus* is widely cultivated in Mexico. A breeding program was undertaken in this study to develop improved strains combining *L. edodes* and *P. eryngii* characteristics. Eleven hybrids, obtained by pairing *P. eryngii* and *L. edodes* neohaplonts were grown on substrates suitable either for *P. eryngii* or *L. edodes*. Six hybrids preserved *Lentinula* phenotype and 3 of them, showed high biological efficiencies (119-153%). Additionally, 28 hybrids were obtained from Di-Mon matings by pairing different *Pleurotus* spp. dikaryons with *P. eryngii* neohaplonts, 19 hybrids were selected for fruiting on *P. eryngii* substrate; 16 hybrids showed biological efficiencies higher than 100%. Hybrids showed a wide variety of morphologies, *i.e.* different pile sizes, colors and shapes were observed though most of them with large, thick and fleshy stipe, resulting hence in suitable strains for a commercial exploitation.

Keywords: *Lentinula*, *Pleurotus*, inter-genera hybrids, neohaplonts, genetic improvement.

INTRODUCTION

Production of edible mushrooms has gained increased interest throughout the world, a multimillion dollar business has been developed producing a high quality food, rich in proteins, fiber, vitamins and minerals. World production of oyster mushrooms (*Pleurotus* spp.) is placed second following bottom mushroom (*Agaricus*). *Pleurotus eryngii* is a specialty mushroom showing a fleshy thick stipe, it is tasty with nice flavor and easy to combine with various types of foods. It is not produced commercially in Mexico nowadays but it is available as an imported "gourmet" mushroom with prices far superior to the bottom mushroom. Therefore, procedures for cultivation of *Pleurotus eryngii* and availability of improved strains are important factors for introducing this fungus into the local market.

Development of hybrid strains of *P. eryngii* by combining characters with commercial *Pleurotus ostreatus* strains and with strains of different genera, *i.e. Lentinula edodes* could yield strains retaining the tasty flavor, nice fleshy, thick stipe and long shelf life of *P. eryngii*. Hopefully, new interesting characteristics could arise, *i.e.* larger caps, new colors, flavors and taste, herewith strains showing a wider spectrum of characteristics would then become available for cultivation.

MATERIALS AND METHODS

Strains. Following strains were used for production of hybrids by mating neohaplonts and dikaryotic strains: A commercial *P. eryngii* dikaryon [1], 11 neohaplonts (designated from PeC9 to PeC45) recovered by dedikaryotization from the commercial *P. eryngii* dikaryon [2], 4 *L. edodes* dikaryons (L9, L10, L18, L21) [3], a dikaryotic *Pleurotus djamor* strain [4], 8 *Pleurotus* spp. dikaryons (Asp14, CP50, CP253, HK3539, IE200, IE201, P401 and *Pleurotus* sp. PB) and 6 neohaplonts (designated from L10-1S to L21-2S) recovered by dedikaryorization of *L. edodes* strains [2]. All strains are stored in the fungal collection of the Department of Food Science and Biotechnology at the Faculty of Chemistry (University of México). The strains were propagated in malt extract agar (MEA) (1.5% malt extract and 2% agar); cultures on MEA plates (Petri dishes) were stored at 2 to 4°C [3].

Hybrids from Matings of *P. eryngii* Neohaplonts with *L. edodes* Neohaplonts. Agar cubes (2 mm) full with growing mycelia were cut from the edge of growing cultures of selected *P. eryngii* and *L. edodes* neohaplonts. They were placed side by side on MEA plates and incubated at 24ºC. Developing colonies were inspected under the microscope during the following 7 days and those showing clamp connections were reseeded on MEA plates for further evaluation.

Hybrids from Di-mon Matings of *P. eryngii* Neohaplonts with *Pleurotus* spp. Dikaryons. Four agar cubes (2 mm) full with growing mycelia were cut from the edge of a growing culture of a selected *P. eryngii* neohaplont and symmetrically distributed on the surface of a MEA plate and incubated at 24ºC until development of 1 cm (∅) colonies. At this stage, a 2 mm agar cube was cut from the edge of a growing culture of a selected dikaryon and placed on the periphery of the growing neohaplont culture. Plates were again incubated at 24ºC and inspected under the microscope every day until appearance of clamp connections on the side opposite to the point of inoculation of the dikaryotic culture. The newly emerging dikaryotic strain (hybrid) was recovered on MEA plates. Mycelium growth of all resulting hybrids as well as of their respective parental dikaryons and neohaplonts was evaluated by placing 8 mm (∅) inocula cut from the edge of a growing culture and placed on MEA, with 3 replicates per strain. Colony diameters were measured after 3, 6 and 9 days incubation and when significant differences were established by variance analysis, strains were classified according to Duncan test.

Fruiting of Hybrid Strains. Two types of substrates were used for fruiting of hybrids, one is recommended for fruiting of *P. eryngii* and the second one for *L. edodes* (Table 1).

Table 1: Substrates for fruiting of *P. eryngii* and *L. edodes* hybrids and parental strains

Components	Substrates (% fresh weight)	
	L. edodes	*P. eryngii*
Sawdust	50.0	20.0
Cottonseed waste	36.0	60.0
Millet	6.0	-----
Sorghum (milled)	6.0	-----
Wheat bran	-----	16.0
Ammonium sulfate	0.5	-----
Citric acid	0.5	-----
Benlate	1.0	-----
Calcium carbonate	-----	3.0
Calcium sulfate	-----	1.0

Both substrates were used for fruiting hybrids of *P. eryngii* with *L. edodes* since each genus requires different substrates for good mycelium growth and fruiting. Hybrids of *Pleurotus eryngii* neohaplonts with *Pleurotus* spp. dikaryons were fruited only on *P. eryngii* substrate. Sawdust, cottonseed waste and millet were soaked in water for 24 h; after draining excess water, ingredients were thoroughly mixed according to formulation and water content of substrate was adjusted to 60%. *Lentinula* substrate (1.5 Kg) was filled into 25 x 35 cm polypropylene bags and *Pleurotus* substrate (1 Kg) was filled into 17 x 45 cm polypropylene bags. Sterilization and inoculation of substrates as well as preparation of spawn was according to Ramírez *et al.* [3]. After incubation for 9 weeks, *Lentinula* substrates were transferred into the fruiting room and polypropylene bags were completely detached in order to expose the whole surface of the substrate to the environment. After incubation for 9 weeks, *P. eryngii* substrates were transferred to the fruiting room, polypropylene bags were folded down in order to leave only the upper surface of the substrate exposed to the environment. Conditions in the fruiting room throughout the experiment were 70 to 80% air humidity, 20 to 23°C air temperature and 700 to 900 ppm CO_2. Fruit bodies developing on the substrates were harvested before pileus edge turned up completely. Harvesting period was 12 weeks for *P. eryngii* substrates and 8 weeks for *L. edodes* substrates. Weight of fresh fruit bodies was registered for each substrate bag and biological efficiency was determined as BE = g fresh fruit bodies/100 g dry substrate.

Statistical Analysis. Variance analysis of biological efficiencies of hybrids and parental strains were performed to evaluate significant differences and Duncan multiple range test was used to identify the highest producing strains (SPSS ver. 17 for Windows was used for both tests).

RESULTS

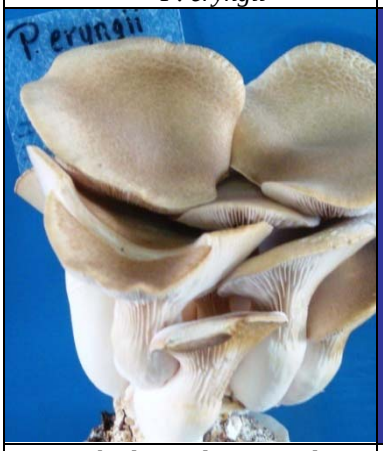
Hybrids from Matings of *P. eryngii* Neohaplonts with *L. edodes* Neohaplonts. Eleven hybrids obtained by mating *L. edodes* and *P. eryngii* neohaplonts were fruited on both types of substrates shown in Table 1 and biological efficiencies of the six hybrids presenting *L. edodes* morphology are shown in Table 2.

Table 2: Biological efficiencies of hybrids with *L. edodes* morphology

Strains	Biological efficiency (g fresh fruit bodies / 100 g dry substrate)							
	L. edodes substrate				*P. eryngii* substrate			
	$\bar{x}$	±	σ	Duncan test	$\bar{x}$	±	σ	Duncan test
L10	33.82	±	9.22	A				
PeC40 / L18-2S	50.63	±	16.25	B	44.17	±	8.04	a
L21	62.51	±	4.21	BC				
L18	67.67	±	7.53	C				
L9	84.13	±	7.84	D				
PeC40 / L18-1S	91.32	±	12.88	D	55.25	±	7.06	b
PeC40 / L10-1S	119.44	±	15.27	E	53.68	±	3.18	ab
PeC40/ L10-4S2	123.64	±	6.98	E	61.43	±	8.45	bc
PeC40 / L10-4S	123.64	±	6.98	E	53.68	±	3.18	ab
PeC40/ L21-2S	153.38	±	9.32	F	68.60	±	11.88	c

Different letters indicate significant differences in the same substrate

All the hybrids produced with *P. eryngii* neohaplont PEC40 produced fruit bodies with *Lentinula* morphology whereas hybrids derived from the other 5 neohaplonts belonging to mating type II paired with *L. edodes* neohaplont L21-3S resulted in fruit bodies with *P. eryngii* morphology. Biological efficiencies of hybrids producing fruit bodies with *Lentinula* morphology after 12 weeks of cropping period are shown on Table 2. Strain PeC40/ L21-2S produced the highest biological efficiency, 153%, though other 3 hybrids yielded high BE, 119-124%, in all cases better BE values than control *L. edodes* strains, whose BE ranged from 34 to 84%. Fig. 1 shows the fruit bodies produced by control *P. eryngii* dikaryon and two *L. edodes* strains as well as 4 different hybrids fruiting with *Lentinula* morphology.

Parental Strains		
P. eryngii	L10	L21
		
Fruit bodies: large. Pileus: brown grayish with regular edges. Stipe: white colored, long and thick	Fruit bodies: large. Pileus: dark brown, thin, slightly convex with scales on edges. Stipe: long and thin	Fruit bodies: medium. Pileus: dark brown, thick, without scales. Stipe: thin and very short

Hybrid Strains			
PeC40/L21-2S	PeC40/ L10-4S	PeC40/L10-4S2	PeC40/ L10-1S
			
Fruit bodies: medium, large and very large. Pileus: dark brown, thick, with few scales. Stipe: beige, long and medium sized, with scales	Fruit bodies: medium, large and very large. Pileus: dark brown, thick, with abundant scales. Stipe: beige with brown spots, long and thick, with scales	Fruit bodies: small, medium and large. Pileus: light brown, thick, with abundant scales. Stipe: thick medium sized, whitish with scales	Fruit bodies: small, medium, large and very large. Pileus: light brown, thick, with few scales. Stipe: light brown, long and thin, with few scales

Figure 1: Parental strains and highest producing hybrids with *L. edodes* morphologies

Biological efficiencies of the 5 hybrids producing fruit bodies with *P. eryngii* morphology are shown on Table 3. Hybrids cultivated on *P. eryngii* substrate had lower

biological efficiencies (13 to 67%) than the parental *P. eryngii* dikaryon (151%). Noticeably, 2 hybrids, PeC20/L21-3S and PeC29/L21-3S, produced higher yields on *P. eryngii* substrate and other 2 hybrids, PeC12/L21-3S and PeC45/L21-3S, conversely produced higher BE on *L. edodes* substrate, the last hybrid exceeding 100% BE. This suggests that nutrient requirements were differentially inherited by each group of strains, the first one similar to *P. eryngii* and the second one to *L. edodes.* The Figure 2 shows the fruit bodies produced by 3 different hybrids fruiting with *P. eryngii* morphology.

Table 3: Biological efficiencies of hybrids with *P. eryngii* morphology

Strains	Biological efficiency (g fresh fruit bodies / 100 g dry substrate)					
	P. eryngii substrate			*L. edodes* substrate		
	$\bar{x} \pm \sigma$		Duncan test	$\bar{x} \pm \sigma$		Duncan test
PeC20/L21-3S	43.61 ±	7.02	B	14.12 ±	1.94	a
PeC29/L21-3S	61.25 ±	8.63	CD	21.42 ±	5.94	ab
PeC12/L21-3S	13.16 ±	1.91	A	33.47 ±	3.61	b
PeC27/L21-3S	67.44 ±	10.96	D	56.38 ±	8.27	c
PeC45/L21-3S	56.13 ±	6.00	C	111.79 ±	17.55	d
P. eryngii	151.42 ±	11.49	E			

Different letters indicate significant differences in the same substrate.

PeC45/ L21-3S	PeC27/L21-3S	PeC12/L21-3S
		
Fruit bodies: large and very large. Pileus: light brown, thick, with undulated edges. Stipe: white, long and thick	Fruit bodies: small and medium. Pileus: white, with undulated edges. Stipe: white, medium, thin to thick	Fruit bodies: small, medium and large. Pileus: light brown, with regular edges. Stipe: white, medium, thin to thick

Figure 2: Hybrids between *P. eryngii* and *L. edodes* with *P. eryngii* morphologies on *L. edodes* substrate

Hybrids from Di-mon Matings of *P. eryngii* Neohaplonts with *Pleurotus* spp. Dikaryons. Mycelium growth rate of the 28 hybrids obtained by this procedure was measured to identify those showing similar growth to the parental strains, and those showing either faster or slower growth than the parental strains. For fruiting experiments, the following 19 hybrids were selected from these 3 groups:

hybrids with faster mycelium growth than parental strains:

PeC9/CP50 PeC20 / IE200 PeC38 / P401

PeC12 / P401 PeC35 / *P. djamor* PeC38 / ASP14
PeC12 / *P. djamor* PeC35 / HK3539 PeC44 / *P. djamor*
PeC20 / *P. djamor* PeC38 / *P. djamor* PeC44 / P401
hybrids with similar mycelium growth than parental strains
PeC11 /CP50 PeC35 /P401
PeC35/ *Pleurotus* sp. PB PeC44 / CP253
hybrids with slower mycelium growth than parental strains
PeC12 / CP253 PeC20 / ASP14 PeC20 / IE201

Biological efficiencies of the 19 hybrids produced by di-mon matings are shown in Table 4. Strain PeC11/CP50 showed up as the highest producing hybrid with an amazing 323% biological efficiency, however, 3 other hybrids produced also very high yields, *i.e.* PeC9/CP50 (271%), PeC12/P401 (207%) and PeC35/*Pleurotus* sp. PB (177%), in all cases, BE were significantly higher than BE of the parental *P. eryngii* dikaryon (151%). Remarkably, 12 more hybrids produced biological efficiencies higher than 100%; among them, 3 hybrids derived from *P. djamor* dikaryon with neohaplonts PeC12, PeC20 and PeC35 had significant higher biological efficiencies than the parental *P. djamor* dikaryon.

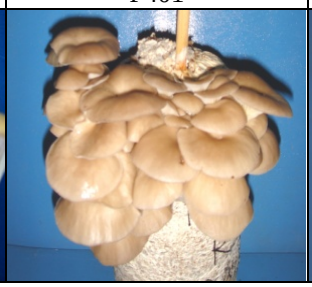
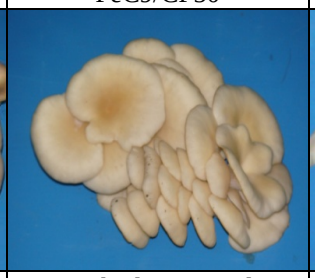
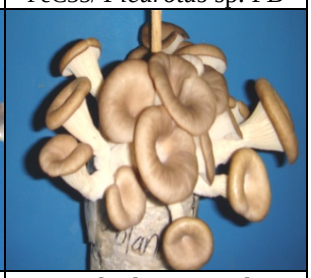
Parental Strains			
P. eryngii	CP50	P401	*Pleurotus* sp. PB
			
Hybrid Strains			
PeC11/ CP50	PeC9/CP50	PeC12/P401	PeC35/ *Pleurotus* sp. PB
			
Fruit bodies: small, medium and large. Pileus: light brown, with regular edges. Stipe: white, medium, thin, trumpet shaped	Fruit bodies: medium and large. Pileus: beige with regular edges. Stipe: white, medium, thin, trumpet shaped	Fruit bodies: large. Pileus: light brown with undulated edges. Stipe: white, medium, long and thin	Fruit bodies: medium and large. Pileus: light brown with regular edges. Stipe: white, medium, long and thin to thick, trumpet shaped

Figure 3: Parental strains and highest producing hybrids from di-mon matings with *Pleurotus* morphologies

Results from Table 4 also allow the identification of PeC35 as a neohaplont generating highly producing dikaryons when mated with different dikaryotic partners. These results also establish that most hybrids are higher yielding than the respective parental dikaryotic strains

employed for di-mon matings, *i.e.* IE201, CP253, IE200, CP50 and P401. Morphologies of fruit bodies produced by these hybrids are very interesting since wide varying characteristics were found among them, *i.e.* medium sized and long stipes of variable thickness and pileus of diverse color, shape and texture. Fruit bodies from 3 different hybrids are shown in Figure 3.

Table 4: Biological efficiencies of hybrids from di-mon matings of *P. eryngii* neohaplonts with *Pleurotus* spp. dikaryons

Strain	Biological efficiency (g fresh fruit bodies / 100 g dry substrate) $\bar{x}$ ± σ			Duncan test
IE201	26.58	±	3.98	a
CP253	35.05	±	2.98	a
IE200	40.67	±	8.73	a
CP50	43.47	±	9.74	a
PeC20/IE201	75.91	±	11.82	b
PeC44/*P. djamor*	78.27	±	3.84	b
Pleurotus sp. PB	78.66	±	17.56	b
P401	85.70	±	8.86	bc
P. djamor	92.11	±	8.75	bcd
PeC38/ *P. djamor*	99.54	±	2.15	bcde
PeC12/CP253	104.90	±	14.50	cdef
PeC44/P401	107.64	±	3.64	cdef
PeC35/P401	108.03	±	3.95	cdef
PeC20/IE200	108.44	±	12.80	cdef
PeC44/CP253	111.38	±	11.22	cdef
PeC20/ASP14	113.27	±	14.01	def
PeC38/ASP14	114.54	±	2.49	def
PeC38/P401	114.85	±	5.80	def
PeC12/*P. djamor*	119.83	±	21.84	ef
PeC20/*P. djamor*	122.87	±	10.52	ef
PeC35/*P. djamor*	127.86	±	9.81	f
P. eryngii	151.42	±	11.49	g
PeC35/HK3539	156.40	±	6.67	gh
PeC35/*Pleurotus* sp. PB	177.20	±	42.92	h
PeC12/P401	207.02	±	34.98	i
PeC9/CP50	271.39	±	43.84	j
PeC11/CP50	323.02	±	29.46	k

Different letters indicate significant differences.

DISCUSSION

Development of improved strains for commercial cultivation of edible fungi has been undertaken seriously by the bottom mushroom industry; *Agaricus* spawn producers perform this task continuously investing important resources. However, other cultivated fungi have not received this attention and thus offering strains with new characteristics for the industry has not occurred. This task is not such complicated as for *Agaricus* at least with fungi like *Pleurotus* spp. and *L.edodes*, both with heterothallic tetrapolar sexuality. For such fungi, dedikaryorization has been proposed as an effective procedure for recovery of the monokaryotic components of selected dikaryons making feasible a directed improvement of fungal strains by combination of monokaryotic cultures containing desirable characteristics [5].

In previous works, hybrids from 2 different genera, *i.e. P. ostreatus* and *L. edodes* have been produced by pairing neohaplonts from these 2 fungi [3, 6]. Hybrids yielding higher

biological efficiencies than the parental strains were then reported in accordance to the results presented in the present study where *P. eryngii* strains were used for the first time. Moreover, hybrids were now obtained yielding fruit bodies either with *Lentinula* or *P. eryngii* morphology contrasting to the previous report by Ramírez *et al* [3] where only hybrids producing fruit bodies with *P. ostreatus* morphology were recovered. Thus, in this study, hybrids with *Lentinula* morphology were obtained for the first time; 5 of these 6 hybrids showed higher BE than the parental *L. edodes* strains and 3 of them produced BE in the range of 119-153% (Table 2), becoming suitable for commercial cultivations. Furthermore, such strains exhibited interesting morphologies (Fig. 1), *i.e.* firmer pileus with fleshy texture as well as larger and thicker stipes. These newly acquired characters were possibly inherited from *P eryngii* whereas the higher yields on *Lentinula* substrate were probably received from parental *L. edodes* strains. On the other hand, although all hybrids with *P. eryngii* morphology showed lower yields compared to their parental *P. eryngii* dikaryon, hybrid PeC45/L21-3S remarkably produced higher BE on *Lentinula* substrate (111%) than on *P. eryngii* substrate, showing up also as an attractive strain for commercial cultivation and this fact again suggests that differential inheritance of nutrient requirements is present in these hybrids. Productivity of hybrids was also markedly increased in those obtained by di-mon matings of *P. eryngii* neohaplonts with *Pleurotus* spp. dikaryons (Table 4). Biological efficiency of 3 hybrids ranged from 323 to 200% while *P. eryngii* yielded 151% BE and 13 more hybrids produced BE higher than 100%.

Strikingly, a large diversity of morphologies are produced when different neohaplonts, derived from the same parental strain are paired with different partners as shown on Fig. 1, 2, 3, in some cases it was observed even in pairing with neohaplonts arising from the same parental strain, *i.e. P. eryngii* neohaplont PEC40 (Table 2). Furthermore, strains consistently showed variations in pileus (size, colors and shapes), and stipe (length, thickness and texture). Even though, this may be a desirable achievement in this study, such phenotypic variations should not be expected since all neohaplonts are supposedly carrying the same genetic information. However, this may be true in regards to the mating-type genes but obviously it is not true regarding the genetic information related to fruit body formation. This observation suggests that during dedikaryorization, some factors involved in the process of fruit body formation are separated into the neohaplonts in an irregular pattern, dissimilar as mating types do, thus arising a large variation in phenotypes. Such an observation is supported by the presence among neohaplonts of strains with abnormal mating types, *i.e.* unable to mate at all or with irregular mating pattern, varying mycelium morphologies and sometimes, the incapability of recovered compatible neohaplonts to reconstruct the original parental strain, when mated.

Finally, hybrids obtained in this study by mating neohaplonts consistently resulted in strains producing higher yields, regularly yielding more than 100% BE, in comparison with the traditional approach of strain improvement by matings of meiotic progenies. Following such an approach, Valencia del Toro and Leal Lara [7] obtained improved *Pleurotus* strains yielding 40-73% BE, while Galván [8] obtained improved *Lentinula* strains yielding 13-73% BE. Meiotic products show a large variation in genotype, making more difficult to find out combinations of monospore cultures giving higher yields. Such a variation was observed by Gharehaghaji et al. [9]; they germinated basidiospores from 5 *P. ostreatus* dikaryons recovering 17 monokaryons, which were paired to produce 27 hybrids. However, only primary mating characters were observed, *i.e.* morphological interaction in contact zones of mycelium, increased growth rate, change of colony morphology and presence of clamp connections to identify dikaryons, but no fruiting experiments were performed to evaluate productivity of the generated hybrids, a crucial step for assessing success of any breeding program since optimization of industrial mushroom production depends on improving the culture process and breeding new strains with higher yields and productivities.

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VALIDITY OF MYCELIAL GROWTH ON MALT EXTRACT AGAR AND COMPOST AS SELECTION CRITERIA FOR INITIAL SCREENING OF GENOTYPES FOR YIELD AND QUALITY IN *AGARICUS BISPORUS*

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ABSTRACT

Sixty-three fertile single spore isolates (SSIs) representing five strains were screened for radial growth on Malt Extract Agar (MEA) medium, downward linear growth on compost and yield. The morphological traits like size of gill, pileus and stipe were also recorded in the above isolates. There was no significant correlation of yield with radial growth on MEA (r= -0.124) suggesting that the growth on MEA in petriplates cannot be an indicator of yield performance of the genotype. In another study on seventeen isolates conducted earlier, no significant association of yield was recorded with radial growth on malt, Coon's and yeast potato dextrose agar. The downward linear growth on compost, however, showed highly significant relationship with yield (r= 0.771; y = 0.33x –10.01; R^2 = 0.59) suggesting that mycelial growth on compost can be used to predict the yield potential and at least can be used to reject the isolates. On the other hand, radial growth on MEA showed significantly negative interrelationship with gill (r= - 0.381) and stipe size (r= -0.310).

Keywords: *Agaricus bisporus*, single spore isolates, inter-relationship, path analysis

INTRODUCTION

Conventional breeding in *Agaricus bisporus* is time consuming due to secondary homothallic nature of the species, absence of clamp connections in mono and dikaryons, and lack of well-defined morphological differences in fertile and non-fertile cultures. One of the simplest techniques for breeding of the strain is to isolate and evaluate single spore isolates. This gains importance while screening SSIs obtained from a hybrid. The frequency of high yielding isolates is low and it is practically impossible to screen all the single spore isolates for their yield performance. It is thus important to look for selection criterion at early stages. Non-significant differences between mycelial growth on media and yield have been reported earlier [1, 2] implying that growth on agar media cannot be an indicator of yield potential. In the present study 63 isolates have been evaluated for their growth on malt extract agar, downward linear growth on sterilized compost and yield, and their inter-relationships have been studied to identify selection criterion for yield and quality of mushrooms.

MATERIAL AND METHODS

Spore print of five strains viz. A-4, A-6, A-94, S-11 and U-3 were taken and in total 129 single spores were isolated. Sixty-three of these, used for the present study, were cultured on Malt Extract Agar (MEA) and their spawn was prepared on wheat grains following standard practices. 20g pasteurized compost was filled in 25 (dia) x 180 mm sized test tubes and sterilized at 22 p.s.i for 1h. About five

gram spawn was overlaid in each tube and incubated in a BOD incubator at 25±1°C in vertical position for study of downward linear growth. Growth was measured on four sides after 20 days of each tube and averaged. Each isolate was replicated thrice for statistical analysis. The radial growth on malt extract agar medium was measured along both horizontal and vertical axis in mm. The yield was evaluated in bags (10 kg pasteurized compost per bag prepared using wheat straw and chicken manure). Three replication of each isolate were maintained. Yield data was taken up to four weeks of cropping. The morphological data i.e. stipe length, pileus diameter, gill size, firmness were recorded on 10 randomly selected mushrooms from each isolate. The correlation coefficients and path analysis of radial growth on MEA, downward linear growth, morphological traits and yield was done using standard methods.

RESULTS AND DISCUSSION

The average, standard deviation and coefficient of variation in 63 isolates for different traits viz., radial growth on malt extract agar, downward linear growth, morphological traits (figure 1) and yield are presented in table 1. The highest variation was observed in radial growth on malt extract agar whereas least variation was observed for pileus size.

Table 1: Mean, standard deviation and coefficient of variation of different traits of 63 single spore isolates from five strains

Mean	SD	CV	
Downward linear growth (mm)	68.899	10.293	14.939
Linear growth on MEA (mm)	16.67	7.43	44.575
Gill size (mm)	20.44	3.50	17.135
Pileus size (mm)	35.40	1.46	4.115
Stipe size (mm)	20.23	3.17	15.671
Yield (kg/100 kg compost)	12.807	4.421	34.516

Figure 1. Variation in morphological traits of single spore isolates

The correlation coefficients among traits viz. yield, downward linear growth, gill size and stipe size were found to be positive and significant/highly significant in all combinations. The radial growth on malt extract agar had significant negative association with gill size and stipe size and negative, though non-significant, with yield.

Table 2: Correlation matrix of different traits of 63 single spore isolates from five strains

	Downward linear growth	**Linear growth on MEA**	**Gill Size**	**Pileus Size**	**Stipe Size**	**Yield**
Downward linear growth	1.000	-0.097	0.301*	-0.082	0.424**	0.771**
Linear growth on MEA		1.000	-0.381**	-0.010	-0.310*	-0.124
Gill size			1.000	-0.161	0.672**	0.467**
Pileus size				1.000	-0.212	-0.073
Stipe size					1.000	0.618**
Yield						1.000

* p< 0.05, ** p<0.01

On partitioning these correlation coefficients using path analysis (Table.3), it can be seen that downward linear growth has a dominant direct effect on yield along with small indirect effect via stipe length. The direct effect of stipe length is only 0.329 and the high correlation coefficient of 0.618 included indirect effect of 0.26 via downward linear growth. The radial growth on MEA has almost zero direct effect on yield and the little non-significant correlation value of -0.124 was due to indirect effect via stipe size.

Table 3: Direct and indirect effects of correlation of different parameters with yield

	Downward linear growth	**Linear growth on MEA**	**Gill Size**	**Pileus Size**	**Stipe Size**	**Corr Coef with yield**
Downward linear growth	**0.616**	-0.008	0.030	-0.005	0.138	0.771
Linear growth on MEA	-0.060	**0.078**	-0.039	-0.001	-0.102	-0.124
Gill Size	0.184	-0.030	**0.102**	-0.010	0.221	0.467
Pileus Size	-0.050	-0.001	-0.015	**0.062**	-0.069	0.467
Stipe Size	0.260	-0.025	0.067	-0.013	**0.329**	0.618

From the correlation coefficients apparently there seems to be highly significant negative association between stipe size and growth on malt extract agar. However, on partitioning, it can be seen that direct effect of radial growth on malt extract agar on stipe size is only -0.104 against the correlation value -0.310 (Table.4).

Table 4: Path analysis for stipe vs other traits

	Downward linear growth	**Linear growth on MEA**	**Gill Size**	**Pileus Size**	**Yield**	**Correlation with stipe**
Downward linear growth	**-0.073**	0.010	0.126	0.010	0.350	0.424
Linear growth on MEA	0.007	**-0.104**	-0.160	0.001	-0.055	-0.310
Gill Size	-0.022	0.039	**0.421**	0.019	0.214	0.671
Pileus Size	0.006	0.001	-0.067	**-0.120**	-0.032	-0.212
Yield	-0.056	0.012	0.198	0.008	**0.455**	0.618

In earlier studies involving former author, it has been observed that there is no significant association of radial mycelial growth on different media (like Malt Agar, Coon's Agar, Compost Agar and Yeast Potato Dextrose Agar) and the yield (Table.5). However, downward mycelial growth had significant positive association. These results were based only on small sample of 17 SSIs of a single strain and were thus only indicative. The present study based upon sixty-three isolates from five strains has verified the earlier results as there is no association of yield with radial growth on media and the correlation coefficient between downward linear growth and yield is highly significant and positive.

Table 5: Correlation coefficients of mycelial growth of 17 single spore isolates of strain S-11 on different media, their downward growth on autoclaved compost and their yield

	Malt agar	**Coon's agar**	**Compost agar**	**YPDA**	**Downward linear growth**	**Yield**
Malt agar	1.000	-0.012	0.368	0.375	-0.152	-0.238
Coon's agar		1.000	0.403	-0.044	0.399	0.110
Compost agar			1.000	0.358	0.207	0.357
YPDA				1.000	-0.317	-0.278
Downward linear growth					1.000	0.713**
Yield						1.000

** $p<0.01$

Analysis of another data of growth rate on media, pileus-stipe-weight-ratio, laccase activity of 21 single spore isolates obtained from a single strain also showed no significant association among these traits (Table-6). Hence, from repeated trials on radial growth on media and yield, it can be inferred that mycelial growth rates on agar are not indicator of yield potential of a culture and thus cannot serve as a selection criteria.

Table 6: Correlation coefficients of Growth rate, Pileus/Stipe weight ratio, Laccase activity and Yield

	Growth rate	**P/S weight ratio**	**Laccase activity**	**Yield**
Growth Rate	1.000	-0.055	-0.398	-0.175
P/S weight ratio		1.000	0.234	-0.159
Laccase activity			1.000	0.148
Yield				1.000

On the other hand, the earlier as well as present studies support that downward mycelial growth on sterilized compost can serve as selection criteria while handling a large number of SSIs. Based on the significant association between downward linear growth and yield, the yield can be predicted (R^2 = 0.595) with fair accuracy using the regression equation given in figure 2.

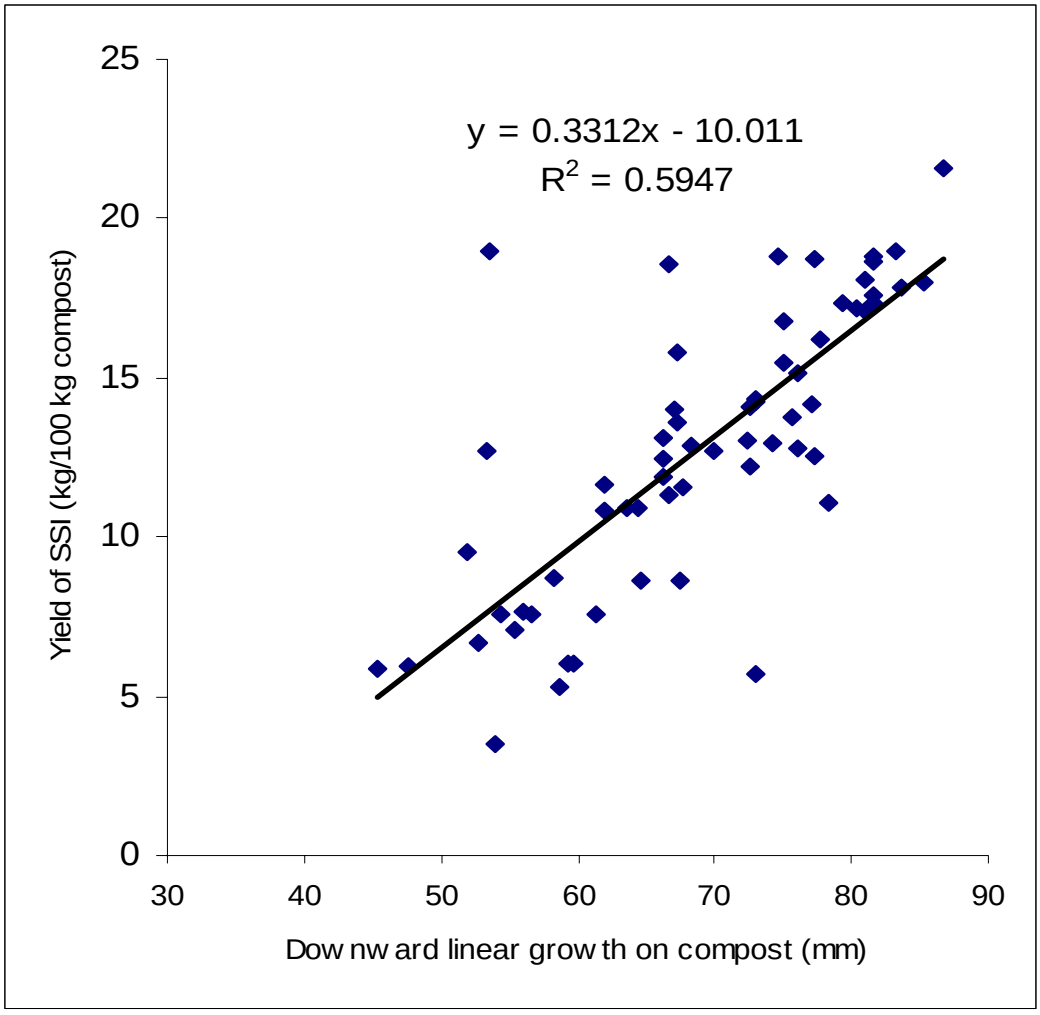

Figure 2. Regression equation and R^2 value of downward linear growth and yield of 63 SSIs from five strains of button mushroom

CONCLUSIONS

The criteria of downward linear growth can be used to reject 80% or more single spore isolates thereby reducing the total work load for evaluation of SSIs for their yield performance. The growth on malt extract agar though apparently appears to have negative association with stipe length and gill size but partitioning this correlation into direct and indirect effects indicates that the association may not be precisely valid as selection criteria for traits like gill size and stipe length. Even though, association of quality traits like gill size and stipe length with radial growth on MEA is significant, but the degree of association is relatively low and path analysis also shows low direct effect of growth on MEA on stipe size. Hence, detailed studies are required to develop selection criteria for quality traits.

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DIVERSITY AND POPULATION BIOLOGY OF WILD MUSHROOMS FROM SOUTHWESTERN CHINA

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ABSTRACT

Wild mushrooms have been a part of the human diet and an important source of essential nutrients and medicine for centuries. While some wild edible mushrooms are artificially cultivable in human-made environments, the majority has resisted human domestication attempts. Southwestern China is a hotbed for wild edible mushrooms. Unfortunately, with increasing consumer demands and changes in environmental conditions, the genetic resources of many indigenous wild mushrooms are diminishing, with some populations on the brink of extinction. We have recently started a large-scale investigation of the diversity and population biology of wild edible mushrooms in this region. Our results indicate extensive diversity and reveal evidence of cryptic speciation, genetic differentiation and geographic structuring. In this paper, we present some preliminary results from our recent surveys.

Keywords: Wild edible mushroom; biodiversity; cryptic speciation; gene flow

INTRODUCTION

Fungi are among the most specious organisms on Earth. Morphologically, they are extremely diverse, from unicellular yeasts, to filamentous hyphal forms and a huge variety of fruiting body structures. They play significant roles in human health, forestry, agriculture, industry, food and the environment. They are an integral component of nutrient and elemental cycling of the global ecosystem. In natural environments, many fungi grow conspicuous fruiting bodies for reproduction and dispersal. Some of these fruiting bodies (mushrooms) have been collected for food and food supplements for hundreds to thousands of years. Over the millennia, humans have developed special preferences towards many of these wild mushrooms, some as expensive delicacies and others as potent medicine. Though different societies showed different preferences, strong relationships between people and wild mushrooms are found across all human racial, ethnical and geographic populations. For example, southern Europeans from France and Italy have a distinctive preference for truffles; the Japanese for the matsutake mushrooms, and those from southwestern China for ganbajun. However, rapid globalization in recent years has brought people from very different backgrounds into close contact with each other, enhancing the exchange and sharing of material goods, cultures, foods and medicine. Consequently, regional wild edible mushrooms are becoming global commodities and regional

resources are susceptible to global demands and exploitations. Such changes are leading to increasing problems for the management of these wild mushrooms, with potentially far-reaching implications for the survival of such resources and for the livelihood of the indigenous people who depend on these resources.

In this presentation and paper, we will describe our recent and current efforts in trying to understand the genetic resources of wild gourmet mushrooms from southwestern China. This region is geographically and ecologically extremely diverse and is one of the world's 34 biodiversity hotspots. For example, over 600 out of 2,000 edible fungal species worldwide occur in this region [1]. Some of the economically important mushrooms from this region include *Thelephora ganbajun* M. Zang, *Tricholoma matsutake* (S. Ito & S. Imai) Singer, and *Russula* spp. Our preliminary analyses of these wild mushrooms identified high and novel phylogenetic and population genetic diversities. In the following sections, we highlight some of our findings and discuss the implications of our results for conservation and management of these genetic resources.

MATERIALS AND METHODS

Since 2006, we have been collecting wild edible mushrooms from forests and farmers' markets in many parts of southwestern China, with a focus on Yunnan Province. Specimens were recorded, dried, brought into the lab, and preserved for taxonomic, systematic, population genetic, and molecular ecological studies. Our collection efforts were primarily on mushrooms with relatively high consumption levels, high economic values, and/or broad geographic distributions.

To study the phylogenetic diversity of wild edible mushrooms in southwestern China, we rely almost completely on DNA sequence information from the collected specimens. To obtain those DNA sequences, we first extracted their DNA and then amplified the inter-transcribed spacer (ITS) regions of the ribosomal RNA gene cluster using the fungal universal primers. The DNA sequences were then compared with each other and with those in databases.

To study the genetic variation among strains and populations from different geographic and/or ecological areas within individual species, whenever possible, species-specific and/or single gene markers were used for genotyping strains. The data were then analyzed for ecological and population genetic patterns using various computer software programs that incorporate ecological, geographical, and other relevant information [2].

RESULTS AND DISCUSSION

Below we summarize our preliminary understanding of the ecology, diversity, and population biology of three selected wild edible mushrooms. When possible, our data are compared with those from other geographic areas and or closely related species.

Ganbajun: diversity, geographic structuring, cryptic speciation. *Thelephora ganbajun* is a gourmet mushroom that forms ectomycorrhizae with pine trees, predominantly *Pinus yunnanensis* endemic to Yunnan Province in southwestern China [3,4]. The mature mushroom produces a unique and attractive aroma and is well liked by locals. In Yunnan, freshly collected mature fruiting bodies of this mushroom are priced similarly to or higher than most other wild mushrooms. Because of consumer demand and our inability to cultivate this mushroom

artificially, there has been noticeable overexploitation and consequent decline of local populations of this species in many areas in Yunnan. However, despite its economic and potential ecological importance, very little is known about its ecology and genetics. In a recent study, DNA sequence variation among strains and populations were analyzed within the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene cluster [5]. The ITS regions were chosen here because there are universal fungal PCR primers for this region that could be used for amplification and sequencing [6]. In addition, the ITS is among the most commonly used gene regions for analyzing relationships among strains within as well as between closely related fungal species [6-8], and it's the recommended DNA fragment for fungal barcoding. Furthermore, no gene sequence information (not even ITS) about this organism was available before the study, thus no primers were known to exist that could allow us to amplify specific gene sequences from this organism. In total, the ITS sequences were obtained from 156 fruiting bodies obtained from 23 sites in nine regions of Yunnan province (Table 1). These regions covered about 600 km from east to west and over 300 km from south to north.

Table 1. ITS sequence types and their distribution among geographic populations of Ganbajun from Yunnan, southwestern China (modified from Sha et al. [5])

Region/ District	County/ Community	Sample size	ITS sequence type (# of isolates in each type)[1]	ITS diversity
Baoshan	Changning	4	1(2); 2(1); 3(1)	0.625
	Baoshan	9	1(8); 3(1)	0.198
Chuxiong	Chuxiong	8	1(4); 7(1); (22(1); 24(1)	0.688
	Nanhua	10	1(3); 7(3); 13(1); 17(1); 20(1); 23(1)	0.780
	Wuding	8	1(2); 7(4); 28(1); 31(1)	0.656
	Lufeng	6	1(4); 21(1); 31(1)	0.500
Dali	Midu	5	1(2); 25(2); 31(1)	0.640
	Wenxian	4	1(4)	0.000
	Dizi	4	2(1); 7(2); 31(1)	0.625
	Fengyi	5	1(2); 13(1); 7(1); 26(1)	0.720
Honghe	Shiping	4	1(1); 5(1); 6(1); 7(1)	0.750
Kunming	Jingning	1	1(1)	N/A
	Anning	1	1(1)	N/A
	Yiliang	5	1(1); 8(1); 9(1); 10(1); 11(1)	0.800
	Songming	9	1(6); 15(1); 25(1); 31(1)	0.519
	Luquan	6	1(2); 7(2); 20(1); 25(1)	0.722
	Xundian	9	1(2); 12(1); 14(3); 16(1); 25(2)	0.765
Pu'er	Pu'er	20	8(13); 18(1); 19(2); 34(4)	0.525
Qujing	Malong	2	1(1); 2(1)	0.500
Wenshan	Guangman	4	1(2); 4(1); 7(1)	0.625
Yuxi	Tonghai	4	1(3); 7(1)	0.375
	E'shan	11	1(5); 2(4); 25(1); 32(1)	0.645
	Yimeng	17	1(12); 25(1); 27(1); 30(1); 31(1); 33(1)	0.484

[1,] The ITS sequence types correspond to those in Figure 1.

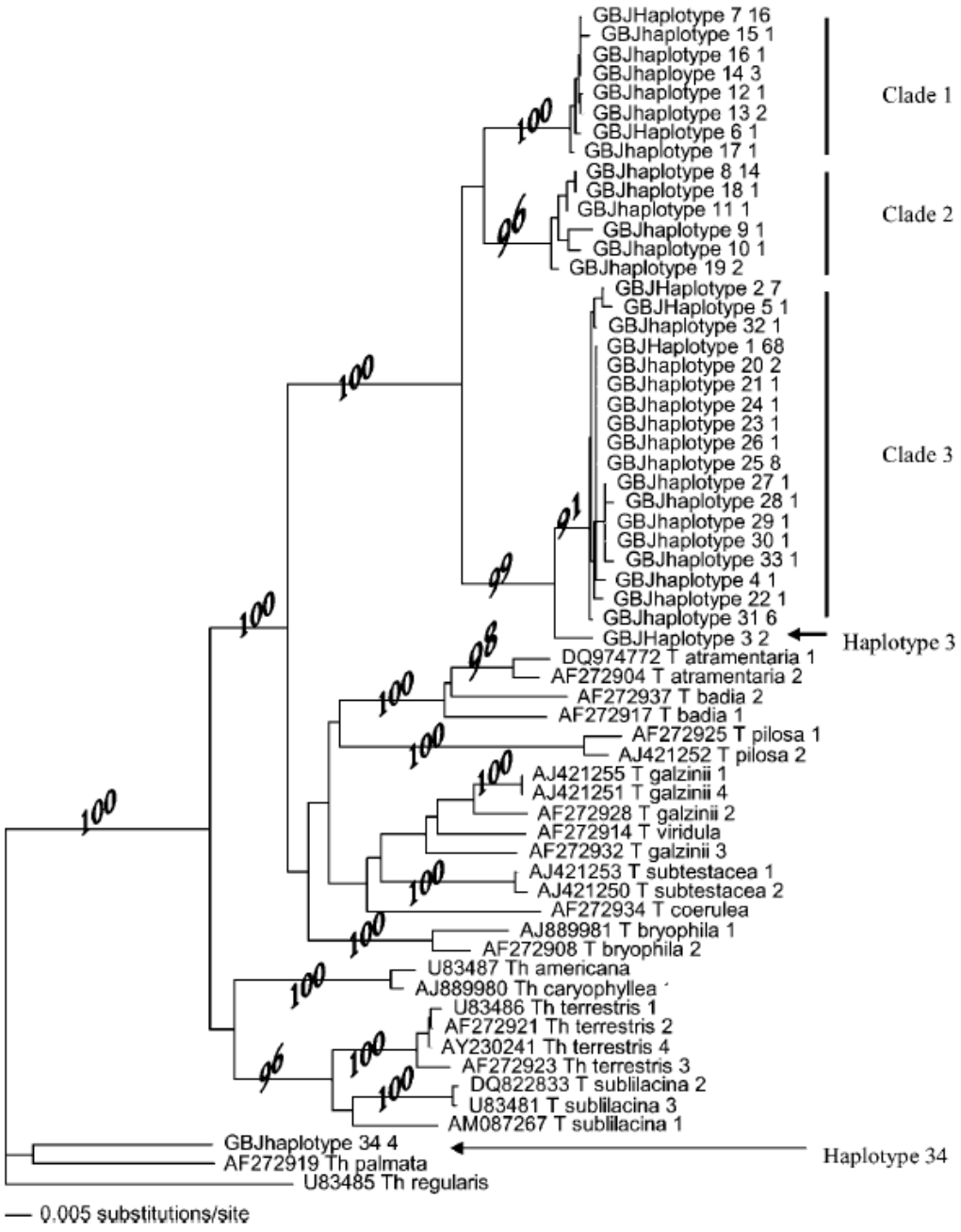

Figure 1. Phylogenetic relationships among ITS sequences of 34 haplotypes of *Thelephora ganbajun* and representative sequences from closely related species of two genera *Tomentella* and *Thelephora*.

For each ITS haplotype of Gan-Ba-Jun (GBJ), the first number represents the haplotype assignment corresponding to those in Table 1; the second number represents the total number of strains belonging to the specific haplotype. The 27 reference strains are each represented by its GenBank accession number, the genus abbreviation (T for *Tomentella* and Th for *Thelephora*), the species name and when multiple strains from the same species were available, the strain code (1, 2, 3, or 4). Numbers across branches are bootstrap values greater than 90% obtained from 1000 replicates. Gaps were treated as missing data. Branch lengths are proportional to the amount of sequence divergence. Tree length = 670; consistency index = 0.610; retention index = 0.882. [Modified from ref. 5]

The 156 aligned sequences were 671 nucleotides long and contained 138 variable sites. Each of the variable sites contained only two alleles. These variable sites included 35 insertions/deletions, 85 transitional substitutions, and 18 transversional substitutions. Among these variable sites, 130 were phylogenetically informative and the remaining 8 sites were phylogenetically uninformative. The analyses of the aligned ITS sequences of all 156 specimens identified 34 unique sequence types (Figure 1). The distributions of these ITS sequence types among the 23 local populations are presented in Table 1. Among the 34 ITS sequence types, 22 were represented by one specimen each and the remaining 12 were each shared by two or more specimens. The most common type, haplotype 1, contained 68 specimens that were distributed in 21 of the 23 sites (Table 1). Similarly, haplotype 7 was also widely distributed – it contained 16 specimens collected from eight of the 23 local populations (Table 1). However, the other major shared halpotype 8 was found in only two regions. The number of ITS haplotypes for each local population ranged between one and seven. Aside from the two local populations where only one specimen each was available for analysis, 20 of the remaining 21 local populations had more than one ITS genotype. Of these 21 local populations, 13 were found to contain unique ITS haplotypes not found in other local populations.

Based on BLAST searches against the GenBank database, 27 representative ITS sequences with species identifications and with high levels of sequence identity (>90 %) to our sequences were included for comparative analyses. These sequences were found belonging to either *Thelephora* or *Tomentella* genera. These sequences were retrieved from the GenBank based on: (i) their high sequence identity to our samples, (ii) their comparable sequence lengths to ours, (iii) their representative phylogenetic positions, and (iv) in several cases, the availability of two or more strains for the same species. The selections of multiple strains from the same species were to compare the potential divergence within other species to that within *Th. ganbajun*. These 27 ITS sequences from GenBank represented 14 species total, nine in *Tomentella* and five in *Thelephora*. The analyses of our sequences and the 27 representative GenBank sequences revealed five phylogenetically distinct clades for the ganbajun samples from Yunnan (Figure 1). Clade 1 contained eight ITS haplotypes represented by 22 strains; clade 2 contained six haplotypes represented by 20 strains; clade 3 contained 18 haplotypes represented by 106 strains; clade 4 contained one haplotype (haplotype 3) represented by two strains; and clade 5 contained one haplotype (haplotype 34) represented by four strains. Our results showed that strains in clades 1-4 had closer evolutionary relationships to representative species from the genus *Tomentella* than to those from *Thelephora*. In contrast, clade 5 was more similar to ITS sequences of in *Thelephora*, with the closest relative in *Th. palmata* and *Th. regularis*.

Taken together, the ITS sequence analyses suggest that the wild collected gourmet mushroom ganbajun in Yunnan are highly heterogeneous, belonging to two different genera *Thelephora* (the minority of our isolates) and *Tomentella* (the majority of our isolates) and contained at least five divergent evolutionary lineages. These lineages showed sequence divergences from each other similar to or greater than those between several known sister species pairs in these two genera. In addition, there are significant differences in the distribution of the sequence types and lineages. While a couple of the ITS sequence types were geographically broadly distributed, the majority of the sequence types were unique to specific local populations. The lack of significant gene flow among local populations suggests that targeted efforts should be made to preserve the significant genetic diversity within individual populations of ganbajun.

The "Big Red Mushroom": diversity, cryptic speciation, and population genetics. The genus *Russula* contains a highly diverse group of ectomycorrhizal fungi that includes over 700 reported species [9]. In natural forests, this genus contributes a significant amount of the ectomycorrhizal biomass with a broad distribution ranging from the tropics to subtropics, temperate regions, and even arctic zones [10]. Except a few species such as *R. emetica* and *R. subnigricans* that are not edible, many species in this genus are enjoyed by humans and mushrooms in this genus are among the most commonly found along roadside markets in southwestern China. While this genus contains species with very diverse morphological features, distinguishing closely related species is often very difficult due to the large number of species, extensive phenotypic plasticity among strains within individual species, and the lack of macro-morphological features to separate them. Consequently, molecular information has been increasingly used to help define and identify species boundaries, especially between morphologically ambiguous species pairs. However, most such studies have focused on European and North American samples [e.g. 11]. The diversity and genetic structure of *Russula* species in other parts of the world, including southwestern China, remain poorly understood.

Among the *Russula* mushrooms collected and traded in southern China, one called 'Dahongjun' [literary means ''Big Red or Bright Red Mushroom''] is probably the most prominent. This mushroom has been harvested and traded in local, national and international markets for over 20 years. Like many other pricey gourmet wild mushrooms such as matsutake, ganbajun and chanterelles, *Russula* cannot be artificially cultivated because of their dependency on a living plant host. Therefore natural populations in the forests are the only sources for the consumer market. Similar to ganbajun discussed above and the matsutakes discussed below, the significant profits, uncontrolled harvesting practices in recent years, human disturbances of the forest ecosystem, and loss of habitats are threatening the wild populations of Dahongjun in the regions.

Historically, mushroom enthusiasts and mycologists have regarded Dahongjun in southern China as *R. vinosa* Lindblad, which was originally described in Europe [12]. Recently, a population of Dahongjun from southern Yunnan was found to have ITS sequences very different from those of the typical *R. vinosa* from Europe, and this group was described as a new species *R. griseocarnosa* X. H. Wang et al. [13].

We recently investigated the diversity of Dahongjun from southern China [14]. A total of 122 samples were collected from five local populations representing the known distribution ranges of this mushroom in southern China (Table 2). We analyzed the genetic diversity and geographic structure of this mushroom using sequences from four DNA fragments: the ITS, the nuclear large subunit of the ribosomal RNA gene (nucLSU rRNA), the mitochondrial small subunit of the ribosomal RNA gene (mtSSU rRNA), and the second largest subunit of the nuclear RNA polymerase enzyme II (RPB2).

Among the 683 aligned nucleotide sites for the ITS regions, 82 were variable with 54 base substitutions and 28 insertion/deletions. All the indels were found between strains and none was found within any of the 122 strains. Our analyses of these ITS sequences suggested that our samples contained at least three phylogenetically distinct lineages (Figure 2). Lineage 1 contained 85 strains from the Ailaoshan (AL) region in central Yunnan and 5 strains from Cangwu (CW) in eastern Guangxi (GX). This lineage included a total of 42 ITS genotypes with 37 from AL and 5 from CW. Lineage 2 contained 17 ITS sequence types and 27 isolates, including 9 strains from Mengla (ML), 8 from Jinuo (JN), 8 from Dadugang (DDG), and 2 from CW. Lineage 3 contained 3 strains from DDG and 2 strains from CW. The ITS genotype

distribution within and among the 5 local populations are presented in Table 2. The analyses of sequences from the three other additional DNA fragments confirmed the existence of three divergent lineages within our Dahongjun samples.

To analyze the relationships between our samples and those of other closely related *Russula* spp., the sequences representing our 63 ITS genotypes identified above were used as queries to retrieve similar sequences in GenBank through BLAST searches. We retrieved 17 sequences with a comparable length to ours and that showed an overall sequence identity ≥90% to our Dahongjun sequences. These included three ITS sequences of *R. griseocarnosa* recently identified based on three specimens of Dahongjun in southern Yunnan [13] and 12 ITS sequences representing 5 closely related species in *Russula* (*R. vinosa, R. claroflava, R. occidentalis, R. decolorans, R. xerampelina*; Figure 2).

Table 2: Geographic distribution and ITS sequence diversity of Dahongjun samples collected from Yunnan and Guangxi provinces in southern China (Modified from Li et al. 2010).

Region/ District	County/ Community	Sample size	ITS sequence type[1] (# isolates in each type)	ITS diversity[2]
Central Yunnan	Ailaoshan (AL)	85	1(2); 2(4); 3(1) 4(1); 5(1); 6(1) 7(2); 8(1); 9(1) 10(1); 11(2); 12(1) 13(3); 14(1); 15(9) 16(1); 17(1); 18(1) 19(1); 20(16); 21(2) 22(1); 23(1); 24(1); 25(5); 26(1); 27 (4); 28(5); 29(1); 30 (2); 31(1); 32(2); 33(1); 34(4); 35(1); 36(1); 37(1)	0.945
Southern Yunnan	Mengla (ML)	9	38(1); 39(1); 41(1); 42(1); 43(1); 44(1); 45(1); 46(1); 54(1);	1.000
	Jinuo (JN)	8	39(1); 43(1); 49(1); 50(1); 47(2); 48(1); 51(1)	0.964
	Dadugang (DDG)	11	38(2); 40(2); 49(1); 50(2); 51(1); 55 (1); 56(1); 57(1);	0.945
Eastern Guangxi	Cangwu (CW)	9	58(2); 53(1); 52(1); 59(1); 60(1); 61(1); 62(1); 63(1)	0.972

[1,] The ITS genotype code correspond to those in Figure 2.

[2,] Genotypic diversity is defined as the probability that two individuals taken at random have different genotypes. It's calculated as $(1-\sum p_i^2)\bullet n/(n-1)$, where p_i is the frequency of the *i*th genotype and n is the number of individuals in the sample.

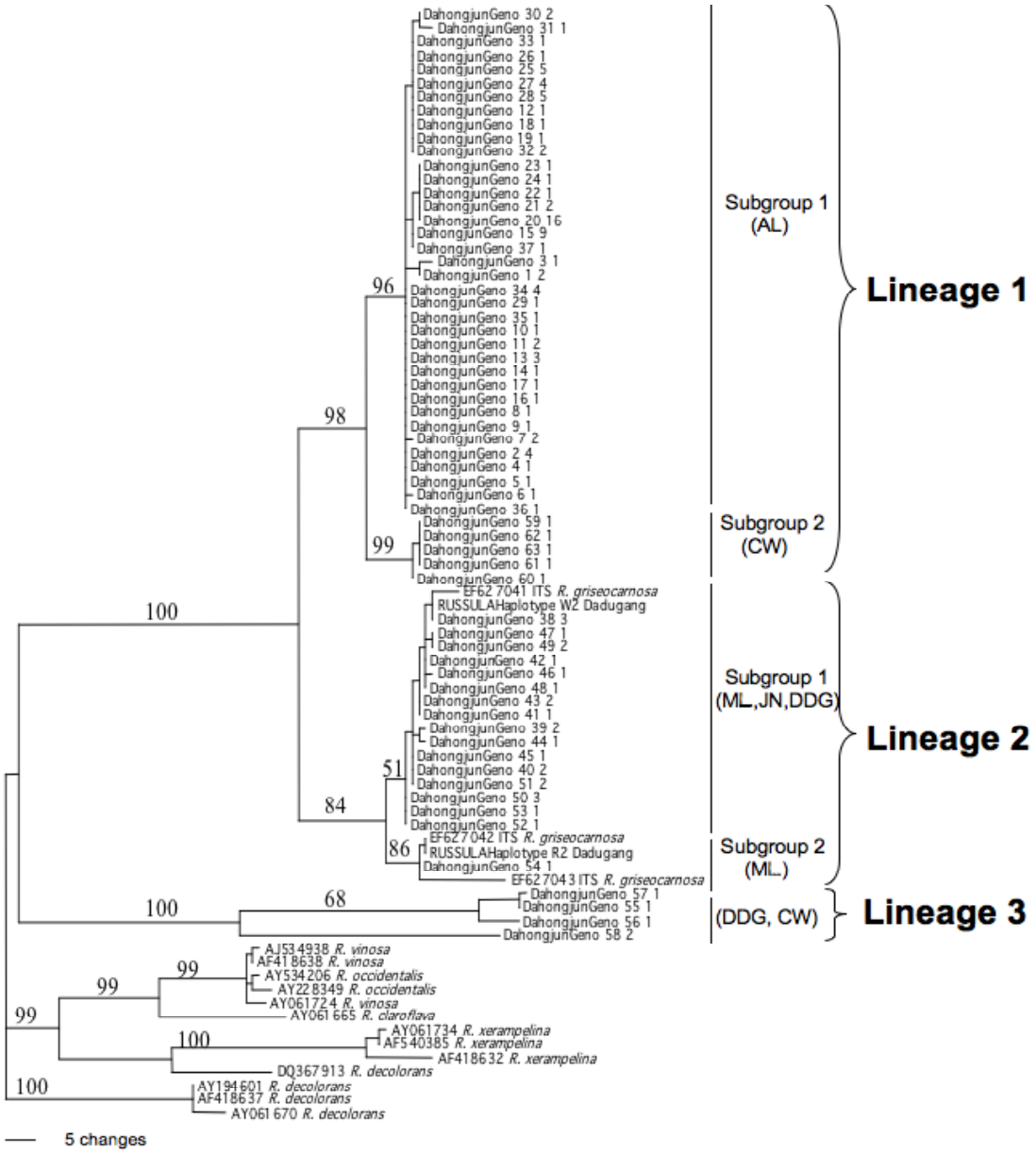

Figure 2: Phylogenetic relationships among ITS sequences from 122 isolates of Dahongjun from southern China and from closely related reference sequences from GenBank.
Each strain label contains its geographic location information [See text and Table 2 for details], followed by a field isolation number. Numbers along branches are bootstrap values greater than 90% obtained from 1000 replicates. Reference sequences contain species identification (when available), followed by the specific GenBank accession number. Sequences of non-Dahongjun samples are used as outgroups. Tree length=368, Consistency index=0.766, Retention index=0.943. [Modified from ref. 14].

The joint analyses of our sequences and those from the GenBank confirmed the distinctiveness of the three lineages within Dahongjun from southern China. One lineage (Lineage 2) corresponded to the known species, *R. griseocarnosa* recently identified from southern Yunnan [13]. The separation of these three lineages was supported by >98% bootstrap

values. Similarly, the separation of the three lineages of Dahongjun from a closely related species *R. vinosa* had 100% bootstrap support, including large phylogenetic distances among them. The divergences among the three lineages were comparable to or greater than those among the closely related known *Russula* species (Figure 2). While these lineages were prominently structured geographically based on ITS sequences, evidence for ancient and/or recent gene flow was also identified within individual lineages. In addition, as expected, the local population of Lineage 1 from Ailaoshan in central Yunnan Province where 85 of our 122 specimens came from showed clear evidence of recombination, consistent with the important roles of sexual spores and sexual reproduction in the ecology and population biology of Dahongjun.

Matsutake: diversity, ecology, population genetics, and counterfeiting. The matsutake (or Pine-mushroom) is among the most revered and valuable mushrooms in the world, especially in Japan. Similar to Ganbajun and Dahongju, high consumer demand, high price, limited natural production areas, and low productivity are threatening its genetic resources. However, unlike Ganbajun and Dahongjun where the consumer pressure is mostly from local communities in southern China, the pressures on matsutake are from both domestic and international sources. These pressures have called for a concerted effort to develop effective management and conservation programs in southwestern China. Understanding the ecology, reproductive biology, and population relationships of the organism would be essential for developing such a strategy.

Broadly speaking, matsutake refers to a loosely defined species complex in the genus *Tricholoma.* Like most mushrooms, the major biomass of matsutake is underground in the soil in which their mycelia form an extensive network. Its mycelia form symbiotic relationships with the roots of conifer and broadleaf trees. However, due to the lack of distinct morphological features, it has been difficult to separate the different species within the *T. matsutake* species complex. Based on DNA sequence information at the ITS, the European specimens of *T. nauseosum* and the Asian *T. matsutake* are considered con-specific and belong to the "true matsutake" (15; Figure 3). Though also consumed in Japan and North America, *Tricholoma magnivelare* is considered a "matsutake-ally", similar to *Tricholoma caligatum* and *Tricholoma fulvocastaneum*. On the other hand, though morphologically similar to *T. matsutake*, a more distantly related species *Tricholoma bakamatsutake* is not typically consumed and is commonly called the "fool's matsutake".

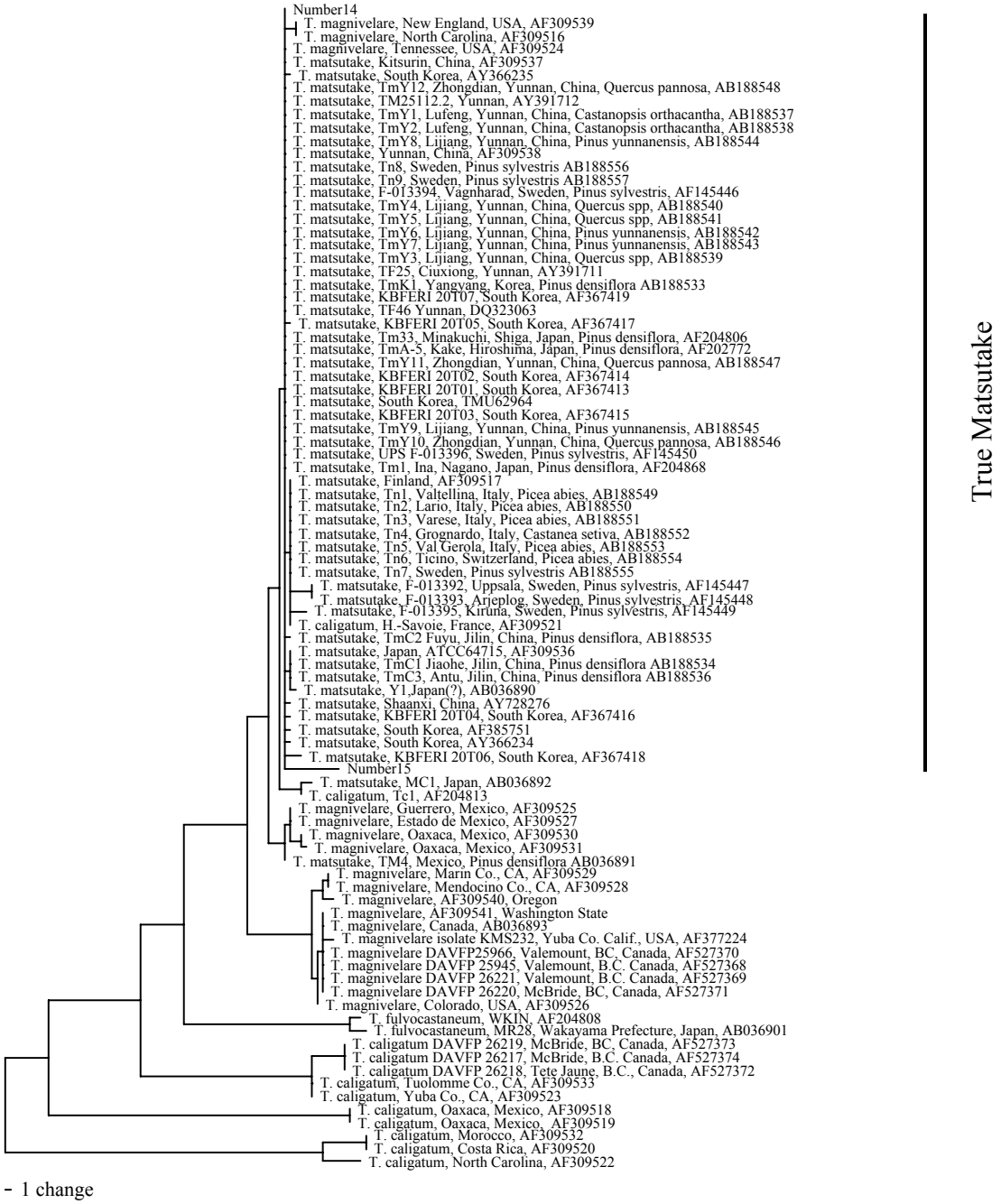

Figure 3: Phylogenetic relationships among strains of *Tricholoma matsutake* and its close allies based on ITS sequences. Each entry contains the species name, followed by strain name, geographic location, likely associated plant host, and the GenBank accession number. Strains labeled "Number 14" and "Number 15" are two strains from Yunnan associated with *Pinus yunnensis* and *Quercus spp* respectively. Note the limited or no ITS sequence variation pattern among populations of *T. matsutake* from different geographic areas. [Modified from ref. 16]

Geographically, the *T. matsutake* species complex has been reported from northern and highland Europe, northwestern Africa, southeastern-Himalaya, the Far East, the Pacific Rim in North America, the Great Lakes, the east coast of the United States, and Mexico. In general, the range of matsutake coincides approximately the distribution of coniferous genera such as *Pinus, Pseudotsuga, Tsuga, Picea, Cedrus* and *Abies*. However, matsutake is often found associated with oak (*Quercus* spp) in southwestern China; and *T. magnivelare* associated with *Lithocarpus densiflora* (tanoak) in the Pacific Northwest of North America. *T. bakamatsutake,* the "fool's matsutake" sympatric with *T. matsutake,* is believed to be associated with *Castanopsis, Fagus, Pasania* and *Quercus* spp., despite its occurrence in forests where conifers also are present [16]. The matsutake-ally *T. caligatum* from the US and Mexico is also suspected to be associated with angiosperm hosts.

Japan is the world's preeminent consumer market for the matsutakes, most prominently for the "true-matsutakes". Matsutakes collected and imported from different parts of the world are priced very differently in Japan, from less than US$100 to over US$4000/kg of fresh fruiting bodies [16,17]. The significant price differences for matsutakes from different parts of the world create conditions for counterfeiting. Two types of counterfeiting are possible. In the first, "matsutake-ally" and "false matsutake" are marketed as "true matsutake". A "trained eye" in morphological identifications and ITS sequencing or PCR-RFLP of the ITS using universal primers can reliably distinguish the "true matsutake" from the "matsutake-ally" and "false matsutake". However, as shown from the ITS phylogeny of the matsutake species complex, ITS sequencing has very limited discriminating power to distinguish different geographic populations of the "true matsutakes" from different regions (Figure 3). At present, there is relatively little information about the genetic and phenotypic differences among the "true matsutakes" from different parts of the world (i.e. northern Europe, southeastern Himalaya, northeastern China, Korea, Japan, and eastern north America). In our analyses of local and regional samples of *T. matsutake* from southwestern China (the main component of the southeastern Himalaya matsutake population) based on 14 single nucleotide polymorphisms, we found plenty of genetic variation within individual samples but very limited genetic differentiation among the 17 analyzed geographic populations [18,19]. On the other hand, comparisons of PCR-fingerprinting profiles using a pair of transposable element-based primers identified that *T. matsutake* from the Far East (Japan, Korea and northeastern China) were heterogeneous and showed consistent difference from those from southwestern China [16]. Below we describe how the simple difference in DNA fingerprinting profile allowed us to identify counterfeit *T. matsutake* in China [20].

Evidence for counterfeiting was found in our analyses of matsutakes from two major natural production and trading regions in China, the northeast (NE) and the southwest (SW) China [20]. In this analysis, we obtained DNA profiles of *T. matsutake* fruiting bodies from matsutake trading companies and compared them with known authentic wild-collected specimens from NE and SW China. The commercially purchased matsutake included 107 fruiting bodies from four companies in NE China and 45 fruiting bodies from three companies in SW China. The geographically authentic matsutake samples included 38 mushrooms from four local populations in Jilin and Heilongjiang in NE China, and 183 samples from 18 local populations in Yunnan, Tibet, and Sichuan provinces in SW China [19]. Our analyses showed that 67% commercial matsutake claimed to be from the northeast were in fact genetically identical at this marker to those from southwest China but different from authentic northeast

Chinese samples [Figure 4]. Such analyses highlight the importance of accurate identification of matsutake mushrooms, not only at the species level but also at population and strain level. Our analysis is similar to those found for counterfeited fish in New York City, USA [21] (Wong and Hanner 2008). Our finding suggests that caution should be applied to authenticate commercial matsutake from northeast China. Similar concerns about the authenticity of other gourmet mushrooms such as truffles, morels, and tubers have also been raised but the identification system is yet to be finalized.

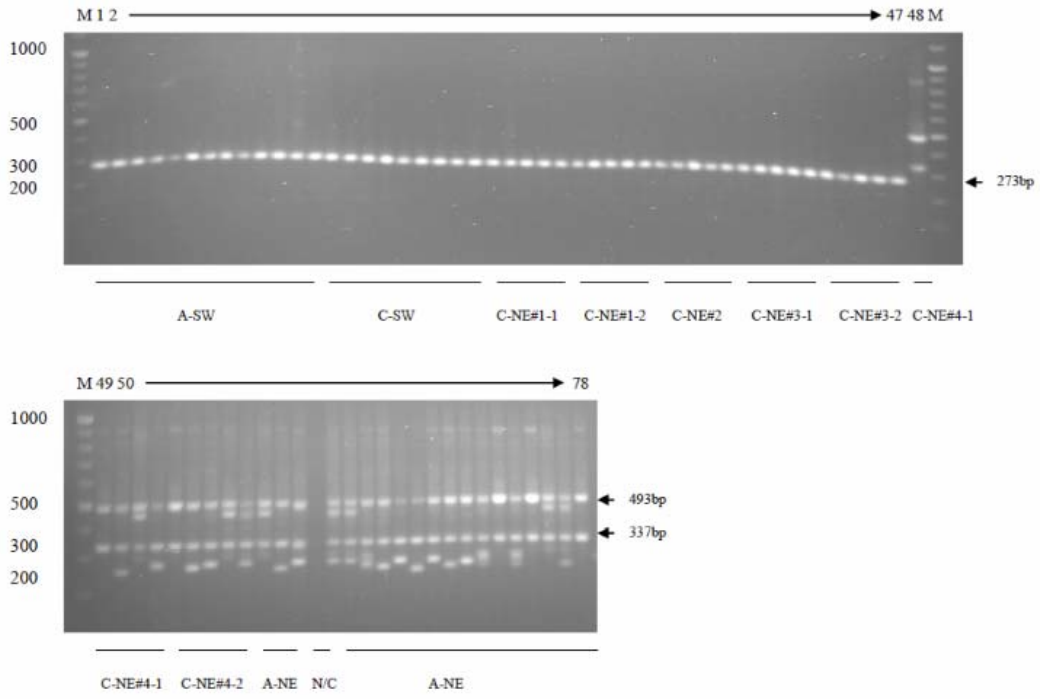

Figure 4: PCR fingerprinting profiles of representative Chinese isolates of *Tricholoma matsutake* based on the pDGSL313-1/pS48 primer typing system.
The lane numbers are indicated at the top and the origins of specimens are given at the bottom of gels: A-SW, authentic southwestern matsutake (lanes 1-13); C-SW, commercial southwestern matsutake (lanes 14-22); C-NE, commercial samples claimed from the northeastern (lanes 23-57), with different sets from different trading companies; A-NE, authentic northeast matsutake (lanes 58-60, 62-78); N/C, negative control (water, no sample DNA). Lanes M, molecular markers (200 to 1000 bp). Right-hand labels indicate signature DNA fragments corresponding to SW (273bp) and NE (493bp and 337bp) Chinese matsutake samples. [Modified from ref. 20].

CONCLUSIONS

The three commercially harvested wild mushrooms share several features: all are ectomycorrhizae, not cultivable, of significant importance to the local and regional economy, and with very limited knowledge about their biology. Our analyses identified that each of these commercial mushrooms contained significant genetic diversities, including multiple evolutionary divergent lineages that likely correspond to several phylogenetic species. These lineages typically show different geographic distribution patterns, with some lineages broadly distributed

while others more limited. Whether such patterns are representative of the wild edible ectomycorhizal mushrooms or wild mushrooms in general in southwestern China remain to be determined. Our results based on the ganbajun and dahongjun samples indicate that the number of edible mushroom species in southwestern China may be underestimated by 3-5 folds. The genetic information obtained here should enhance our ability to develop strategies for effective conservation and management programs of these genetic resources.

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FROM THE COMPARATIVE ANALYSIS OF FUNGAL MITOCHONDRIAL GENES TO THE DEVELOPMENT OF TAXONOMIC AND PHYLOGENETIC TOOLS

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ABSTRACT

The complete sequence of the mitochondrial *cox1* gene, encoding the largest subunit of the cytochrome oxidase of the Basidiomycota *Agaricus bisporus* has been achieved. It has the longest *cox1* gene (29,902 nt) with the largest number of group I introns (18 group I introns) reported to date in any eukaryote. The group I introns in the *A. bisporus cox1* gene are similar to those reported in other Basidiomycetes includeing: 3 of the 4 introns in *Agrocybe aegerita*, 7 of the 9 introns in *Pleurotus ostreatus*, 3 of the 6 introns in *Moniliophthora perniciosa*, and 10 of the 15 introns in *Trametes cingulata*. constituting 18 of the 23 introns described in all Basidiomycota available genes, Moreover, the *A. bisporus cox1* gene possesses two introns specifically reported in this gene (*iAbi1* and *iAbi14*) and one intron (*iAbi 18*) possessing orthologous sequences only in the Ascomycota phylum but unknown to date in the Basidiomycota. Hence, *A. bisporus cox1* gene contains three-quarters (18/24) of the fungal introns described in all the fungal *cox1* genes from Dikarya (Ascomycota and Basidiomycota). From the *A. bisporus* sequence, primers were designed to evaluate the potential of rare and widely distributed introns to act as taxonomic and/or phylogenetic markers of species belonging to the genus *Agaricus*. Indeed, we found that the rare introns could be specifically recovered in some strains and/or species. Sequences of the widely distributed fungal introns provide information on the phylogeny and spread of introns among distant as well as closely related species.

Keywords: *Agaricus* genus; mitochondria; *cox1* gene; group I intron; phylogeny

INTRODUCTION

Although the fungal Dikarya subkingdom (including the Ascomycota and Basidiomycota phyla) contains a broad range of taxa with a great variety of morphologies, ecologies and life cycles, it is sometimes difficult, even for specialized mycologists, to define distinct and unambiguous species boundaries based on their phenotypic differences. In this context, there is an urgent need to obtain improved and cost-effective molecular markers to help discriminate closely related species, especially for species belonging to the same genus and with commercial interests.

To date, molecular identification of fungi relies mostly on nuclear DNA markers, such as the conserved LSU-rDNA (18S), SSU-rDNA (28S) or the frequently studied variable spacers (ITS1 and ITS2) within the nuclear ribosomal gene cluster. Currently, the 18S and 28S are used to discriminate high taxonomic levels such as family and genera while the internal transcribed spacers (ITS) allow the characterization of organisms at the species level [1]. However, in recent

years, it has been necessary to develop several additional nuclear markers to resolve the inefficiency of ITS to discriminate some well-characterized species or to identify cryptic species in some fungal species complexes, as well as to clarify phylogenetic relationship between related as well as distant species. The β-tubulin gene (BenA) [2], the elongation-factor EF-1-α [3], the second largest subunit of RNA polymerase II (RPB2) [4] have often been used for these purposes.

Besides these nuclear DNA sequences, the potential use of mitochondrial DNA specific markers has become increasingly common. Indeed, mitochondrial DNA markers possess several interesting features such as their high copy number allowing an easy recovering of the sequences and the paucity of repetitive regions often involved in misleading results. Furthermore, mitochondrial DNA (mtDNA) has been reported to be less affected by genetic recombination, mainly due to a predominantly uniparental heredity, and to show a higher rate of evolution than the nuclear genome. These features make mitochondrial DNA a potentially powerful source of molecular markers to identify species [5]. Hence, a 648 nt sequence located at the 5' end of the *cox1* gene encoding the subunit I of the "cytochrome c oxydase" (complex IV of the respiratory chain) has been widely used in a successful "DNA barcoding" method in several animal groups such as insects or birds [6]. With a taxonomic resolution higher than 95% in most of the Metazoa group, this mitochondrial region was proposed as the core of global bio-identification systems for eukaryotes [7].

In this way, in the Metazoa related kingdom of fungi, the *cox1* gene was recently shown suitable for discriminating fungal species in the taxonomically challenging genus *Penicillium* [8]. However, in most genera of the fungal kingdom, this approach is hampered by the presence of several large group I introns, frequently occurring in mitochondrial coding sequences and, especially in the *cox1* gene which is the mitochondrial gene showing the highest number of introns [9]. Owing to this wealth of introns, few fungal complete *cox1* gene sequences are available in database, especially for species belonging to the Basidiomycetes where only six sequences have been reported and correctly annotated to date (*Moniliophtora perniciosa, Pleurotus ostreatus, Agrocybe aegerita, Schyzophyllum commune, Agaricus bisporus* and *Trametes cingulata)* [9].

Here, we report the analysis of three types of mitochondrial sequences with the aim to define molecular markers suitable as taxonomic and/or phylogenetic tools at different taxonomic ranks and/or for strain fingerprinting: (i) Variables domains of the SSU-rDNA of the mitoribosome, (ii) the sequences of a" rare" group I intron, and (iii) a widely distributed intron carried by the *cox1* gene. The taxonomic and phylogenetic potentials of these mitochondrial markers will be discussed by comparing with the sequences of the conventional nuclear ribosomal cluster.

The comparative analysis was carried out with six strains representing five *Agaricus* species (*A. boisseletti, A. gennadii, A. arvensis, A. subrufescens* and *A. bisporus*) belonging to four different taxonomic sections of this genus. Two *A. subrufescens* strains from two geographical origins (France and Brazil) were included in the analysis for comparison.

MATERIALS AND METHODS

***Agaricus* species sampling and determination.** Sporophores representing six *Agaricus* strains (Table 1) were collected and morphologically identified. Sequences of the nuclear ribosomal unit obtained from these stains were established and compared with sequences available in the GenBank (data not shown).

Table 1: *Agaricus* strains and sequences

Section	*Agaricus* species	strain N°	ITS Acc N°	V6 domain	V9 domain	intron i7	intron i18
Sanguinolenti							
	A. boisseletti	CA369		CA369	nd	CA369	0
	A. boisseletti	CA123	DQ182531				
Chitonioides							
	A. gennadii	CA387		CA387	CA387	CA387	CA387
	A. gennadii	Gn17	AF432881				
Arvenses							
	A. arvensis	CA640		CA640	CA640	CA640	CA640
	A. arvensis	strain 15	AJ887993				
	A. subrufescens	CA516		CA516	CA516	CA516	CA516
	A. subrufescens	CA454		CA454	CA454	CA454	CA454
	A. subrufescens	I_101_S1	AY818660				
Bivelares							
	A. bisporus	BS518		BS518	BS518	BS518	BS518
	A. bisporus	ATCC MYA-4626	GU327642				

***In vitro* DNA manipulation and sequencing.** Sequences used as molecular markers were obtained by conventional procedures from cloned PCR products.

Total DNA of fungal strains were extracted from 0.1 g of dried carpophores after grinding in liquid nitrogen to generate a fine powder. Nucleic acids were extracted according to the *N*-cethyl-*NNN*-trimethyl ammonium bromide (CTAB) procedure adapted to small quantities of basidiomycete mycelia by [10]. DNA (OD_{260}) was quantified using a NanoDrop spectrophotometer (NanoDrop ND-1000, Nanodrop technologie, DE, USA), diluted in deionized sterilized Milli-Q water (Milli-Q water system production, Millipore, Saint-Quentin en Yveline, France) and stored at -20°C.

PCR amplifications were carried out using the Go *Taq* polymerase from Promega Corp. (Madison, Wis, USA) and with corresponding primer pairs synthesized by Eurofins MWG Operon (Germany). PCR were performed in a Programmable Thermal Cycler PTC 200 (MJ Research Inc., Watertown, Mass., USA). Each reaction contained 10 to 100 ng of fungal genomic DNA, 1 µM of both primers, 200 µM of each dNTP, 1 unit of *Taq* DNA polymerase, in a final volume of 50 µl of the appropriate buffer. Reactions were run for 30 cycles at 95 ° C for 30s, then two degrees below the lowest Tm of both oligonucleotides for 30s, 72° C for 1 to 2 min, and one final cycle at 72° C for 5 min. An aliquot of 10 µl of each PCR product was analysed by agarose (1%, w/v) gel electrophoresis containing 200ng/ml of ethidium bromide, in TEB buffer [11].

DNA Sequencing. PCR products were purified with the Wizard SV gel and PCR Clean-Up System (Promega Corp.Madison, WI, USA) before they were sequenced by the primer walking methods using the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Courtaboeuf, France) and corresponding primers used for the initial PCR amplifications. Sequence reactions were carried out, according to the supplier recommendations, in a final volume of 10 µl containing 100 ng of PCR product and 0.5 µM of the specific primer. Sequence reactions were conducted in a thermocycler by applying an initial denaturation step at 95°C for 1 minute; 27 cycles each composed of the three following steps: 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. The reaction products were ethanol precipitated, dried then separated by capillary electrophoresis (on an automated sequencer ABI 3130x1, ABI Prism Corp., France) at the genomic platform of the University Bordeaux Segalen (France). Sequencing profiles were edited and corrected using the BioEdit sequence alignment editor v7.0.9 free software (Ibis Biosciences Carlsbad, CA, USA).

Sequence analyses. Comparisons with sequences of the GenBank and EMBL databases were performed using the search algorithm BLAST [12]. Multi-alignments of nucleic acid and/or proteins were performed using Clustal W algorithm [13] or Muscle [14] for multiple alignment and Gblocks for automatic alignment curation [15]. For phylogenic analyses, the sequence data were aligned and checked for accuracy manually. Pairwise evolutionary distances based on unambiguous nucleotides were computed using the dnadist (Jukes and Cantor option) and neighbor-joining programs in the PHYLIP suite. Seqboot was used for Bootstrap analysis [18], using algorithm version 3.572c. One thousand Bootstrap replicates were employed to determine confidence in the branches order. (The phylogenetic softwares used were part of the PHYLIP package version 3.572 Mac executables [19].

The alignments were also submitted to the PhyML tree building program [16] and TreeDyn for tree drawing [19]. PhyML was run with the aLRT statistical test of branch support. These programs were obtained on line at: http://www.phylogeny.fr/ [20, 21]. In this case, confidence in the branches order was measured by the ratio test developed by [22] working with the PhyML tree building program at the phylogeny site (http://www.phylogeny.fr/) [20, 21].

RESULTS AND DISCUSSION

Comparison of the molecular organization of the *cox1* gene in *A. bisporus* (section Bivelares) and in other Basidiomycete species

The complete sequence of the mitochondrial *cox1* gene of *Agaricus bisporus* was achieved. This gene is both the longest mitochondrial gene (29,902 nt) and the largest intron reservoir reported to date in an eukaryote [9]. It possesses 18 group I and one group II introns. An exhaustive analysis of the group I introns available in *cox1* genes shows that they are ancestral mobile genetic elements, whose frequent events of loss (according to the "late theory") and gain by lateral transfer ("early theory") would combine to obtain the observed wide and patchy distribution extending on several kingdoms [23]. Its distributions are consistent with both the "early" and "late" paradigms, which are still matters of debate [24, 25]. However, the overview of the intron distribution in eukaryotes indicates that they are mainly evolving towards elimination and, in such a landscape of eroded and lost intron sequences, the *A. bisporus* largest intron reservoir, by its singular dynamics of intron keeping and catching, would constitute the most fitted relic of an early split gene [9]. However, the analysis was carried out on phylogenetically distant organisms extending on several kingdoms.

When the analysis was limited to the Dikarya, results show that the *Abi cox1* gene possesses most of the group I introns available in other Basidiomycete *cox1* genes: it contains 3 of the 4 introns in *Agrocybe aegerita*, 7 of the 9 introns in *Pleurotus ostreatus*, 3 of the 6 introns in *Moniliophthora perniciosa*, 10 of the 15 introns in *Trametes cingulata, and* 18 of the 23 introns described in all basidiomycota available genes (Fig. 1). Moreover, the *A. bisporus cox1* gene possesses two introns only reported in this organism (*iAbi1* and *iAbi14*) and one intron (*iAbi 18*) possessing orthologous sequences found only in the Ascomycota phylum so far but unknown to date in the Basidiomycota. Hence, *A. bisporus cox1* gene contains three quarters(18/24) of the fungal introns described in all the fungal *cox1* genes from Dikarya, i. e. from all the available Ascomycota and Basidiomycota *cox1* genic sequences [9].

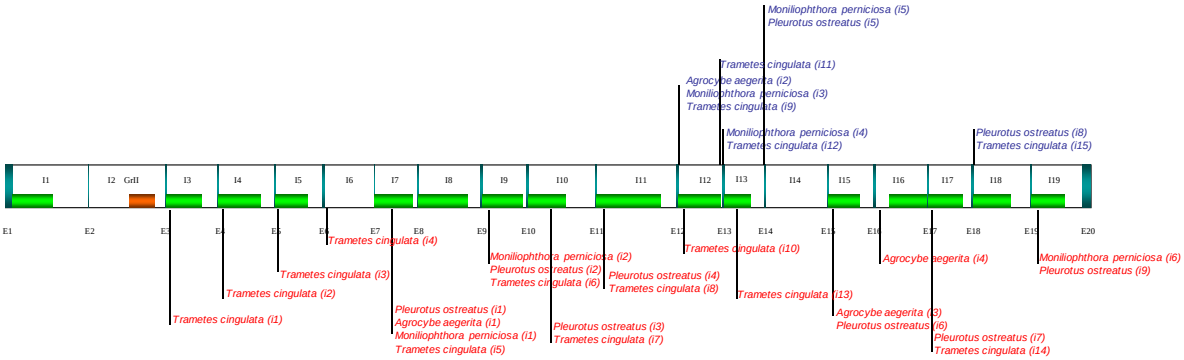

Figure 1: Molecular organization of the *A. bisporus* strain BS518 *cox1* gene.
The position of orthologous (red) and not orthologous (blue) introns reported in other Basidiomycota are indicated below and above the *A. bisporus cox1* gene, respectively. Blue boxes represent exons E1 to E20. The names of the introns are indicated in the empty boxes. The putative functional *heg* in group I introns are shown by green boxes; the eroded reverse transcriptase gene carried by the *iAbi 2* group II intron by an orange box.

In other to investigate the evolution (loss and/or gain), the ancestral feature and the correlated potentiality of *cox1* group I introns to act as phylogenetic markers, we have studied the occurrence and phylogenetic relationships of two group I introns reported in the *Abi cox1* gene (strain BS518) in five additional *Agaricus* species belonging to four different sections. The evolution of these intronic sequences was compared with that of the nuclear ribosomal unit and with that of variable domains of another mitochondrial gene, namely the SSU-rDNA, encoding the small rRNA (16S) of the mitoribosome.

Occurrence and phylogenetic analysis of the widely distributed *iAbi7* group I intron. The first intron studied was *iAbi7* (1207 nt) which is the most widely distributed group I intron in eukaryotes (present in all the divisions of the fungal kingdom and also in the Viridiplantae kingdom). All the available Basidiomycete *cox1* genes reported to date possess orthologous sequences of the *iAbi7* intron, with the exception of *Schizophyllum commune* whose mitochondrial genome does not possess any intron.

From the *A. bisporus cox1* gene, a couple of specific primers located in the upstream (primer U7: 5'ACAGGGTGGACGGTA3') and downstream (primer R7: 5'GATTCCTGATAAAGGAGG3') exon regions flanking *iAbi7* were defined and used in PCR to amplify six *Agaricus* strains as matrix. As shown in Table 1, all the studied strains generated a PCR product of a large size around 1,200 nt. For each strain, this PCR product was purified and sequenced. The resulting sequences confirmed the presence in the *cox1* gene of each strain of an *iAbi7* orthologous sequence. Moreover, all these orthologous introns possess a large ORF corresponding to the *heg* encoding a putatively functional Homing Endonuclease (HE) involved in the transfer and site-specific integration (homing) of the mobile intron. These results suggest that the *iAbi7* intron is a mobile genetic element with likely conserved functions in the *Agaricus* genus.

A phylogenetic tree was constructed by two methods: a distance (Neighbor-Joining) method (Fig. 2C) and the maximum likelihood method. The trees were obtained from the MUSCLE alignment of a 754 nt sequence read on both strands and located in the central part of the intron sequence and in the central part of the *heg* (Homing Endonuclease Gene) carried by these orthologous introns.

These trees confirm the close relationship between the six *iAbi7* orthologs. The deduced relationship among the strains are in agreement with those deduced from the trees based either on the nuclear ribosomal cluster (figure 2A) or on the compiled sequences of two variable domains

(V6 and V9) of the mitochondrial SSU-rDNA (figure 2B). Particularly, sequences of the intron 7 of both strains of *A. subrufescens* (CA454 from Brazil and CA516 from France) were identical to each other and to the *A. Arvensis* strain CA640 which belongs to the same section Arvenses. When these sequences were compared with *iAbi7* sequence of *A. bisporus* which belongs to the second clade of the tree, they showed 82,6 % of nt identity.

The agreement between the *iAbi7* phylogeny and those based on the nuclear ribosomal gene cluster (figure 2A) or based on the compiled sequences of two variable domains (V6 and V9) of the mitochondrial SSU-rDNA (figure 2B) argues for an ancestral feature of the *iAbi7* intron. This result, along with the high conservation of the *i7* intron within the *cox1* genes, and the wide distribution of *heg* within the *i7* intron represent a powerful tool for phylogenetic studies of the genus *Agaricus*, and more particularly at the section level.

Occurrence and phylogenetic analysis of the rare *iAbi18* group I intron. The second studied intron is *iAbi18* (1148 nt), a rare intron only reported in *A. bisporus* in the Basidiomycota and two species in the Ascomycota, *Gibberella zea* and *Penicillium marneffei*.

From the *A. bisporus cox1* gene, a couple of specific primers located in the upstream (primer U18: 5'TGCAGGTTTCTATTATTGG3') and downstream (primer R18: 5'AAGTGTTGAGGGAAAAATG3') exon regions flanking *iAbi18* were defined and used in PCR to amplify the six *Agaricus* strains. As shown in table 1, only one of the six strains, *A. boisseletti* CA123 did not possess orthologous sequence to *iAbi18*. In this case, the PCR product with a size of 120 nt corresponded to the size of the COX1 CDS located between both primers. The exon nature of the PCR product was verified by sequencing. This confirms that the presence of a group I intron in a strain or species is optional. However, the study of several *A. boisseletti* strains will be needed to determine if the observed intron loss concerns the strain level or has to be extended to the species level.

The large size PCR product (around 1,300 nt) obtained with the five other strains was sequenced. The resulting sequences confirmed the presence in the *cox1* gene of each strain of an *iAbi18* orthologous sequence, carrying a putative functional *heg*.

Moreover, the two orthologs carried by both *A. subrufescens* strains from Brazil and France were identical, with a 100% of nt sequence identity.

Similar to the analyses for iAbi7 sequences above, trees were constructed by the Distance and PhyML programs. The trees (figure 2D) were obtained from the MUSCLE alignment of a 896 nt sequence read on both strands, and located in the central part of the intron sequence and in the central part of the *heg* (Homing Endonuclease Gene) carried by the intron. These trees confirmed the close relationships between the five *iAbi18* orthologs. It is to be noted that the *iAbi18* ortholog harboured by the *A. Arvensis* strain *(iAarv18*) follows the phylogenetic relationships deduced from the trees based on the nuclear ribosomal unit and on the mitochondrial variable domains as well as on the *iAbi7* orthologous sequences. However, in the Arvenses section, the sequence of *A. arvensis* was highly diverged from the two sequences of *A. subrufescens*. Indeed, *iAarv18* possesses 91.4 % nt identity with *iAbi18* and 92.3% with *iAsub18*, although *A. arvensis* and *A. subrufescens* are two phylogenetically closely related species (belonging to the same Arvenses section).

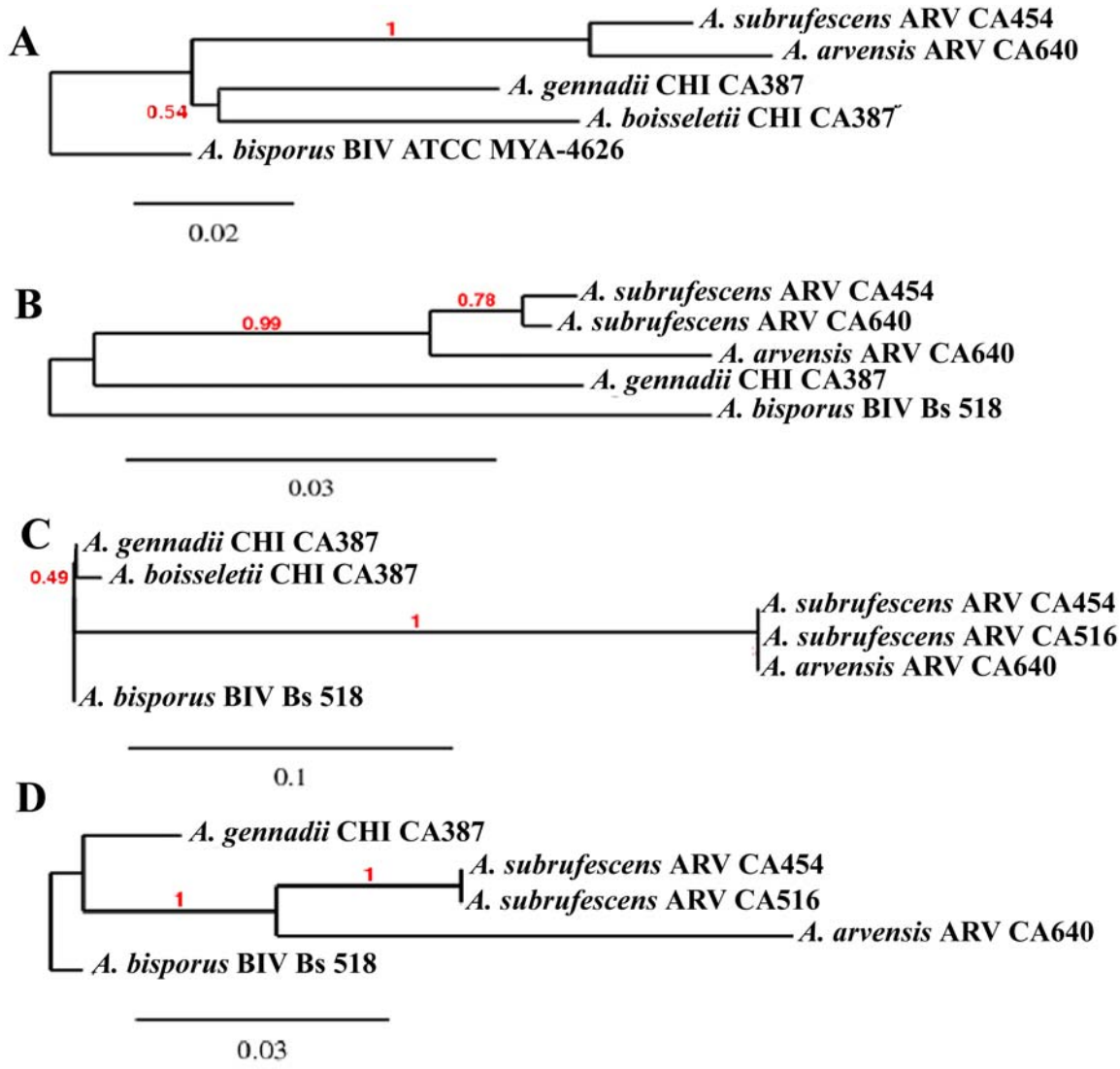

Figure 2: Distance (Neighbor) cladograms based on the nuclear ribosomal unit (A), a compilation of the V6 and V9 variable domains (B), the orthologs of the *iAbi7* intron (C) and *iAbi18* intron (D).
The Bootstraps values indicated above branches were obtained with 1,000 replicates. The cladograms based PhyML program (maximum likelihood) led to similar trees with branches supported by comparable SH-like Branch supports.

CONCLUSION

This report focused on three different mitochondrial molecular markers and six strains representing five species and four sections of the *Agaricus* genus. From the preliminary results, two types of mitochondrial sequences appear as potentially suitable tools to add phylogenetic and/or taxonomic information to the well-established nuclear ribosomal units.

The first one is the compiled sequences of two variable domains (V6 and V9) of the SSU-rDNA, encoding the 16S RNA of the small-subunit of the mito-ribosome. Indeed, these domains mainly evolve by length mutations involving indel (insertion/deletion) sequences and, consequently, can easily lead to CAPS markers for species determination.

The second kind of sequences is constituted by the orthologs of the *iAbi7* intron. Indeed, this group I intron appears widely distributed in the eukaryote kingdom, but also in the *Agaricus* genus. Moreover, this mobile genetic element carries a structural gene, encoding a Homing Endonuclease (HE) which seems to have maintained its function during evolution, and consequently can constitute a permanent phylogenetic marker, to replace the “barcoding region ”

of the *cox1* gene which is split too much by several large group I introns in the fungi, and especially in the *Agaricus* genus (data not shown).

The third studied sequence, the "rare" group I intron *iAbi18*, was shown to be frequent but not universally distributed in the *Agaricus* genus. Additionally, one of its orthologous sequences described in an *A. arvensis* strain reveals an unexpected phylogenetic behavior, suggesting that its evolution might not strictly follow evolution by descent but could involve lateral gene transfer between a distantly related species. This behavior does not allow to consider it as an easy phylogenetic marker but opens the way to the discovery of still unknown transfers of mitochondrial sequences.

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POSITIONING OF INTRONS IN DIFFERENT LACCASE GENES, A RELEVANT TOOL FOR SOLVING PHYLOGENETIC POSITION AMBIGUITY OF *VOLVARIELLA VOLVACEA* LACCASE GENES

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ABSTRACT

Volvariella volvacea (paddy straw mushroom) is a high temperature-loving mushroom with the shortest cropping cycle in the basidiomycete family Pluteaceae. This mushroom is cultivated extensively in many South-East Asian and African countries by employing both the outdoor and indoor cultivation techniques. The objective of this study is to analyze intron positions in different laccase genes of this mushroom. Primer pairs for laccase 1, 2, 3, 4, 5 and 6 were designed by using the mRNA sequences of these genes available in NCBI nucleotide database and the Primer 3 free software. These primers were used to amplify different laccase genes in one heterozygotic strain, Vv-01 and one putative homozygotic strain, BBSR-003 at an annealing temperature range of 53 to 58ºC to obtain amplicons of requisite sizes. PCR products were then sequenced and annotated using the BioEdit and Artemis softwares. Out of 6 laccase genes, partial sequences of laccase 1, 2, 3 and 6 in the heterozygotic strain Vv-01 and partial sequence of laccase 3 in homozygotic strain BBSR-003 were obtained. The lcc 3 sequences of two strains, Vv-01 and BBSR-003 have been submitted to NCBI GenBank with accession numbers HQ687205 and JF313903, respectively and they showed differences at 10 different base positions. In lcc 3, there was an intron 8 found earlier in laccase genes from *Coprinopsis cinerea*, but same has not been spliced in published mRNA sequence of *V.volvacea*. This intron has to be spliced in strain Vv-01 to give a functional protein and by virtue of which, the phylogenetic position of lcc3 in laccase dendrograms is probably wrong. In lcc 1, 16 different introns have been recorded and the amino acid sequence of Vv-01 strain differs by one amino acid less than the published sequence of lcc1. No evidence of heterozygocity has been recorded in this sequence. The lcc 2 sequence also revealed 16 different introns. Although only a small portion of sequence could be validated but evidence for heterozygocity was found in strain Vv-01. The small sequence of lcc 2 in strain BBSR-003 also differed at some bases from strain Vv-01. At the protein sequence level, 26 amino acids of the published mRNA seemed to be derived from the wrong reading frame. This could be due to errors in sequencing, or that the strain used had a pseudo gene or a very original allele for lcc 2. Based on these observations, the position of laccase 2 in the dendrogram is likely to be changed. The sequence of lcc 6 helped in identifying 5 introns. The positions of introns in different laccase genes (lcc 1, lcc 2 and lcc 3) of *V. volvacea* grouped them to sub-family A.

Keywords: *Volvariella volvacea*; Phylogenetic; Laccase; Introns; Heterozygotic

INTRODUCTION

Volvariella volvacea (Bull ex Fr.) Sing. belongs to the family Pluteaceae Kotl. & Pouz. of the Basidiomycetes [1]. It is an important edible mushroom of the tropics and subtropics. Literature available on this mushroom has emphasized more on its cultivation technology than its morphology [2], cytology [3], physiology [4, 5] and molecular biology [6]. This mushroom can produce an array of extracellular hydrolytic and oxidases enzymes - e.g. endo-1,4-β-gucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21) and laccase (EC 1.10.3.2) [6-11]. The copper binding regions and the N-terminal amino acid sequences have also been used to generate complete sequence of six laccases from this mushroom [12, 13] and out of these, lac1 and lac4 have been suggested to play roles in morphogenesis in this mushroom [12, 13]. The complete mRNA sequences of six laccases in this mushroom have also been obtained in the past, which are also available in NCBI database.

The genus *Volvariella* comprises about 50 species worldwide [14], including the cultivated *V. volvacea* (paddy straw mushroom). Using the 'strips' recognized by Singer [15], several morphological/ecological groups have been differentiated within this genus. Specifically, the *V. volvacea*-group has been identified with pileus >50 mm in diameter, with darkly colored and usually grey-brown basidiospores. Based upon the nLSU data, Moncalvo *et al*. [16] placed *Volvariella* in a very distinct position to *Pluteus*, clustering with *Fistulina* Bull. and *Schizophyllum* Fr. Similarly in a later study [17] using six genes, *Volvariella* (*V. gloiocephala*) was placed as the sister group of *Pluteus* along with *Melanoleuca*, traditionally classified in the *Tricholomataceae* R. Heim ex Pouzar [15], and were together placed with members of *Amanitaceae*, *Pleurotaceae* Kuhner, the aquatic basidiomycete *Limnoperdon* G.A. Escobar and some other 'orphan' agaric genera (*Tricholomopsis* Singer, *Cantharocybe* H.E. Bigelow & A.H. Sm., *Macrocystidia* Joss) in one major group named the 'Pluteoid' clade. Likewise Binder *et al*., [18] got a similar topology for *Pluteus*, *V. gloiocephala* and *Melanoleuca* with *Amanita* Pers. as members of the core Pluteoid clade with *Tricholomopsis* and *Cantharocybe* outside of the clade. In a recent study based upon molecular data from nuclear ribosomal genes (nSSU, ITS, nLSU), *Volvariella* has been described as polyphyletic, and *Volvariella sensu stricto* has been placed with some hygrophoroid genera (*Camarophyllus*, *Cantharocybe*), representing as a sister group of the Pluteoid clade and a new generic name, *Volvopluteus* has been proposed with species as *earlei* and *gloiocephalus*, keeping these last ones in Pluteoid clade [19]. Compared to the Agaricoid clade, the respective positions of *Pleurotus ostreatus* and *V.volvacea* are still unclear.

Recently, the laccase genes have been used for species identification and establishment of evolutionary relationships, where other molecular markers have failed [20]. This group of enzymes (benzenediol: oxygen oxidoreductase, EC 1.10.3.2.) play important role in catalyzing the single electron transfer from various organic compounds and concomitant four-electron reduction of oxygen to water, and they are found widely spread in plants, bacteria, insects and fungi. Laccase gene has also been used for studying presence of basidiomycetes in a forest soil and saprotrophic fungi have been reported to be less spread through the soil horizons than the mycorrhizal ones. [21]. The diversity of the laccase genes have again been used in phylogenetic perspectives of *Botryosphaeria rhodina* (Ascomycota: fungi) and some related taxa [22], and likewise the multi-gene family of *Coprinopsis cinerea* has been studied, which has been divided into two sub-families A and B [23]. In the laccase phylogeny, three principal clades, comprised

of sub-family A and B from *C. cinerea*, and sub-family C from *Pleurotus ostreatus* PoxA3 and *A. bisporus* lcc1 and lcc2 laccases have been proposed [24-26]. In clade A, the position of *V. volvacea* is very peculiar, which suggests that these laccases can belong to a new clade, distinct from clade A and supports the idea of an early divergence of these genes from the other laccase genes of clade A. The present study was conducted to determine the variations in the positions of introns in laccase genes of *V. volvacea*, and identify their relationship with the positions of introns in laccase genes of other related species, and to draw the evolutionary relationships between the laccase genes of related species.

MATERIALS AND METHODS

***V. volvacea* strains and their DNA extraction**. The strains Vv-01 and BBSR-003, originally collected from Coimbatore (Tamil Nadu) and Bhubaneswar (Orissa), the Southern costal region of India, were used in the study. The strains were grown separately at 32 ± 2ºC for 5 days on Malt Extract Agar medium petridishes and the well grown mycelia was scarped and used for genomic DNA extraction. DNA was extracted by using Illustra DNA extraction Kit from GE Healthcare, UK by following the protocol provided by the manufacturer.

Primers for laccase gene amplification: The primer pairs for six laccase genes (lcc1, lcc2, lcc3, lcc4, lcc5 and lcc6) of *V. volvacea* were designed based on the mRNA sequences of these genes (Accession Nos. lac1-AY249052.1, lac2-AY338483.1, lac3-AY338484.1, lac4-AY338486.1, lac5-AY338485.1 and lac6-AY338487.1) available in NCBI nucleotide database using Primer 3.0 (version 0.4.0) free software available on internet. These primers were synthesized from Sigma Life Science, France (Table 1).

PCR amplification of laccase genes: PCR amplification of different genes was performed in reaction mixture of 25 µl; comprising 5X PCR buffer 5 µl (Promega, Medison, WI), dNTP 3.5 µl (1.2 mM each), bovine serum albumin 0.5 µl (10 mg/ml), Taq DNA polymerase 0.2 µl (5 U/µl, Promega M830B), primer pairs @ 2 µl each (10 µM) and DNase/RNase- free water 10.8 µl and template DNA 1 µl (50 ng/µl). The PCR reaction was performed in Master Cycler Gradient (Eppendorf) with initial denaturation at 95ºC for 5 min, followed by 35 cycles of 95ºC for 1 min, 55ºC for 5 min and 72ºC for 2 min 30 sec, and by final extension at 72ºC for 4 min, with a lid heating option at 110ºC. The PCR amplified products (amplicons) were run on 1.2% agarose gel prepared in 1.0% TBE buffer and the gel was run at 70 V for 1 hour 30 min in 0.5% TBE buffer. The gel was visualized under gel doc system from BioRad.

PCR amplification of different genes was optimized by using the annealing temperature gradients of 2 to 6ºC depending on the performance of the initial PCR amplification of the different laccase genes. The annealing temperature giving the highest product intensity at appropriate position (product size) under UV light was selected as the annealing temperature for further PCR reactions.

Sequencing of PCR amplicons: The amplicons along with the corresponding primer pairs (forward and reverse) were sent to BECKMAN COULTER GENOMICS, UK for sequencing and the received sequences were further processed.

Table 1: Source and sequences of primers designed for different laccase genes

Laccase gene	mRNA Accession No. in NCBI GenBank	mRNA GI No. in NCBI GenBank	Primers	Sequence (5´ to 3´)
Laccase 1	AY249052.1	37732219	Vv1FL1	CCGATGAAGTTGGGACATTC
			Vv4RL1	TCGCAATCACAATCACAGTTC
Laccase 2	AY338483.1	37791150	Vv1FL2	CCTTGCTCAACACCCTTACC
			Vv4RL2	GCCAGAGACTGGGTTAGACG
			Vv12FL2	CTGCTGCACAACAGAGCTTC
			Vv42RL2	AATACCAGAGGACGCGAGAC
Laccase 3	AY338484.1	37791152	Vv1FL3	TGCGAGCGTAACTCAATGTC
			Vv4RL3	GCAAAGCTCATCCCAAGAAG
Laccase 4	AY338486.1	37791156	Vv1FL4	ATATCTGCTCGGCCATCTTG
			Vv4RL4	GACAGAGCTGCTTCCATTCC
			Vv15FL4	TCATTCTGGGGGTTATCCTG
			Vv45RL4	TAGAAGCGGGTTTCTCCTTG
			Vv05FL4	CTCGTCGCGGGTAAAATATC
Laccase 5	AY338485.1	37791154	Vv1FL5	CAGTGCAATTTTGGTCAACG
			Vv4RL5	AAGTGTCCGATCCACTGTCC
Laccase 6	AY338487.1	37791158	Vv1FL6	TGGAACAGCCTCTCACACAC
			Vv4RL6	ATTCGACACCAGGTCTGAGG
			Vv01FL6	CAACACCCTCACTGTCGTTC
			Vv35RL6	CACAAAGGTCATCCCACTCC
Laccase 3 (for remaining portion)	Received genome sequence of lcc3	NA	Vv2FL3	AGCCCGTTGACAACTACTGG
			Vv3RL3	GGGACGGCATTTTCTATGAG
			Vv2RL3	AGTTGTCAACGGGCTGATTG
			Vv3FL3	TTGATTGGGATATGCTGCAC
Laccase 1(for remaining portion)	Received genome sequence of lcc1	NA	Vv18FL1	GGCTGATTTCGATCTCTTGC
			Vv2FL1	CCGTTATCAATAGGCCAACG
			Vv18RL1	AATCAGCCTCATGCGATACC
			Vv2RL1	TTGATAACGGAATGCGTCAG
			Vv3RL1	CCTAGGGTCTTGCTCGAGTTC
			Vv08FL1	TTGAGCTCGCATACGTTGAC
			Vv45RL1	TCTGAAGCGGGGATAGAAAA

Analysis of sequences and annotation for intron positioning: The received sequences were edited using the BioEdit free software. This software facilitates visualization of the nucleotide sequences along with their peaks on chromatogram. The poor quality sequences were first deleted, followed by blast searches to verify the genes. The confirmed cleaned sequences were saved.and compared with the available mRNA sequence using CAP contig. The comparison involved opening of the cleaned sequences from both forward and reverse primers of respective laccase genes along with mRNA sequence together inside the same window. The sequences obtained from both forward and reverse primer were matched with the sequence of mRNA to identify the location of different introns in the laccase genome. Further annotations for finding amino acid sequences and the intron positions at the amino acid level were performed using the Artemis free Software. The annotated nucleotide and amino acid sequence obtained from different laccase genes were prepared in Sequin for their submission to NCBI data base as the new sequences.

Statistical Analysis: The intron profile of each laccase gene was scored as '1' for presence and '0' for absence of intron. A putative ancestor laccase gene with no intron was added. A

combined binary data matrix for all the laccase genes was constructed. The binary data matrix was entered in the Paup package version 4.0 beta [27] and data was analysed using DOLLO parsimony [28]. Bootstrapping was carried out with 1000 replications.

RESULTS AND DISCUSSION

Amplification of laccase genes: Out of the six pairs of primers corresponding to the six different laccase genes of strain Vv-1, unique single bands of desired sizes were obtained only for laccase genes 1 and 3. For laccase 2, only low molecular weight fragments (probably primers dimers) were obtained, while in the case of laccase 4, multiple amplicons were obtained, which indicates non-specificity of the primers. For laccase 5 and 6 genes, the amplicons of desirable size were obtained but were in very low concentration. In order to improve the amplification efficiency, the PCR was performed at an annealing temperature of 57 ± 2ºC and amplicon's band intensities increased with the increase in the annealing temperature (Fig 1). Similarly, attempts were made for laccase 4 and 5 genes at annealing temperatures of 57 ± 2ºC and 54 ± 1ºC, respectively but no further improvement in amplicon quality (intensity /purity) was recorded, hence the primer pairs were rejected due to low specificity. The redesigned primers of laccase 2 and 6 genes were tried at annealing temperatures of 58 ± 2ºC and laccase 2 exhibited fairly good amplification at almost all temperatures (56-60ºC), while laccase 6 gene exhibited amplification at only 59 and 60ºC annealing temperatures. In case of homozygotic strain BBSR-003, out of 4 pair of primers (laccase1, 2, 3 & 6) attempted at 57 ± 1ºC annealing temperatures, appropriate amplification were obtained only for laccase 3 gene.

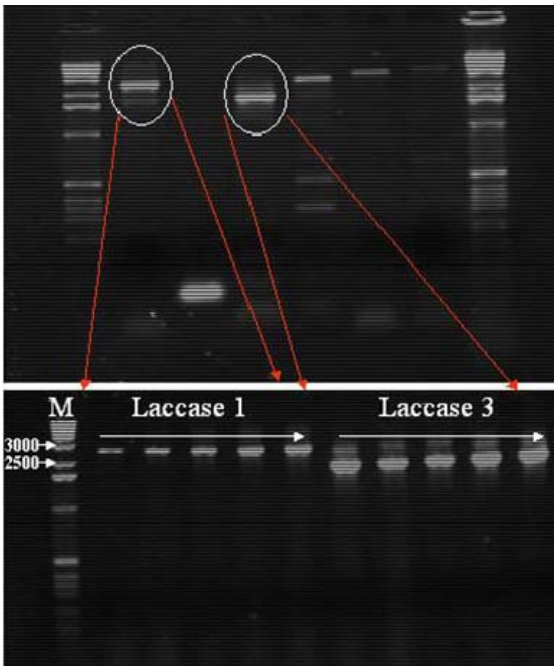

Figure 1: PCR amplification of different laccase genes of *V.volvacea*.
a) From left to right, surrounded by 1 Kb ladder (Life Technologies), Laccase 1 to 6. b) DNA ladder, Laccase 1 annealing temperature from 55 to 59ºC, Laccase 3 annealing temperature from 55 to 59ºC

Intron positions in different laccase genes: The CAP contig of laccase 1 gene revealed a total of sixteen introns in addition to the possibility of additional introns at the beginning of the gene. The number of introns was also sixteen in laccase 2 gene. However, in same stretch of gene length, it had intron 12, which was absent in lcc 1. The three introns (70, 72 and 75), which existed in lcc1 were missing in this gene. Laccase 2 exhibited two introns (3 and 4) in the genome region, where sequence is missing in lcc1 and lcc3 (Fig. 2). A total of eleveen introns could be documented in the same gene length of laccase 3 from strain Vv-1, out of which only six matched with the positions of introns in lcc1 and seven in lcc2 (Fig. 3). The small partial sequence of lcc6 we obtained exhibited only five introns (position 72, 39, 73, 74 and 77, a new position between 62 and 75) and their location was not considered in laccase gene classification nor in the phylogenetic analyses between laccases of different mushroom species. In a similar study carried out on *Coprinopsis cinerea*, the number of introns in different laccase genes varied from what we have recorded in the present study [23]. It was 7 in lcc1, while 19 in lcc17 [23, 29].

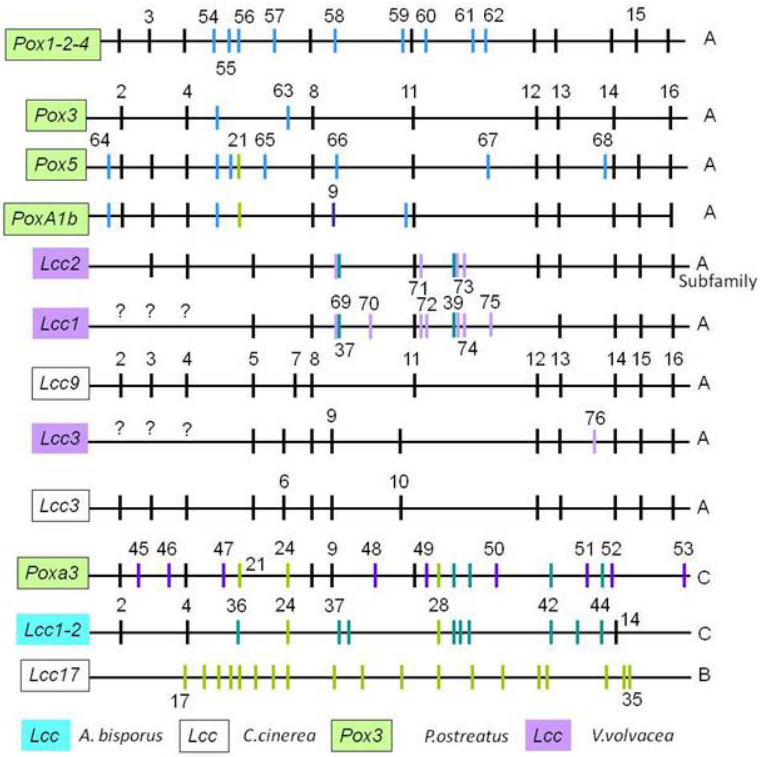

Figure 2. Relative positions of introns in different laccase genes of different sub-families. Nomenclature of introns 2 to 35 [23], 36 to 44 (*A.bisporus* lcc1 and lcc2), 45 to 68 (*P. ostreatus* Poxa3, Pox1-2-4, Pox3, Pox5), 69 to 76 (*V.volvacea* lcc1 and lcc3). *P. ostreatus* gene nomenclature corresponds to: Pox1(LACC9), Pox2 (LACC10), Pox3 (LACC4), Pox4 (LACC1), Pox5 (LACC11), PoxA1b (LACC6) and Poxa3 (LACC2)

The position of introns in different laccase genes of different mushrooms was compared and the laccases belonging to sub-family A (Lcc3 and Lcc9) from *Coprinopsis cinerea* exhibited a closer relationship with respect to positions of introns in their genes than laccase genes belonging to sub-family B or C (Fig. 2). Fig. 3 presents the comparative sequences of laccase 2 gene of strain Vv-01 of *V. volvacea* with positions of 16 introns to the published mRNA

sequence from some other strain. In this figure the gray boxes indicate bases that still need to be confirmed. Some of the ambiguous results were due to the heterozygocity of this gene (two sequences of gene) in the heterokaryotic strain Vv-01.

Figure 3: Sequence of laccase 2 gene of strain Vv-01 of *V. volvacea* with positions of 16 introns, compared to published mRNA sequence.
a) Letters in bold indicate the position of forward and reverse primers (Vv1FL2 and Vv42RL2). b) Grey boxes indicate bases that must be confirmed

A gap underlined corresponds to a base pair defect in the published mRNA sequence, which might have arisen in consequence of frame shift resulting from a first gap in the sequence (probably localised 40 to 80 bp before in the sequence missing in this work). The amino acid translation of the sequence before the identified gap should be LGSLMPSGSYIEL (which is very similar to laccase 1 homologous sequence: VSSLLPSGSYIEL) instead of IRLAHAQRILYRV.

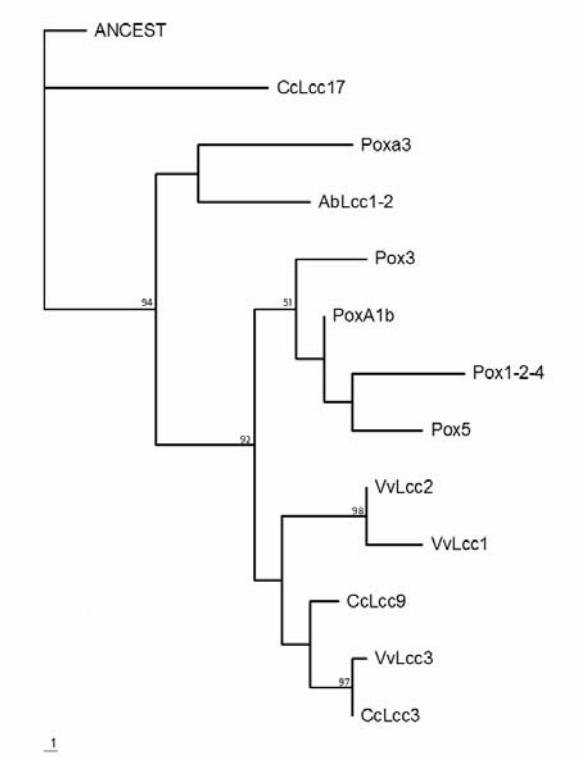

Figure 4: One of the eight most parsimonious cladograms obtained by Dollo parsimony deduced out of 75 introns positions in laccases genes of three subfamilies of four basidiomycetes.
Thirty intron positions were parsimony-informative characters. Bootstrap values are from 50 % majority-rule consensus tree. Sub-family B - CcLcc17, sub-family C- Poxa3 and AbLcc1-2, sub-family A – all other genes, ANCEST – hypothetical ancestor gene with no intron.

Eight most parsimonious cladograms deduced from the positions of introns in twelve laccase genes belonging to three sub-families from four different basidiomycetes, revealed formation of eleven different clades (Fig. 4). In this dendrogram, Lcc3 from *V. volvacea* occupied the same clade as Lcc3 from *C. cinerea* in 97% of the bootstraps. Lcc1 and Lcc2 from *V. volvacea* also formed one clade with more than 98% of the bootstraps. Lcc9 from *C. cinerea* belonging to sub-family A formed a separate clade from Lcc3 from same mushroom [23]. The four laccases (Pox1-2-4, Pox5, PoxA1b and Pox3) of *P. ostreatus* belonging to sub-family A formed a clade in 97% of the bootstraps. All sub-family A laccase genes formed a clade supported by 92 bootstraps and sub-family A and C laccases formed clade supported by 94 bootstrap value. The only laccase (Lcc17) from *C. cinerea* belonging to sub-family B shared only 3 intron positions with genes of sub-family A and C (Fig. 2). The results indicate high

similarities between laccases of one sub-family belonging to a particular species and their distinctness from laccases of other sub-families either from the same or other related species. Laccases from *V. volvacea* share into two groups as already shown with protein sequences. In neighbour joining tree of the deduced amino acid sequences of *C. cinerea*, lcc2, lcc3, lcc12, lcc13 and lcc14 were placed in one clade [23]. In Hoegger's study [24], the neighbour joining tree of basidiomycete laccases based on realigned sequences has also clustered Vvo lac3 with Cci Lac3, as in present study. In Fig. 4, VvLcc2 and VvLcc1 clustered outside of the clade along with CcLcc9, VvLcc3 and CcLcc3, but another of the eight most parsimonious cladograms obtained, have placed these two genes as the sister group of the remainder of the sub-family A laccases.

Sequence variations in lcc3 of two strains: The nucleotide sequence obtained for laccase 3 genes of two strains (Vv-01 and BBSR-003) varied at ten different nucleotide positions. Strain Vv-01 exhibited heterozygocity at 10 different places, which are presented as R, Y, R, Y, R, Y, R, Y, R and Y, which mean A or G for R and C or T for Y. However, at these locations, the nucleotides in strain BBSR-003 were A, T, G, T, G, C, A, C, G and C respectively. This proves heterozygocity of this mushroom at the nucleotide level of this laccase gene (Fig 5). However, at the amino acid sequence level, there was only one difference: strain Vv-01 had D or N (i.e. Aspartic acid or Asparagine) noted X (i.e. any amino acid) in Fig. 6, while strain BBSR-003 had N means Asparagine.

The present study has mainly analyzed the variations in number and position of introns in different laccases of *V. volvacea* and compared that variation to other known laccases of *A. bisporus*, *P. ostreatus* and *C. cinerea*. The study has generated some valuable information like the grouping of *V. volvacea* laccases (lcc1, lcc2 and lcc3) in sub-family A and confirmation of clustering VvLcc3 with CcLcc3. In the present study, there was an intron 8, found earlier in lcc3 from *C. cinerea* but has not been spliced in the published mRNA sequence. This intron has to be spliced in strain Vv-01 to give a functional protein and by virtue of which, the published phylogenetic position of lcc3 in the laccase dendrograms is probably wrong. In lcc1, 16 different introns have been recorded and the sequence of Vv-01 strain differs by one amino acid from the published sequence of lcc1. No evidence of heterozygocity has been recorded in this sequence. The lcc2 gene also revealed 16 different introns. Although only a small portion of sequence could be validated but that also proved some heterozygocity in strain Vv-01. The small sequence of lcc 2 in strain BBSR-003 also revealed differences at some bases from strain Vv-01. At the protein sequence level, there were clearly 26 amino acids of the published mRNA, which were on the wrong reading frame. Maybe it is due to errors in sequencing, or the strain used had a pseudo gene or a very original allele for lcc 2. Due to these factors, the position of laccase 2 in the dendrogram will be changed. The sequence of lcc6 has helped in identification of 5 introns. The positions of introns in different laccase genes (lcc1, lcc2 and lcc3) of *V. volvacea* showed that they belong to sub-family A. Although not the main objective, the present study has proved heterozygocity in strain Vv-01 of *V.volvacea*, which has not been shown in earlier studies carried out on this species.

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lcc3_Vv1        TTGCGAGCGTAACTCAATGTCCTATTGTCCCATCAGAGTCTTTCCTCTATRATTTCACCG
lcc3_BBSR003      ----------TCAATGTCCTATTGTCCCATCAGAGTCTTTCCTCTATAATTTCACCG
                           *********************************** *********

lcc3_Vv1        TTCCAGACCAGGCTGGGACTTTCTGGTATCATTCTCATYTGTGTTAGTGGGRCCCGCCTA
lcc3_BBSR003    TTCCAGACCAGGCTGGGACTTTCTGGTATCATTCTCATTTGTGTTAGTGGGGCCCGCCTA
                ************************************ ************ ********

lcc3_Vv1        GTACCCTGCATTCGGATCGGTTYATTGACGTCGACCCTRACAGCTACACAGTATTGCGAT
lcc3_BBSR003    GTACCCTGCATTCGGATCGGTTTATTGACGTCGACCCTGACAGCTACACAGTATTGCGAT
                ********************* *************** ********************

lcc3_Vv1        GGTCTCAGGGGCCCGCTYGTCGTTTATGATCCTGATGACCCTCATCGACACCTGTACGCC
lcc3_BBSR003    GGTCTCAGGGGCCCGCTCGTCGTTTATGATCCTGATGACCCTCATCGACACCTGTACGCC
                ***************** ********************************************

lcc3_Vv1        GAGTTAGTTCATTTATTGCAAGGCRGACTCTAACCATATAATAGATATGATATAGACGAY
lcc3_BBSR003    GAGTTAGTTCATTTATTGCAAGGCAGACTCTAACCATATAATAGATATGATATAGACGAC
                ************************ ***********************************

lcc3_Vv1        GGTGCGCATCATGATGTGGCTGAATCAATGCCACCCCGATTCTTACGTTGCCCATAGAAT
lcc3_BBSR003    GGTGCGCATCATGATGTGGCTGAATCAATGCCACCCCGATTCTTACGTTGCCCATAGAAT
                ************************************************************

lcc3_Vv1        CCACAATCATAACTCTTTCGGACTGGTATGAGTTCCACTGGACTAATCCCGCTCATAGAC
lcc3_BBSR003    CCACAATCATAACTCTTTCGGACTGGTATGAGTTCCACTGGACTAATCCCGCTCATAGAC
                ************************************************************

lcc3_Vv1        TGAGCTTCTGCACAAGGTACCATGTAAAGGCACCGGTTGCAGGACTTCARCCTACACCTA
lcc3_BBSR003    TGAGCTTCTGCACAAGGTACCATGTAAAGGCACCGGTTGCAGGACTTCAGCCTACACCTA
                ********************************************** ***********

lcc3_Vv1        TGTCCACGCTTATAAACGGAAGGGGGAGATATGCCGGAGGCCCTAACGTGCCTCTTGAGG
lcc3_BBSR003    TGTCCACGCTTATAAACGGAAGGGGGAGATATGCCGGAGGCCCTAACGTGCCTCTTGAGG
                ************************************************************

lcc3_Vv1        TGATTAACGTAGTCCCTGGAACAAGATATCGCTTCCGCCTAGTATCAATGGTTTGCGATC
lcc3_BBSR003    TGATTAACGTAGTCCCTGGAACAAGATATCGCTTCCGCCTAGTATCAATGGTTTGCGATC
                ************************************************************

lcc3_Vv1        CYAACTACATCTTTTCCATAGACGGACACGAACTGGTATGCTATGCTCTTGCATAAAAGG
lcc3_BBSR003    CCAACTACATCTTTTCCATAGACGGACACGAACTGGTATGCTATGCTCTTGCATAAAAGG
                * ************************************************************
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Figure 5: Variations in partial nucleotide sequences of laccase 3 from strains Vv-01 and BBSR-003 of *V. volvacea*

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lcc3_BBSR003    ----QCPIVPSESFLYNFTVPDQAGTFWYHSHLSTQYCDGLRGPLVVYDPDDPHRHLYDI
lcc3_Vv01       ASVTQCPIVPSESFLYXFTVPDQAGTFWYHSHLSTQYCDGLRGPLVVYDPDDPHRHLYDI
                    *********** ******************************************

lcc3_BBSR003    DDESTIITLSDWYHVKAPVAGLQPTPMSTLINGRGRYAGGPNVPLEVINVVPGTRYRFRL
lcc3_Vv01       DDESTIITLSDWYHVKAPVAGLQPTPMSTLINGRGRYAGGPNVPLEVINVVPGTRYRFRL
                ************************************************************

lcc3_BBSR003    VSMVCDPNYIFSIDGHELTIIEADGVNTKPLTVDSIQIFAGQRYSFILNANQPVDNYWIR
lcc3_Vv01       VSMVCDPNYIFSIDGHELTIIEADGVNTKPLTVDSIQIFAGQRYSFILNANQPVDNYWIR
                ************************************************************

lcc3_BBSR003    ANPNLGTTGFTNGINSAILRYEGAPLQDPTTPLVPSVNPLVESNLRNLIENAVPGQPFPG
lcc3_Vv01       ANPNLGTTGFTNGINSAILRYEGAPLQDPTTPLVPSVNPLVESNLRNLIENAVPGQPFPG
                ************************************************************

lcc3_BBSR003    GADININLFTDFDWDMLHFTVNGSPYIPPNAPVLLQILSGTYNAQDLMPAGSIIELPSNK
lcc3_Vv01       GADININLFTDFDWDMLHFTVNGSPYIPPNAPVLLQILSGTYNAQDLMPAGSIIELPSNK
                ************************************************************

lcc3_BBSR003    VIEISMPGGSPGSPHPFHLHGHTFDVIRSTDTQEYNFVDPVRRDVVNTGFETDNVTIRFT
lcc3_Vv01       VIEISMPGGSPGSPHPFHLHGHTFDVIRSTDTQEYNFVDPVRRDVVNTGFETDNVTIRFT
                ************************************************************

lcc3_BBSR003    TDNPGPWILHCHIDWHLEVGLAVVFAEDIPRISTPAPPPSW----
lcc3_Vv01       TDNPGPWILHCHIDWHLEVGLAVVFAEDIPRISTPAPPPSWDELC
                ****************************************
```

Figure 6: Variations in amino acid sequences of laccase 3 from strains Vv-01 and BBSR-003 of *V. volvacea*

We compared intron positions present in sub-family A laccase genes in *A. bisporus* and *C. cinerea* from Agicoid clade (VI) and *P. ostreatus* and *V. volvacea* from Pluteoid clade (III) [17]. From this comparison, it is clear that only intron position 2 was shared exclusively by *P. ostreatus* and the Agaricoid clade laccases. However, data in the present study is insufficient to indicate whether this intron position is present in *V. volvacea*. In contrast, intron positions 5, 6 and 10 are shared exclusively by *V. volvacea* and Agaricoid clade laccases. VvLcc3 clustered with Agaricoid clade VI sub-family A genes while *P. ostreatus* laccase genes formed a sister clade. This could indicate that ancestor genes from sub-family A laccases from *V. volvacea* and Agaricoid species could have diverged more recently than from ancestor genes of *P. ostreatus* sub-family A laccases. However VvLcc1 and VvLcc2 share intron position 37 and 39, which are present in laccase sub-family C and in Polyporus sub-family A laccases (Billette C. unpublished result). Then they could belong to the clade containing Polyporus laccase in sub-family A. Moreover, position of these two genes vary in the different cladograms we have obtained. Present finding is not sufficient to elucidate the respective phylogenetic positions of *V. volvacea*, *P. ostreatus* and Agaricoid clade species. To build further species phylogenetic trees, it is important to use sequences from *V. volvacea* and from *Volvopluteus gloiocephalus*, since they don't cluster together as demonstrated by Justo *et al*. [19].

The present findings have implications on other issues such as the role of intron positions in phylogenetic studies and the use of nucleotides sequence variability in exons flanking the specific introns for species identification, as it was done in species discrimination in Sclerotiniaceae [20]. The sequence of conserved copper binding amino acids of novel laccase genes also has the potential to help in resolving speciation conflict in several closely related species, where other molecular techniques have yielded confusing inferences, like in the case of Sclerotiniaceae [20]. The concept suggests redefining different genera by identifying these genes and concomitantly supports reclassification of misleading fungal species [30]. The study carried out by Castilho *et al*. [22] has reinforces the laccase diversity and interspecific variation in this genetic pool. As one of the most important criteria in phylogenetic systematics is the issue of homology [31], and out of different types of homologies, laccases fall in the category of paralogy, which means that they originate from gene duplication events. For example, the laccase amino acid sequences have been used for phylogenetic study and the molecular evolution studies can still further help in finding the laccases of industrial importance even in still unknown species.

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AN ASIAN COMMERCIAL STRAIN OF *AGROCYBE CHAXINGU* AND A EUROPEAN WILD STRAIN OF *AGROCYBE CYLINDRACEA* EXHIBITING MORPHOLOGICAL DIFFERENCE AND HIGH GENETIC DIVERGENCE ARE INTERFERTILE

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ABSTRACT

Three molecular markers were compared between a cultivated strain of *Agrocybe chaxingu* from South-Eastern Asia (SM960903) and a French wild strain of *Agrocybe cylindracea* (SM47): (i) the nuclear ribosomal unit, (ii) the mitochondrial SSU-rDNA and (iii) the mitochondrial *cob* gene. All markers reveal extensive variations: the ribosomal units possess up to 67 polymorphic sites (89 % of nt identity), the variable domains of the mitochondrial SSU-rDNA differ by large indel sequences and the *cob* gene molecular organization is characterized by non-orthologous group I introns. In fruiting tests, sporophores of the two strains were macroscopically distinguishable, especially by a thicker and stronger annulus in *A. chaxingu*. In inter-stock mating tests, hyphal clamp connections were detected in mycelia of the junction lines for all the confrontations. The dikaryotic status of the presumed hybrids was confirmed by the presence of both *A. chaxingu* and *A. cylindracea* nuclear ribosomal units. Dikaryotization was also observed in distal regions from the junction lines, indicating that nuclei migration occurred in these matings. Four selected hybrids containing each of the four *A. cylindracea* mating type genotypes were put in fruiting conditions. These hybrid sporophores were molecularly verified and shown to be fertile based on the presence of mature basidiospores. Despite extensive variations at the molecular and phenotypic levels, the complete inter-fertility observed between the Asian *A. chaxingu* and the European *A. cylindracea* strains strongly argues for con-specificity of both taxa. *A. chaxingu* would be a variety of the *A. cylindracea* species which was firstly described.

Keywords: *Agrocybe chaxingu*, *Agrocybe cylindracea*, interfertility, sporophores, molecular markers

INTRODUCTION

Agrocybe cylindracea (DC.: Fr) Singer, syn. *A. aegerita* (Brig.) Singer (Basidiomycota, Agaricales) is a species of mushroom cultivated in Europe since antiquity, but sporadically cultivated today. In contrast, in Asia, mushrooms called *A. cylindracea* but also *A. chaxingu* Huang are largely cultivated today. As published in the Edible Fungal Flora of China [1], the diagnosis of *A. chaxingu* does not allow a clear distinction between this species and *A. cylindracea*.

During the last thirty years, based on its ability to easily achieve a complete life-cycle in laboratory conditions, *A. cylindracea* was largely studied as a laboratory model of mushroom-producing Basidiomycete. Numerous reports on its genetics and molecular biology were produced. Particularly, several reports have focused on the mitochondrial genes and genome of *A. cylindracea* wild-type strains. In 1997, the sequence and secondary structure of the *A. cylindracea* mitochondrial SSU-rDNA was described [2]. This led to the characterization of variable domains (V4, V6 and V9) of the SSU-rRNA showing species-specific length variations within the genus *Agrocybe*, including European wild-type strains of *A. cylindracea* and Asian cultivated strains of *A. chaxingu* [3]. In the same way, a comparative analysis of the mitochondrial *cob* gene of *A. cylindracea* and *A. chaxingu* strains revealed important genetic variations between the two species and the presence of non-orthologous group I introns in this gene [4, 5, 6]. More recently, the study of Uhart et al. [7], mainly based on comparison of mitochondrial ribosomal SSU-rDNA variable domains, confirmed the strong genetic divergence between the European wild specimens of *A. cylindracea* and strains cultivated in Asia under the name *A. chaxingu*. At that time, these two entities were considered as belonging to a species complex but, taking in account some inter-sterility (not reported in details) between the two entities, the authors suggested that these strains could finally belong to two distinct species. However, in another paper, based on morphological comparisons, Uhart & Alberto [8] suggested that these two species should be finally synonymised but that the type specimen would have to be examined first.

Here, we intend to complete previous studies about the genetic divergence between the two entities and to test the inter-fertility between two strains already studied in the papers cited above: a European wild specimen of *A. cylindracea* and a strain cultivated in Asia under the name *A. chaxingu.* We expect to clarify the situation not only at the taxonomic level but also to obtain a better understanding of species circumscription and of their geographical range in order to help manage their genetic resources and future breeding programs.

MATERIALS AND METHODS

Strains. The European wild–type strain *Agrocybe cylindracea* (= *Agrocybe aegerita*) SM47 from South-West of France and the industrially cultivated *Agrocybe chaxingu* SM960903 from Eastern Asia were previously described [3-7]. Both strains are permanently deposited in the collection of the Laboratory (INRA UR 1264, Villenave d'Ornon, France).

In vitro DNA manipulation and sequencing. Sequences used as molecular markers (nuclear ribosomal unit, mitochondrial *cob* gene) were obtained by conventional procedures from cloned PCR products.

Total DNA of fungal strains were extracted from 0.1 g of dried carpophores or 0.2g of fresh mycelium, after grinding in liquid nitrogen to obtain a fine powder. Nucleic acids were extracted according to the *N*-cethyl-*NNN*-trimethyl ammonium bromide (CTAB) procedure adapted for small quantities of basidiomycete mycelia [9]. DNA (OD_{260}) was quantified using a NanoDrop spectrophotometer (NanoDrop ND-1000, Nanodrop technologie, DE, USA), diluted in deionized sterilized Milli-Q water (Milli-Q water system production, Millipore, Saint-Quentin en Yveline, France) and stored at -20°C.

PCR amplifications were carried out using the Go *Taq* polymerase from Promega Corp. (Madison, Wis, USA) and respective primer pairs synthesized by Eurofins MWG Operon (Germany).

The primers used to amplify the nuclear ribosomal unit were the conventional ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'GGAAGTAAAAGTCGTAACAAGG3')

described by [10]. A CAPS test was developed by using the *Hae*II restriction enzyme from Promega Corp. (Madison, Wis, USA), according to the manufacturer's recommendations.

The primer pairs used to verify, by PCR, the presence of the *A. chaxingu* (E1C/E2C) and *A. cylindracea* (EU1A/ER1A and EU2A/ER2A) introns have been previously described [5].

E1C (nt532 5′CGTAATTACTAATTTATTATC3′ nt552);
E2C (nt1811 5′ACAAAAGGTAATAAATAGTG3′ nt1792);
EU1A (nt267 5′ACCAAATGTAGATTTTGCA3′ nt285);
ER1A (nt2976 5′CTTCCAATATGTATATATAC3′ nt2957);
EU2A (nt3448 5′TTATATTCCAGCAGATCC3′ nt3465);
ER2A (nt4675 5′TAGTTTGTTAGGAATAGATC3′ nt4656).

The sizes of the V4, V6 and V9 variable domains of the mitochondrial SSU-rDNA were verified by electrophoresis analyses of the specific PCR products in 1.2 % agarose gels. Specific primers were as previously described [3, 7].

PCR were performed in a Programmable Thermal Cycler PTC 200 (MJ Research Inc., Watertown, Mass., USA). Each reaction contained 10 to 100 ng of fungal genomic DNA, 1 µM of both primers, 200 µM of each dNTP, 1 unit of *Taq* DNA polymerase, in a final volume of 50 µl of the appropriate buffer. Reactions were run for 30 cycles at 95 ° C for 30s, then two degrees below the lowest Tm of both oligonucleotides for 30s, 72° C for 1 to 2 min, and one final cycle at 72° C for 5 min. An aliquot of 10 µl of each PCR product was analysed by agarose (1%, w/v) gel electrophoresis containing 200ng/ml of ethidium bromide, in TEB buffer [11].

For DNA sequencing, PCR products were purified with the Wizard SV gel and PCR Clean-Up System (Promega Corp.Madison, WI, USA) before they were sequenced by the primer walking methods using the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Courtaboeuf, France) and the corresponding primers used for the PCR reaction. Sequence reactions were carried out according to the supplier's recommendations, in a final volume of 10 µl containing 100 ng of PCR product and 0.5 µM of the specific primer. Sequence reactions were carried out in a thermocycler by applying an initial denaturation step at 95°C for 1 minute; 27 cycles with each composed of the three steps: 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. The reaction products were ethanol precipitated, dried, and then separated by capillary electrophoresis (on an automated sequencer ABI 3130x1, ABI Prism Corp., France) at the genomic platform of the University Bordeaux Segalen (France). Sequencing profiles were edited and corrected using the BioEdit sequence alignment editor v7.0.9 free software (Ibis Biosciences Carlsbad, CA, USA).

Fruiting test, spore germination, and monospore culture. Polypropylene bags containing 250 g of chopped humidified wheat straw + 25 g of oat flour + 50 ml of glucose solution (15g / l), were sterilized in an autoclave. Malt agar medium colonized by mycelium was used to inoculate the bags: the culture medium of two Petri dishes of 9 cm in diameter were deposited at the top of each bag. Incubation lasted 15 days at 25 ° C; then, the temperature was lowered to 18 ° C. After 20 days, bags were opened and fruiting began after one week.

Sporeprints were recovered from mature sporocarps on tracing paper. Spore suspensions at different dilution were spread on complete yeast medium [12]. Eight days later, spores began to give rise to young mycelia that were isolated. Single spore isolates (SSIs) were subcultivated individualy on malt agar medium.

Mating tests. Small pieces of agar culture of two different SSIs were placed at about 2 cm from each other in Petri dish containing malt agar medium. The inoculated mycelia were allowed to grow together at 25°C. Positive mating was indicated by the formation of aerial fluffy mycelium at the junction line between two monokaryons. The fluffy mycelium appearing at the junction line is a presumed dikaryon resulting from a plasmogamy between the two sexually compatible

monokaryotic SSIs. For each confrontation two replicates were made. The fluffy mycelium was isolated and subcultured on malt agar medium.

Cytological method for positive mating confirmation. A simple method was used to confirm the positive mating. SSIs of *Agrocybe* are monokaryotic and without clamped hyphae, while fertile dikaryons exhibit clamp connections. For each positive mating, a fragment of mycelium from the junction zone of any replicate was examined under microscope (x100, congo red staining). Observation of clamp connections confirmed the dikaryotic status of the mated mycelium.

Figure 1. Sporophores of the parental strains differentiated in cultivation.
1a: *Agrocybe chaxingu* strain SM960903, cap after opening; 1b: *Agrocybe chaxingu* strain SM960903 cap before opening; 1c. *Agrocybe cylindracea* strain SM 47 at various stages of maturation.

RESULTS

Morphological comparison of the parental strains. Ten culture bags were performed for each strain. Strain SM47 of *A. cylindracea* began to fruit 35 days after the inoculation of the substrate, while strain SM960903 of *A. chaxingu* began one week later in all replicates. Morphological differences between the sporophores of the two strains were noticeable (Fig.1). These differences include cap color that was much more palish for SM47, or the longer stipe of SM960903.

However, these differences likely characterize the strains and not the species in which these criteria have shown to be highly variable and also sensitive to environmental conditions. However differences were also noted for two other traits that may be taxonomically pertinent: the color of the stipe and the aspect and behavior of the partial veil: (i) the color of the stipe was light brown in *A. chaxingu* SM960903 as in other strains of *A. chaxingu* that we have cultivated (data not reported here) along with the white color of tiny scales that make them more visible. In *A. cylindracea*, the stipe was almost uniformly white; (ii) the partial veil is larger and thicker in SM960903 with the underside layer breaking of in crown wheel before it comes off the cap. Moreover, it fits in the upper stipe; the hymenial cavity is very narrow; the cap opening occurs lately and often after spore maturity. At the opening of the cap, we put some bags on the outside and saw the large membranous ring floating in the wind, likely facilitating the spread of spores that had settled on it. In *A. cylindracea*, the cap opened earlier and the partial veil contracted, forming a shorter and thicker annulus that was not covered by spores at this time. In other respects, we did not observe significant difference in spore sizes between the two specimens. They were on average 10.2 × 5.9 µm (n = 30) for SM47 and 10.4 × 5.9 (n = 30) for SM 960903.

Genetic divergence between the parental strains of *A. cylindracea* SM47 and *A. chaxingu* SM960903. The genetic variation between the parental strain *A. cylindracea* SM47 and *A. chaxingu* SM960903 was studied using three different molecular markers: (i) the nuclear ribosomal unit, (ii) the variable domains V4, V6 and V9 of the mitochondrial SSU-rDNA encoding the 16S RNA of the small sub-unit of the mito-ribosome, and (iii) the molecular organization of the *cob* gene which encodes the unique mitochondrial protein of the Complex III of the respiratory chain. All these markers revealed high divergence between the two strains and it is to be noticed that the specific feature of each strains for all these markers were shared with all the studied European *A. cylindracea* strains (10 different strains) for *A. cylindracea* SM47 and with three additional Asian *A. chaxingu* strains for A. chaxingu SM960903 (data not shown). In details, the nuclear ribosomal region of the *A. cylindracea* SM47 strain, corresponding to the ITS1/ITS4 PCR product had a size of 637 nt, while that of *A. chaxingu* SM 960903 had a size of 652 nt. When aligned (Clustal W), the two sequences possessed 67 polymorphic sites (distributed in 39 non contiguous loci), i.e. showing a percentage of nt identity slightly lower than 90 %.

When cleaved by the restriction endonuclease *Hae*III, the ITS1/ITS4 PCR product of *A. cylindracea* generates three fragments of size 341 nt, 180 nt and 116 nt. On the contrary, the *Hae*III restriction profile of the ITS1/ITS4 PCR product of *A. chaxingu* is constituted by four fragments of size 341 nt, 201 nt, 84 nt and a small one of 26 nt not detectable in agarose gel electrophoresis. From this, the use of the *Hae*III restriction endonuclease allowed the development of a CAPS test to easily discriminate both strains by agarose (1.2 %) gel electrophoresis analysis of the *Hae*III-digested PCR product generated by the ITS1/IIT4 couple of primers. The amplified nuclear ribosomal region *A. cylindracea* strain had a profile with 3 fragments (341, 180 and 116 nt), the *A. chaxingu* one possess the same large fragment of 341 nt accompanied by two fragment of 201 nt and 84 nt. The length variations between the two polymorphic fragments of *A. cylindracea* (180 nt and 116 nt) and *A. chaxingu* (201 nt and 84 nt) were easily detectable by agarose gel electrophoresis analysis.

As previously reported, there were also important genetic variations between mitochondrial genes of *A. aegerita* SM47 and *A. chaxingu* SM960903 strains.

The length of the V4 domain varied from 170 nt in *A. cylindracea* to 281 nt in *A. chaxingu*; the length of the V6 domain varied from 172 nt in *A. cylindracea* to 158 nt in *A. chaxingu* and the length of the V9 domain varied from 221 nt in *A. cylindracea* to 246 nt in *A. chaxingu*. These size variations were mainly due to insertion/deletions events of large sequences occurring in these variables domains [3].

In the same way, the previously established [5] molecular organization of the *cob* gene, shown in figure 2, was verified in the parental *A. cylindracea* SM47 and *A. chaxingu* SM960903 strains by PCR with specific primer pairs located in the flanking exon sequences of each gene. As shown in Figure 2, the two strains differed by the presence/absence of three non-orthologous large group I introns: two in *A. cylindracea* and one in *A. chaxingu*.

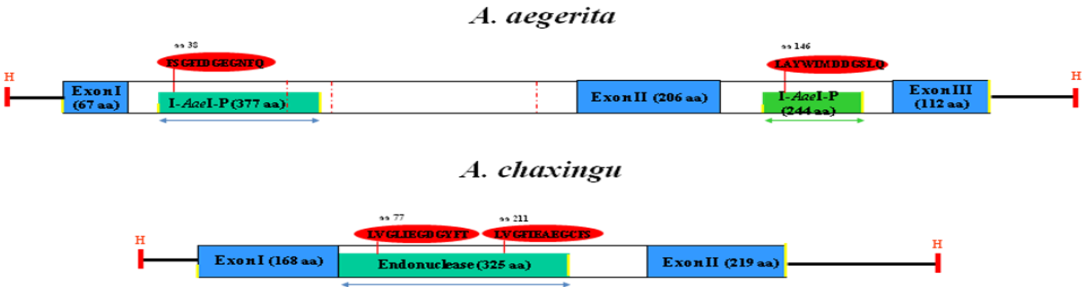

Figure 2: Comparison of the molecular organization of the *cob* gene in *A. chaxingu* (GenBank Acc. N° AY772389) and *A. aegerita* (GenBank Acc. N° AY781064). The *cob* exonic sequences and the intronic *heg* are in blue and green boxes, respectively. The typical LAGLIDADG motif of each HE are indicated. From [5].

Intra-stock and inter-stock mating tests. Parental strains SM47 of *A. cylindracea* and SM960903 of *A. chaxingu* exhibited hyphal clamp connections, in agreement with their dikaryotic status. Spores from SM47 easily germinated, while only few spores of SM960903 germinated. For SM47, 60 SSIs were isolated, but only 15 SSIs for SM960903. All the SSIs were monokaryotic and usable for intra-stock mating tests, except for two SSIs of SM960903 for which clamped hyphae were detected. For SM47, 12 SSIs chosen at random were mated in all pairwise combinations and four groups of incompatibility were recovered based of the detection of hyphal clamp connexions: all the confrontations between group I (1 SSIs) and group II (4 SSIs), or between group III (2 SSIs) and group IV (5 SSIs) were positive and produced clamped mycelia. These data agreed with the heterothallic life cycle and the bifactorial system of sexual incompatibility already described in *A. cylindracea* [13]. For SM960903, 12 SSIs were confronted in all pairwise combinations but only few positive reactions were observed, so, the inter-compatibility groups remain fully identified.

For the inter-stock mating tests, each strain of the seven SSIs of the SM47 offspring, including the single SSI of the group I and two SSIs of each of the three remaining incompatibility groups, were confronted with each of the 12 SSIs of the SM960903 offspring. Positive reactions were generally not clearly visible but rather ambiguous. After six weeks, mycelia at the junction line were examined and hyphal clamp connections were detected in all 84 (= 7 x 12) confrontations.

Isolation of 56 presumed hybrid mycelia exhibiting clamp connections. For 48 confrontations performed between the 12 SSIs from SM960903 and 4 SSIs from SM47 (SM47-6, SM47-7, SM47-11, SM47-13) that belonged to each of the four incompatibility groups I, II, III and IV, hybrid dikaryotic mycelia were isolated from the junction line, and they were then subcultured for further genetic analyses and fruiting tests. In other respects, in the dishes of confrontation between SSIs from the two parental strains, we also tried to detect hyphal clamp connections in the growing area of each monokaryon: they were almost always detected in the area of monokaryons deriving from strain SM960903, but less frequently in the area of the monokaryons deriving from strain SM47. In four dishes corresponding to four different confrontations, hyphal clamps were detected in the areas of both monokaryons, fragments of

mycelia were isolated from each of the two areas, additionally to the mycelium isolated from the junction line. Finally, a total of 56 hybrid mycelia were isolated.

Genetic confirmation of the hybridization. DNA of the 56 presumed hybrid dikaryotic mycelia (isolated from 48 strains collected on the junction lines and 8 strains coming from regions distant from these lines) were extracted. For each strain, the nuclear ribosomal unit was amplified as described in the Materials and Methods section by using the conventional ITS1/ITS4 couple of primers. The resulting PCR products were digested by *Hae*III and the restriction fragments analysed by agarose gel electrophoresis. As expected, this CAPS marker revealed for each strain, a typical heteroallelic profile. In details, all the 48 presumed hybrids from the junction lines, as well as the 8 strains isolated from the areas of the confronted homokaryons, distant from these junction lines possessed both largest *Hae*III restriction fragments characterizing each parental strain: the 342 nt fragment of *A. Cylindracea* and the 422 nt fragment of *A. Chaxingu*. This pattern confirms in all tested cases the presence of both parental nuclei. All these 56 isolates were therefore assumed to be dicaryotic. The presence of both parental nuclei in the 8 strains coming from regions distant from the junction lines suggests that, as deduced from the presence of clamp connections on these mycelia, a migration of the compatible nuclei has occurred in the recipient homokaryotic hyphae.

Cultivation and morphology of the hybrids. Among the 48 isolated hybrid strains, we chose four representatives as follow: all the parental homokaryons from SM960903 that were different and all the parental homokaryons from SM47 that belonged to the four different incompatibility groups. From the information we had, these four hybrids were potentially the most different that we could find: SM47-13 × SM960903-6, SM47-7 × SM960903-3, SM47-11 × SM960903-12, and SM47-6 × SM960903-2. All of them easily fruited and sporulated. Sporophores of three of them are showed on Fig. 3. The cap color at the disc was dark brown like the *A. chaxingu* parent or palish brown like the *A. cylindracea* parent; however, the cap color at the edges was always palish like the *A. cylindracea* parent. In all cases, the stipe had a white brown color like the *A. chaxingu* parent. The annulus was rather small and did not open lately as for the *A. cylindracea* parent but a crown wheel is more or less visible but less pronounced than in the *A. chaxingu* parent.

Molecular characterization of the hybrid sporophores. Sporophores produced by the four hybrids were molecularly characterized by nuclear and mitochondrial markers. The CAPS test described above allow to verify that the sporophores differentiated by the four fruiting hybrids possessed the heteroallelic pattern of the nuclear ribosomal units (restriction fragments of both parental strains), and consequently were also dikaryotic.

The molecular organization of the mitochondrial *cob* gene of a sporophore differentiated by each of the four fruiting hybrids was studied by PCR as described in the Materials and Methods section with three primer pairs allowing the evidence of each of the three non-orthologous group I introns to be detected: *iAeI* and *iAeII* carried by the *A. cylindracea* parental *cob* gene and *iAchax* carried by *A. chaxingu*. Interestingly, all the four sporophores possess only the *A. chaxingu iAchax* intron, This suggests that the molecular organization of the mitochondrial *cob* gene present in the differentiated sporophores was that of the parental *A. chaxingu* strain. This was also confirmed by amplification of the V4, V6 and V9 variable domains of the mitochondrial SSU-rDNA whose size corresponded to the *A. chaxingu* SSU-rDNA. As both genes (*cob* and SSU-rDNA) are located in distant regions of the mitochondrial genome (data not shown), the differentiated sporophores were assumed to possess the mitochondria of the parental *A. chaxingu* strain. This is in accordance with the previously reported uniparental heredity of mitochondria observed in sporophores of *A. cylindracea* [14].

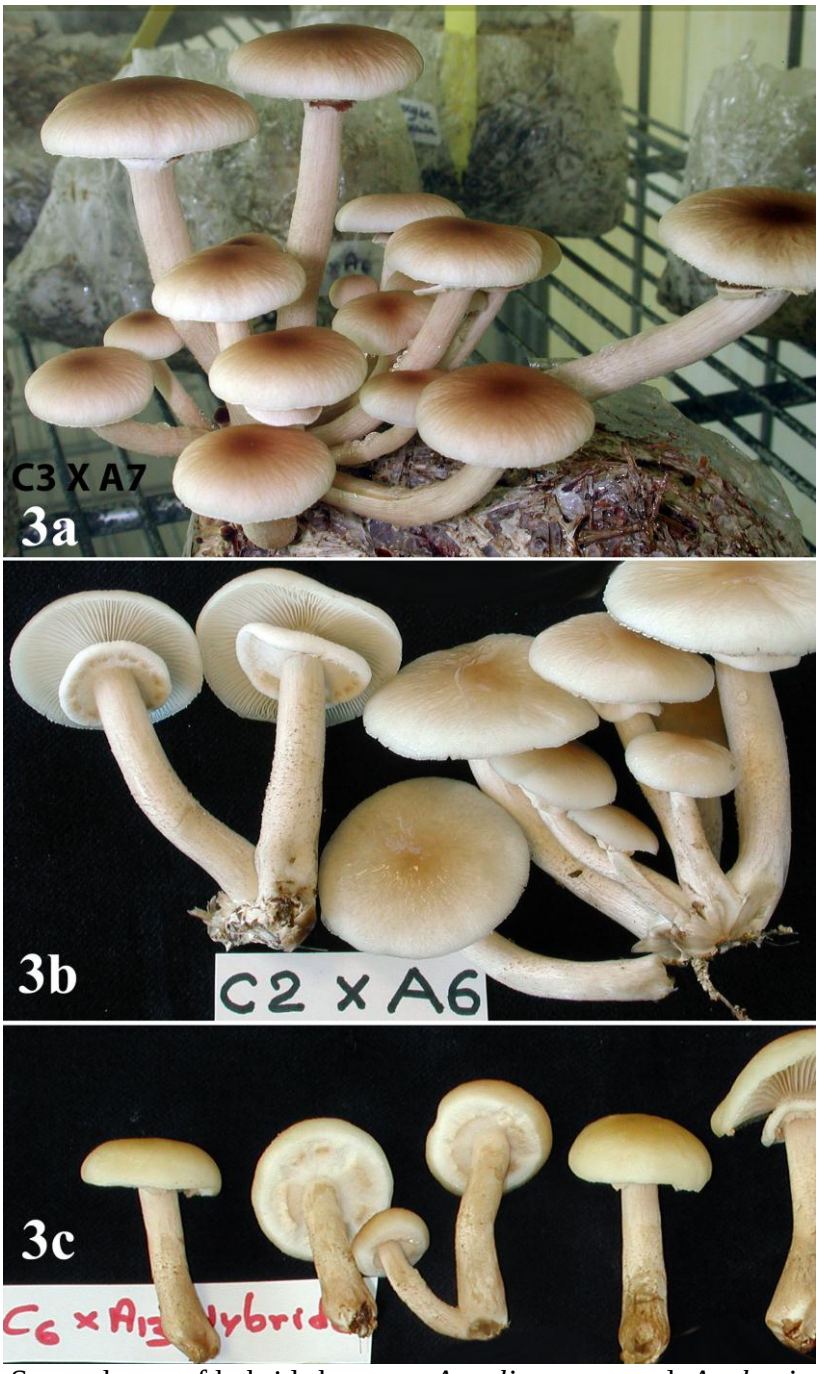

Figure 3. Sporophores of hybrids between *A. cylingracea* and *A. chaxingu*.
3a: SM47-7 × SM960903-3; 3b: SM47-6 × SM960903-2; 3c: SM47-13 × SM960903-6.

DISCUSSION

Although the main difference reported in the literature between *A. cylindracea* and *A. chaxingu* is their natural habitat, our comparison between a wild type strain of *A. cylindracea* and a strain cultivated in Asia under the name *A. chaxingu* showed that the sporophores of the latter had a more colorful stipe and a partial veil that separated later from the cap, forming a wider ring covered with spores. Such differences are not so visible on dried specimens. On the other hand, we are not sure that the habitat is a strong criterion in this complex because, in Europe, *A.*

cylindracea grows easily on various *trees*, including *Acer negundo* which was introduced from North America. Moreover, both strains were able to grow and form fruiting bodies on wheat straw. At the molecular level, the 39 differences between the two parental strains were detected. This is much greater than the number of polymorphic positions that can be found between strains belonging to the same species in the genus *Agaricus*, which rarely exceeds 2 or 3. Such a difference suggests that mutation rate would be higher in the ITS of *Agrocybe* and/or that reproductive barrier more easily takes place in *Agaricus*.

Intra-stock mating tests revealed a bifactorial system of sexual incompatibility for the strain of *A. cylindracea*; although the reproductive system of the other strain was not resolved. In inter-stock mating tests, monokaryons from the two parents were all sexually compatible, indicating that the mating type alleles of tested monokaryons of *A. chaxingu* differ from the alleles of the strain of *A. cylindracea*. Systematic detection of clamped mycelia in the area of the monokaryon of *A. chaxingu* suggests that nuclei from *A. cylindracea* may have migrated, dikaryotizing the monokaryon of *A. chaxingu*. Many hybrid strains were isolated from the interstock mating tests and confirmed cytologically and molecularly. Four representatives of these hybrids were cultivated on fruiting medium: they fruited easily and abundantly. In the four cases, sporophores were fertile with a veil similar to the veil of the *A. cylindracea* parent, but the stipe was colored similarly as *A. chaxingu*. Moreover we showed that the mitochondria were inherited from the *A. chaxingu* strain. Such a uniparental inheritance of the mitochondria is frequent in basidiomycota and especially in *Agrocybe cylindracea* [14]. Since we also noted that nuclei of the monokaryon of *A. cylindracea* apparently easily invaded the monokaryon of *A. chaxingu* and since the putative hybrids were isolated relatively late (6 weeks after the beginning of the co-culture), it is possible that most of the isolates were in fact dikarotized monokaryons of *A. chaxingu*, bearing the mitochondria of *A. chaxingu*. We finally conclude that both entities are completely interfertile although they highly diverge genetically. These data on one hand show that the two entities are conspecific, but, on the other hand, support the concept of a varietal rank for the Asian entity. The type specimen of the latter remains to be studied to determine if the commercial strains named *A. chaxingu* are truly related to this species. Morphological differences being not easy to detect on dried specimens, the best solution should be to compare DNA sequences. Such data would be useful but are not necessary to describe and to name a new variety. To describe such a variety, specimens with morphological and molecular characteristics similar to those of commercial strains used here, but of known origin would be more appropriate.

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DIVERSITY OF BOLETES IN PAKISTAN – FOCUS ON *SUILLUS BREVIPES* AND *SUILLUS SIBIRICUS*

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ABSTRACT

During the exploration of diversity of non-gilled fungi and their ectomycorrhizal morphotypes from Pakistan, *Suillus brevipes* was found ectomycorrhizal with *Quercus incana* while *Suillus sibiricus* was found associated with roots of *Pinus wallichiana* and *Salix alba*. Basidiomes and ectomycorrhizae of the latter are characterized morpho-anatomically and by using rDNA-ITS and 5.8S gene while for the former, both the basidiome and the ectomycorrhizae have been identified and characterized morpho-anatomically. Previously, *S. sibiricus* was found associated only with *P. wallichiana*. In the present work *S. alba* has been found as new host for *S. sibiricus*.

Molecular analysis of *S. sibiricus* was used to describe the phylogenetic position of the fungus using Maximum Parsimony analysis. *S. brevipes* and morphotypes of both boletes are new records for Pakistan.

Keywords: Ectomycorrhizae; Non-gilled fungi; Pakistan; PCR-ITS; Sequence alignment

INTRODUCTION

Boletes (Basidiomycota; Agaricomycetes) are characterized by the presence of tubes instead of gills [1, 2]. There are 35 genera and more than 1,000 species of Boletes known worldwide [3] with only ten (10) genera and forty four (44) species reported from Pakistan [4-6]. They are distributed mostly in Europe, Asia, North America, in the temperate latitude in the northern hemisphere [7, 8] while mostly confined to moist temperate coniferous forests in Pakistan [4, 9]. More than 90% of members of this group form symbiotic associations with the root system of forest trees, which is the most important tool for germination and good development throughout the life of tree [10]. The plant families *Betulaceae, Caesalpiniaceae, Casuarinaceae, Dipterocarpaceae, Ericaceae, Fagaceae, Mimosaceae, Myrtaceae, Pinaceae* and *Salicaceae* form ectomycorrhizal symbioses with Boletes [11, 12]. This group is economically important because it contains edible, medicinally-important and poisonous mushrooms [13,14, 15].

Suillus P. Micheli ex Adans. is a genus of Boletes belonging to *Suillaceae* represented by 54 species worldwide [3] with only four species known from Pakistan [4, 5, 6]. Wu et al. [16] discussed the bio-geographic pattern and phylogenetic relationship of *Suillus* species from Eastern Asian (China and Nepal) and North American territories. During an investigation of the

diversity of boletes in Pakistan, *S. brevipes* and *S. sibiricus* were isolated and in the present work they are described morpho-anatomically along with their ectomycorrhizal morphotypes from Pakistan. *Suillus sibiricus* and its ectomycorrhizae have been described up to molecular level and identified by using nrRNA tandem repeats. Molecular phylogeny of *S. sibiricus* has also been discussed with Eastern Asian and Eastern North American isolates retrieved from Genbank.

MATERIALS AND METHODS

The study area is located in Himalayan moist temperate forests of Pakistan that are evergreen forests of conifers mixed with some oaks and other deciduous broad-leaved trees. Their undergrowth is rarely dense, and consists of both evergreen and deciduous species. These forests occur between 1,500 m and 3,000 m elevation with mean annual rainfall about 1,700 mm. Average aerial humidity is 57% and the mean annual temperature is 12ºC [17]. Many Boletes form symbiotic association with the roots of these plants for their better growth [4].

Sampling was done during the rainy season from July to August 2008 - 2010. Sporocarps and soil samples containing their ectomycorrhizal morphotypes were collected. Small parts of both the fruiting body and ECM were stored in 2% CTAB buffer for DNA analysis. Soil blocks were wrapped in polythene bags to avoid evaporation and crumbling. Fresh characters of sporocarps were recorded in the field and photographed. Sporocarps were dried properly and the soil samples were washed to separate the ECM. Both sporocarps and ECM were characterized morpho-anatomically. The identification was done with the help of available literature [18, 19, 20]. The nomenclature was made according to the Index Fungorum. Special designation numbers were given to each of the samples. The specimens were deposited in Punjab University herbarium, Botany department (LAH).

Molecular Analysis. DNA was extracted by modified CTAB method following Gardes & Bruns [21]. The hymenial tissue was removed with sterile forceps and rinsed with sterile H_2O. Fresh and healthy ectomycorrhizal morphotypes from *Pinus wallichiana* A.B. Jack. and *Salix alba* L. were selected manually under a stereomicroscope (Olympus, SZ30, Japan). The extraction was modified for silica emulsion binding and purification (Gene-Clean; Q-Biogene, Irvine, CA, USA).

The ITS regions of nuclear rDNA were amplified by Polymerase Chain Reaction (PCR) using universal primers i.e. ITS1 and ITS4 primers [21, 22]. Purified PCR products were sequenced using a DNA sequencer (3730ABi). DNA sequence was submitted to BLAST and used to query the nucleotide collection using default settings. rDNA-ITS sequences of *S. sibiricus* were submitted to GenBank.

Phylogenetic position of *S. sibiricus* was deduced by Maximum Parsimony with bootstrapping using PAUP* Version 4.0b10. For that purpose, a tree was made by using seven (7) sequences obtained from *S. sibiricus* from Pakistan and 23 closely related rDNA ITS sequences of *Suillus* spp. were retrieved from the DNA database using the BLAST program from GenBank (Table 1). All sequences were aligned and corrected manually using MacClade 4.08 and Bioedit (version 7.0.9).

RESULTS

***Suillus brevipes* (Peck) Kuntze**, *Revis. gen. pl.* (Leipzig) **3**(2): 535 (1898) (Fig. 1. A-F). Pileus convex to hemispheric, 2.5-6 cm, chocolate brown, shiny, smooth, glabrous, sticky, flesh thick and off-white, margins slightly incurved, entire, smooth, of same color like pileus surface. Hymenium adnate and ascending, frequent pores, creamish to light yellowish, no color change upon bruising, pores rounded to irregular in shape. Stipe centric, clavate, 3.5-6.8 cm long, 1.3-2.2 cm wide, smooth, whitish with brown small patches at some points, semi-hollow, ring or volva absent. Basidia clavate, thin walled, contents brown in Meltzer's, 18-26 x 6-8 µm, 2-4 sterigmate. Basidioles clavate, thin walled, 15-23 x 5.5-8 µm. **Cystidia** cylindrical to clavate to subfusoid to ampullaceous, thick walled, dark brown contents, 35-49 x 5-9 µm. Basidiospores ellipsoid to fusiform to subfusiform, smooth, thick walled, 6-10 x 3-6.5 µm, honey brown to light yellowish.

Figure 1. *Suillus brevipes*, A-F. Views of the Sporocarp (A & B), Basidia (C), Basidioles (D), Cystidia (E), Basidiospores (F). **Scale Bars** for A & B = 1 cm, C = 6 µm, D = 6 µm, E = 8 µm, F = 3 µm.

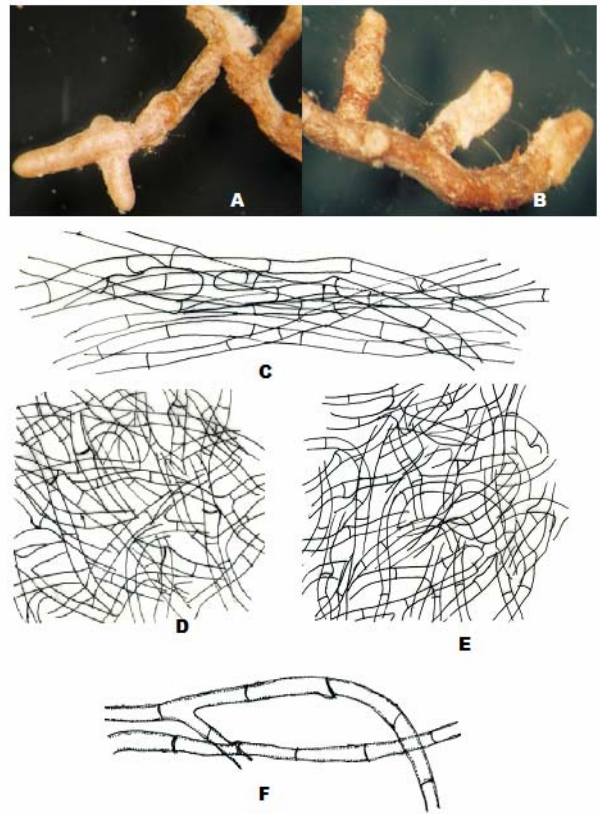

Figure 2. A-F. Ectomycorrhizae of *Suillus brevipes*. Views of ECM under stereomicroscope (A & B). Anatomical features of ECM under light microscope (C-F). Rhizomorph (C), Outer mantle (D), Inner mantle (E), Emanating hyphae (F). **Scale Bars** for A & B = 0.6 mm, C = 4.5 µm, D = 7 µm, E = 7.5 µm, F = 5 µm.

Habitat and Distribution of *Suillus brevipes*. On ground, near and solitary under *Quercus incana*, at 2250 m. a. s. l., Pakistan, KPK, Khanspur, 19th June, 2008. S. S. B. # 12.

Description of ectomycorrhizae of *Suillus brevipes*

Morphological characteristics***:*** (Fig. 2. A & B). Mycorrhizal system: monopodial pinnate, system 3.5-4 mm long, main axis 0.5 mm wide, texture smooth, tips beaded to straight, tip length about 0.5 mm and <0.5 mm wide, young tips honey brown, older tips black and apices light honey brown, host tissue visible under mantle surface. Rare Rhizomorphs, at restricted points, whitish brown. Common emanating hyphae, straight, whitish.

Anatomical characteristics of mantle in plan views (Fig. 2. D & E). Plectenchymatous outer mantle layer (type E, Agerer) [23], matrix material granular, cells 3 µm wide and 11 µm long, honey brown, cell contents: granular, common septa and clamped septa, hyphal junction angle about 60˚, rare anastomose. Plectenchymatous inner mantle layer (type E, Agerer) [23], granular cell contents, cells 5 µm wide and 12 µm long, yellowish brown, common septa and clamped septa, common hyphal junction, hyphal junction angle about 45˚, rare anastomose and H-shaped. Anatomical characteristics of emanating elements (Fig. 2. C & F). Rhizomorphs highly differentiated (type B, Agerer) [23], cells 4 µm wide and 9 µm long, light brown in color, cell contents oil like bodies, common septa and clamped septa, common hyphal junction and Y-shaped, anastomose H- shaped. Rare Emanating hyphae, cells 5 µm wide and 8 µm long, reddish brown, septa and clamped septa present.

***Suillus sibiricus* (Singer) Singer**, *Farlowia* **2**: 260 (1945) (Fig. 3. A-F)
MYCOPAK: EA15194, EA23904, EA24040, EA24104, EA17193, SS142010, SS022010.
GENBANK: JN119748-54

Pileus 4-8 cm, pulvinate to obtuse, sticky, slimy, shiny, glabrous, yellowish brown, smooth, margins smooth, entire, slightly darker color than pileus surface, deflexed. Hymenium: adnate and ascending, pores frequent, yellowish, angular pores. Stipe: 10 cm long, 1.8 cm wide, central, equal, ring present, yellowish to off-white from apex to ring, reddish brown from ring towards base, whitish near base, reticulated, reticulations white, rough, solid, curved. Basidia: long clavate, 2-4 sterigmate, thick walled, brownish contents visible in Meltzer's, 25-39 x 7-9 µm. Basidioles: clavate, thick walled, contents visible, (20-) 26-31 x 7.5-8 µm. Cystidia: cylindrical to subfusiform, thick walled, yellowish brown in Meltzer's, yellowish brown contents, 33-45 (-62) x 7-9 µm. Pileocystidia: a tangled layer of repent hyphae, hyaline to light honey in Meltzer's, thin walled, granular contents, septate, 60-74 x 8-10 µm. Caulocystidia: long, subclavate- clavate-cylindrical, some with pointed ends, septate, thick walled, hyaline in Meltzer's, 68-99 x 13-20 µm. Basidiospores: ellipsoid-fusiform, thin walled, amyloid, smooth, 10-12 x 3.5-5 µm.

Description of Ectomycorrhizae of *Suillus sibiricus* with *Pinus wallichiana*

Morphological Characters (Fig. 4. A & B). Ectomycorrhizal system frequently found under the fruiting bodies, dichotomously branched, 3-5 mm long, 0.5 mm in diam., length of tips 1.5 mm, dark honey brown when older. *Very tips* honey brown to reddish brown, tips straight. Mantle surface long-spiny to cottony; host tissue not visible under the sheath. Rhizomorphs:

common, connecting distinctly to the mantle surface, off-white to light brown, differentiated. Emanating hyphae: light brown, common, giving cottony appearance, straight.

Anatomical characteristics of mantle in plan views. (Fig. 4. C & D). Mantle: pseudoparenchymatous in all layers. Outer mantle layer: pseudoparenchymatous (type L, Agerer) [23], cells rounded to angular, cells 7-9 µm long, 7.3-10 µm in diam., no matrix material observed, cell contents clear, cell color hyaline to light yellowish. Inner mantle layer: pseudoparenchymatous (type H; Agerer) [23], without any ring like structures; cells colorless to yellowish, cells 6.5-7.4 µm in diam., cell walls 5.4-7.2 µm thick; cell contents not observed.

Anatomical characteristics of emanating elements (Fig. 4. E & F)

Rhizomorphs: highly differentiated, cells about 5.4 µm in diameter and about 87 µm length, light brown color, septa rare, clamps absent, cell contents clear. Emanating hyphae: common, 5 µm in diameter, 67 µm long hyphal cells, only clamped septa present.

Habitat and Distribution of *Suillus sibiricus.* On ground, in groups, under *Pinus wallichiana*, at 2347 m. a. s. l., Pakistan, KPK, Khera gali, 7th August, 2010. S. S. B. # 53.

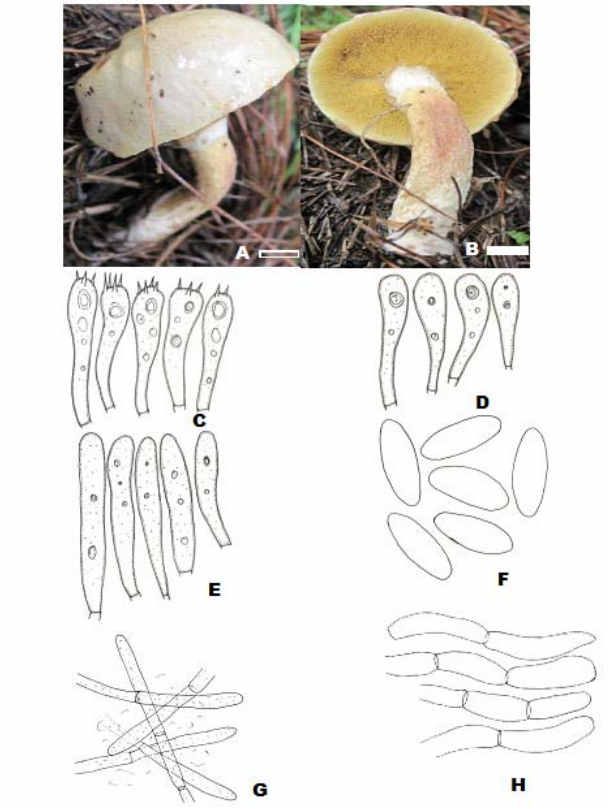

Figure 3. *Suillus sibiricus*, A-F**.** Views of the Sporocarp (A & B), Basidia (C), Basidioles (D), Cystidia (E), Basidiospores (F), Pileocystidia (G), Caulocystidia(H) . Scale Bars for A & B = 1.4 cm, C = 7 µm, D = 7 µm, E = 8.5 µm, F = 4.5 µm, G = 12 µm, H = 24 µm.

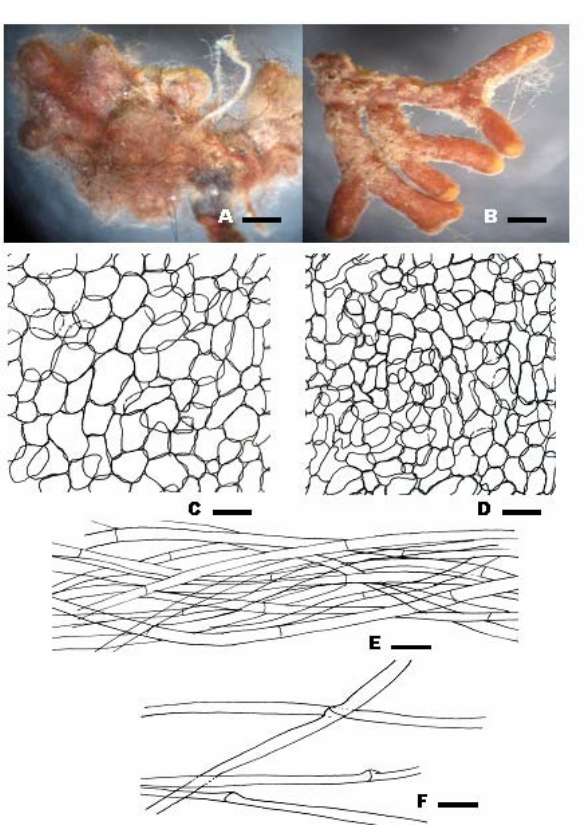

Figure 4. A-F. Ectomycorrhizae of *Suillus sibiricus* with *Pinus wallichiana***.** Views of ECM under steriomicroscope (A & B). Anatomical features of ECM under light microscope (C-F). Outer mantle (C), Inner mantle (D), Rhizomorph (E), Emanating hyphae (F). Scale Bars for A & B = 1 mm, C = 7.5 µm, D = 10 µm, E = 16 µm, F = 9.5 µm.

Description of ectomycorrhizae of *Suillus sibiricus* with *Salix alba.*

Morphological characteristics (Fig. 5. A). Mycorrhizal system found under the fruiting bodies: monopodial pinnate, system up to 5.5 mm long, with 0.6 mm thick main axis, unramified tips 2.2 mm long and about 0.5 mm thick, color of system dark brown, younger tips honey brown, rounded and slightly curved, surface of mycorrhizal system smooth, host tissue visible under mantle surface. Rhizomorphs: rare, attached at restricted points, dark brown to black.

Anatomical characteristics of mantle in plan views (Fig. 5. C & D)
Mantle parenchymatous in all layers. Outer mantle layer: parenchymatous, (type L, Agerer) [23]; cells irregular in shape, 10 µm in diameter and 12.5 µm in length, light brown color of cells, no cell contents, no septa and clamps. Inner mantle layer: parenchymatous, (type M, Agerer) [23]; cells angular, smaller than outer mantle, 9 µm in diameter and 11 µm in length, honey brown color of cells, no matrix material, no septa and clamps.

Anatomical characteristics of emanating elements (Fig. 5. B). Rhizomorphs: differentiated (type B, Agerer) [23]; septa present and H-type anastomose type, cells 5 µm in diameter, 26 µm in length, cells thin walled, septa common, clamps and clamp septa absent.

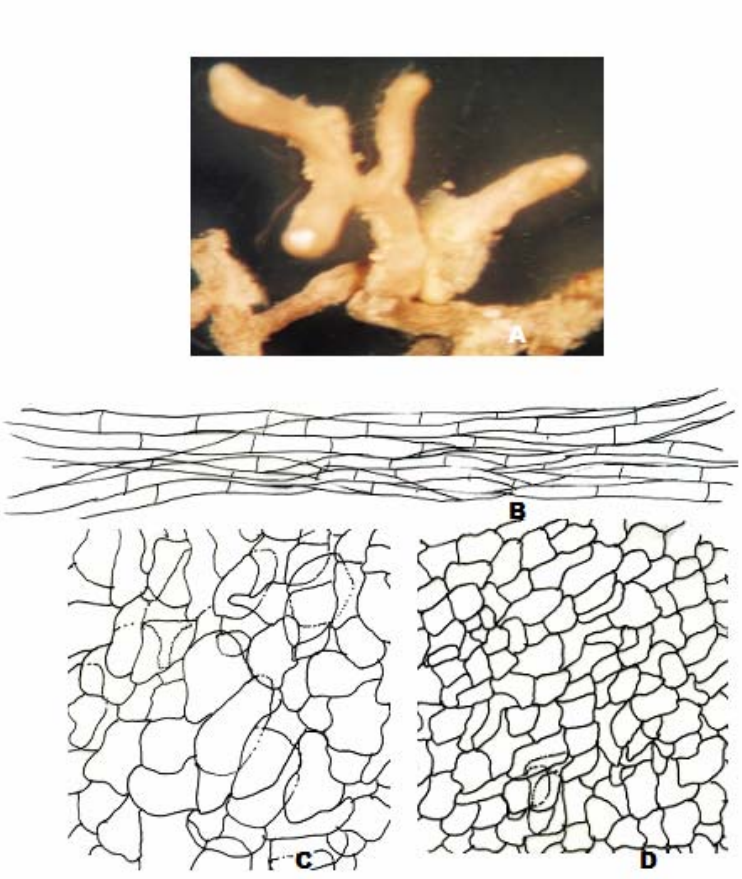

Figure 5. A-F. Ectomycorrhizae of *Suillus sibiricus* with *Salixalba*.
View of ECM under stereomicroscope (A). Anatomical features of ECM under light microscope (B-D). Rhizomorph (B), Outer mantle (C), Inner mantle (D). **Scale Bars** for A = 1 mm. B = 17 um. C = 8.5 um. D = 16 um.

Molecular identification and characterization of *S. sibiricus* and its ectomycorrhizae. PCR-ITS products of rDNA obtained from basidiomes and their ectomycorrhizae with *Pinus wallichiana* and *Salix alba* were sequenced. The sequences showed 99% similarity with isolates of *S. sibiricus* from China and America, confirming the morphological identification.

The phylogenetic analysis for genus *Suillus* was carried out using parsimony as optimality criterion. The sequences included in this analysis had around 659 characters, from which 494 characters were used for further analysis after alignment and trimming from both 3' and 5' sites of rDNA-ITS. After that, none of characters were excluded from final analysis.

All characters were of type 'unord'. There were 53 parsimony-informative sites, 415 constant sites, 26 variable characters were parsimony-uninformative. All the gaps were treated as "missing" data. Multistate taxa were interpreted as uncertainty. The starting tree(s) was obtained via stepwise addition with random addition of sequence and 1000 number of replicates. There were 49145891 starting seeds for the tree generated. Only 01 tree held at each step during stepwise addition of the sequences. Tree-bisection-reconnection (TBR) was used as branch-swapping algorithm. A total of 6,457,051 rearrangements were tried for the best tree. Only 27 trees were retained for analysis. The genetic distance matrix was derived from Maximum Parsimony (MP) analysis generated a consensus tree from the best 144 trees showing the following scores: Tree length (TL) = 146, consistency index (CI) = 0.6438, homoplasy index (HI) = 0.3562, CI excluding uninformative characters = 0.5517, HI excluding uninformative characters = 0.4483, retention index (RI) = 0.7977, rescaled consistency index (RC) = 0.5136. Phylogenetic analysis showed the various species of *Suillus*. Maximum Parsimony consensus tree indicated three major clades and one independent clade. Maximum Parsimony consensus tree was constructed exclusively for *Suillus* species from geographically different localities, mostly from Eastern Asia (China and Nepal), Eastern North America and from Pakistan to resolve exact identification. The cladogram represents a major polytomous clade formed by *S. americanus* and *S. sibiricus* species (Table 01). All of the species of this clade shared 98-99% of characters studied so far for this analysis and thus identified as *S. sibiricus*. Both *S. sibiricus* and *S. americanus* occupied topologically different positions in the same polytomous clade (Fig.6).

The Maximum Parsimony analysis resulted in major polytomous clade comprising sixteen isolates of *S. americanus* and *S. sibiricus*. All these species are monophyletic along with *S. flavidus* (Accession No. FJ845439.1), *S. megaporinus* (Accession No. GQ249400.1) and *S. umbonatus* (Accession No. L541115.1).

DISCUSSION

Boletes are an important component of Himalayan Moist Temperate Forests of Pakistan and make ectomycorrhizal association with gymnosperm and angiosperm trees of this area. Out of ten (10) genera of 44 species of Boletes, *Suillus* is represented by 4 species viz. *S. granulatus* (L.) Roussel, *S. placidus* (Bonord.) Singer, *S. sibiricus* (Singer) Singer and *S. tomentosus* (Kauffman) Singer [4, 5, 6].

Suillus is very important genus of Boletes. Its phylogenetic and bio-geographic relationship comprehensively described by Wu et al. [16]. *Suillus sibiricus* was originally described by Rolf Singer in 1938 [24]. While *S. brevipes* was first described by Charles Frost in 1874 [25].

In the present studies *S. brevipes* and *S. sibiricus* have been found forming ectotrophic mycorrhizae with different host trees of Himalayan Moist Temperate Forests of Pakistan. The

basidiomata and ECM morphotypes of both mushrooms have been described and illustrated using morpho-anatomical methods. The *S. sibiricus* and its ECM mycorrhizae have been identified using rDNA-ITS and 5.8S gene. *S. sibiricus* was described as mycorrhizal from two different hosts viz; *Pinus wallichiana* and *Salix alba* while *S. brevipes* was found associated with *Quercus incana.*

Table 1. List of *Suillus* species sequences, length in base pairs, their Geographic Origin, Collection and Accession Numbers.

Scientific names	Genbank Accession No.	Origin country	Length (bp)
Suillus americanus	AF166503.2	USA	623
Suillus americanus	L54103.1	USA	644
Suillus americanus	AF166500.2	USA	619
Suillus americanus	AF166501.2	USA	648
Suillus bresadolae	GU187544.1	USA	711
Suillus brevipes	FJ845440.1	CANADA	947
Suillus caerulescens	EU486453.1	CANADA	1260
Suillus cavipes	AF166505.2	CHINA	721
Suillus flavidus	FJ845439.1	CANADA	943
Suillus granulatus	AY898617.1	SPAIN	799
Suillus intermedius	L54074.1	USA	643
Suillus lakei	DQ367917.1	CANADA	899
Suillus luteus	DQ440568.1	SPAIN	802
Suillus megaporinus	GQ249400.1	JAPAN	698
Suillus paluster	AB284451.1	JAPAN	792
Suillus quiescens	GQ249402.1	USA	792
Suillus sibiricus	AF166512.1	USA	709
Suillus sibiricus	AF166515.2	CHINA	677
Suillus sibiricus	AF166514.2	CHINA	617
Suillus sibiricus	L54117.1	NEPAL	644
Suillus sibiricus	AF166516.1	USA	694
Suillus sibiricus	MYCOPAK EA15194 (*Salix alba* Root) JN119748.1	PAK	644
Suillus sibiricus	MYCOPAK EA17193 (*Salix alba* Root) JN119752.1	PAK	635
Suillus sibiricus	MYCOPAK EA23904 (*Pinus wallichiana* Root) JN119749.1	PAK	370
Suillus sibiricus	MYCOPAK EA24040 (*Pinus wallichiana* Root) JN119750.1	PAK	580
Suillus sibiricus	MYCOPAK EA24104 (*Pinus wallichiana* Root) JN119751.1	PAK	588
Suillus sibiricus	MYCOPAK S2 (*Pinus wallichiana*) JN119754.1	PAK	507
Suillus sibiricus	MYCOPAK S14 (*Pinus wallichiana*) JN119753.1	PAK	642
Suillus subalutaceus	L54075.1	USA	644
Suillus umbonatus	L54115.1	USA	644

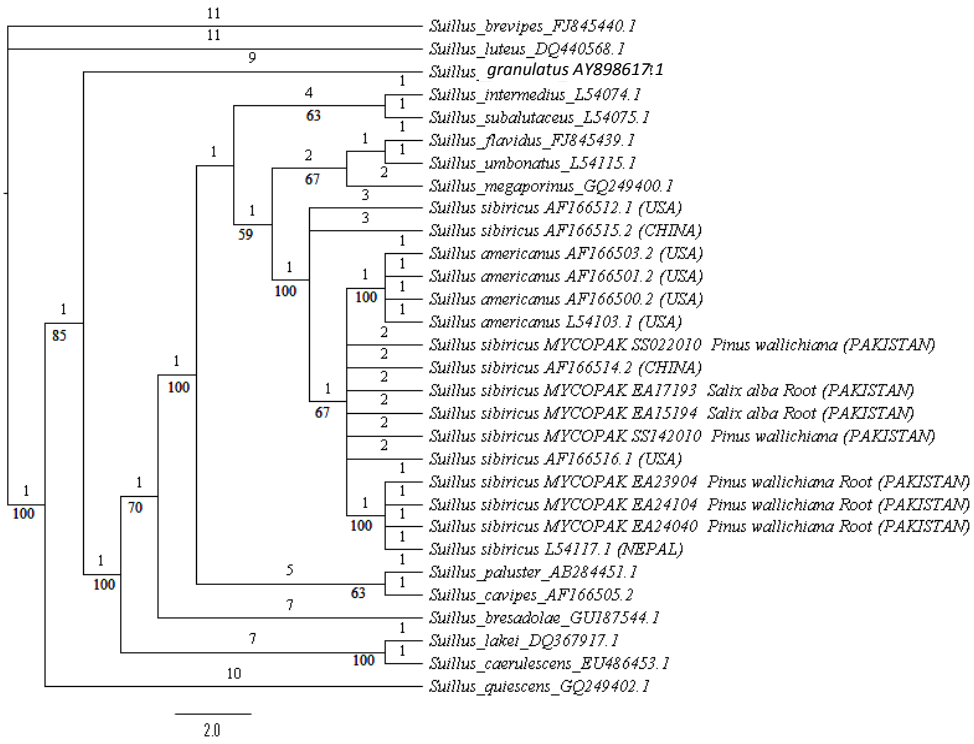

Figure. 6. Bootstrap 50% majority-rule consensus tree. Cladogram based on parsimony analysis of rDNA-ITS region of different species of *Suillus*.

MP tree generated by parsimony analysis of rDNA-ITS with 5.8s gene. The numbers above brackets refer to number of changes, those below to Bootstrap values. The accession numbers of analyzed sequences are sown after each taxon name.

S. brevipes has similarity with *S. granulatus*, *S. albidipes* and *S. pallidiceps*. But the major differences from these species are *S. granulatus* has shorter stem, and distinct raised granules on the stem while *S. brevipes* has smooth white stipe. Similarly, *S. brevipes* is differentiated from *S. albidipes* by not having a cottony roll of velar tissue at the margin when young. A major difference of *S. brevipes* from *S. pallidiceps* is a pale yellow cap color of the latter while the former has a chocolate brown cap [26]. Previously, it was found associated with *Pinus contorta* and *P. ponderosa* [27] and there is no report regarding their distribution in South Asia. The mycorrhizae of *S. brevipes* were described with *P. contorta* which form tuberculate system and tortuous tips. While in present study, with *Q. incana* it forms monopodial pinnate and possess plectenchymatous type of mantle which is in contrast with synchymatous type of mantle in *P. contorta*.

S. sibiricus often confused with North American species, *S. americanus*. The latter has larger sporocarps as compared with *S. sibiricus*. Despite this minor difference, other macro and micro features resemble each other. The only thing separating them is their geographical distribution [16]. *Suillus flavidus* and *S. umbonatus* are also close relatives of *S. sibiricus* that form sister clade with *S. sibiricus* in constructed Cladogram (Fig. 6). The ectomycorrhizae of *S. sibiricus* were previously described with *Pinus cembra*, *P. banksiana*, *P. monticola*, *P. peuce, P. pumila* and *P. sibirica* [24, 28-32]. According to Agerer [23], *S. sibiricus* forms coralloid mycorrhizal with *P. cembra*. Mantle is typically of plectenchymatous type with club-shaped cells. Hyphae of mantel embedded in the pigment matrix and possess large hyphae in

rhizomorphs. In contrast to *P. cembra*, in present investigation *S. sibiricus* forms dichotomously branched mycorrhiza with *P. wallichiana*. The mycorrhizal system bears honey brown coloration with long spiny to cottony appearance. Mantel resembles with *P. cembra* mycorrhizal in its shape but cells varied from round to angular. The symbiotic range of *S. sibiricus* is revised in this investigation. A deciduous host, *Salix alba* L. is found symbiotically with *S. sibiricus*. It possesses monopodial pinnate type of mycorrhizal systems. These are different from *P. wallichiana* and *P. cembra* mycorrhiza. It posses parenchymatous mantle with irregular cells. Rare H-type rhizomorphs are present with dark brown- to black-colored septate hyphae.

During present investigation, it is concluded that *S. brevipes* and its ectomycorrhizae are new reports for Pakistan. In addition, molecular analysis of *S. sibiricus* and its ectomycorrhizae are being done for the first time in Pakistan and association of this fungus with *Salix alba* is new to science world and it seems that *S. sibiricus* extends its host range from conifers to deciduous trees.

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PRELIMINARY SURVEY OF THE DIVERSITY OF THE GENUS *AGARICUS* IN MEXICO

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ABSTRACT

Mushroom cultivation was introduced to Mexico around 1930 and since then mushroom production has been increasing, setting the country as the principal mushroom producer in Latin America. Despite of its commercial importance, the diversity of the genus *Agaricus* in Mexico has been little studied; only one study has been conducted in Mexico which recorded 13 species; although there have been several studies, mainly floristic, that include some species of the genus. The aim of this study is to produce a preliminary *Agaricus* checklist based on the collections of four main Mexican herbaria (ENCB, IBUG, MEXU, XAL), and at the same time gather information on poor explored regions and places with potential presence of *Agaricus* species. At present, 204 specimens have been revised and, after checking the synonyms, 32 species taxonomically valid are recognized, which has been collected in 27 of the 32 states of the Mexican Republic. The most common and wide spread species are *A. campestris* (present in 18 states), *A. placomyces* (present in 12), *A. augustus* (present in 9) and *A. arvensis* (present in 8). Undoubtedly, the revision of the genus *Agaricus* in Mexico will allow to add new records to the Mexican fungal flora, and also to describe new species to science (especially from subtropical and tropical regions, so far poorly explored). These studies must rely primarily on the molecular analysis of the species and it would be desirable to perform, at the same time, genetic studies of productivity in the most popular edible species.

Keywords: Mushroom biodiversity; Herbaria revision; Taxonomy; Species distribution.

INTRODUCTION

The consumption of edible fungi in Mexico represents a culinary tradition that descends from pre-Hispanic times. Popular markets offer during the rainy season a wide variety of edible species estimated at more than 200 species [1-3], many of these fungi have their names in Indian languages and some of them are consumed almost exclusively in the central region of Mexico, as is the case of the "huitlacoche" (corn smut), a species prized for its delicate flavor.

Some species of the genus *Agaricus* L., are traditionally eaten in Mexico and received several popular names: “sanjuanero”, “llanero”, among others [4]. Different publications have emphasized the ethnomycological importance of this fungi genus among indigenous people [5-10]. *A. bisporus* (J.E. Lange) Imbach is the most cultivated and consumed species in Mexico and it is popularly called "champiñon", a word derived from the French term to designate all fungi: champignon. The cultivation of *A. bisporus* was introduced in Mexico in the 1930s [11] and since then its production has been increasing. Mexico is currently the largest producer of mushrooms in Latin America with more than 43 000 tons of fresh mushrooms per year [12].

Although it is difficult to estimate the number of species of the genus *Agaricus* extant [13], it is considered that Europe alone has more than 130 species [14] of the 200 species mentioned at world level [15]. Taking into account that one third of the species analyzed by Kerrigan et al. [16] resulted to be new species to science from the best known mycological regions (Europe and North America), one could predict that the number of *Agaricus* species in subtropical and tropical regions must be substantial.

Paradoxically, the genus *Agaricus* has been little studied in Mexico with only one published work that specifically described 13 species of this genus collected in Mexico [17], although several studies, especially floristic, cite species of the genus. Martínez-Carrera et al. [11] reported the production of 10 wild Mexican *Agaricus* species, but this study was focused on basidiomes production, the authors indicating that the identification of the species cultivated was tentative. By the end of the 1980s it was assumed that all specimens of *A. bisporus* used for the isolation of strains for mushroom cultivation, were exclusively European. However, upon finding wild specimens of this species in the Californian desert, in the United States [18], the concept of distribution has changed. During 2001, one of the authors of the present work and P. Callac (INRA, France) made some excursions in Mexican territory and found wild *Agaricus* specimens with basidia typically bisporic [19]. Recently two new Mexican species have been described, one belonging to the section Xanthodermatei, *Agaricus tollocanensis* [20] and another to section Duploannulati, *A. tlaxcalensis* [21].

The objective of the present work was to compile a list of *Agaricus* species deposited in the major herbaria of Mexico, in addition to documenting species previously mentioned in the literature, in order to identify some scarcely explored regions that could potentially host species of *Agaricus* in this country.

MATERIALS AND METHODS

A review of the *Agaricus* genus specimens deposited in the 4 major herbaria of Mexico was made: ENCB (Instituto Politécnico Nacional), IBUG (Universidad of Guadalajara), MEXU (Universidad Autónoma de México) and XAL (Instituto de Ecología, A.C.). It took data from the locality, collector and species from specimens found in good condition. A bibliographic review of papers published in Mexico relating to the *Agaricus* genus was carrying out. At the same time the valid names for the species mentioned in the Index fungorum were reviewed (http://www.indexfungorum.org/names/names) in order to determine the synonyms in the studied species. Some *Agaricus* specimens were microscopically reviewed to corroborate their identifications. The microscopic study of the specimens was riding hand from various parts of the basidioma temporary cuts made preparations to observe structures, which were revised in a compound microscope (Carl Zeizz Stemi 2000 model), using KOH to 5%, phloxine, Congo red and solution of Melzer.

RESULTS AND DISCUSSION

Two hundred and four specimens from herbaria were reviewed; this material was ascribed to 32 taxonomically valid species after confirming the possible synonyms (Table 1). The specimens were collected in 25 of the 32 States of Mexico, the most common and widely distributed species were: *A. campestris* (collected in 18 states), *A. placomyces* (present in 12), *A. augustus* (present in 9) and *A. arvensis* (present in 8). The states with the largest number of species are Veracruz, Mexico and Tlaxcala, each one with 9 species. The State of Aguascalientes has a single registered species and the territories with no records of *Agaricus* are: Campeche, Coahuila, Colima, Nayarit, Sinaloa, Tamaulipas and Yucatan. In general the majority of the collections

were made from central regions of the country, while in tropical and subtropical zones of South and Southeast and temperate zones of the North, expeditions have been scarce.

Table 1. Species of *Agaricus* genus that have been recorded in the major herbaria of Mexico (ENCB, IBUG, MEXU and XAL)

Species	States in which it has collected	*
Agaricus altipes (F.H. Møller) F.H. Møller	14	
A. arvensis Schaeff	6, 8, 11, 12, 14,17, 23	[22, 24, 30, 31, 32]
A. augustus Fr.	4, 5, 6, 8, 11, 12, 14, 17, 23	[11, 22, 24, 32, 35]
A. benesii Pilát, A	14	[17]
A. benzodorus Heinem. & Gooss	19	[23]
A. bisporus (J.E. Lange) Imbach	1. 6, 7, 8, 12, 22, 23, 24	[22, 30, 32, 33]
A. bitorquis Saccardo, P.A.	6, 11, 14, 17	[22, 32]
A. californicus Peck, C.H.	3, 23, 24	
A. *campestris* L.	3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 21, 23, 24, 25	[22, 24, 30-34, 36-37, 39-40]
A. cretaceus Bull.	6	
A. crocodilinus Murrill, W.A	6, 18	[23, 32]
A. endoxantus Berkeley, M.J.; Broome, C.E.,	19	[23]
A. lividonitidus Møller, F.H.	20	
A. moelleri Wasser	6, 24	[17]
A. nivescens Møller, F.H.	14	
A. osecanus Pilát, A.	17	[23]
A. placomyces Peck, C.H	6, 7, 8, 9, 11, 12, 13, 14, 16, 17, 23, 24	[22, 24-25, 30, 32, 34-35]
A. porosporus Heinem	19	[23]
A. porphyrocephalus F.H. Møller	12	[23. 30]
A. purpurellus (F.H. Møller) F.H. Møller	19	
A. silvaticus Schaeff.	4, 5, 12, 17, 18, 23, 24	[22, 31-32, 35-37]
A. silvicola (Vittad.) Peck	2, 8, 12, 24	[22,31-33, 35-37]
A. singeri Heinemann, P. (	19	[23]
A. solidipes Peck	5	
A. subperonatus (J.E. Lange) Singer	12	[17, 23. 30,32]
A. subrufescens Peck	6, 8	[11]
A. subrutilescens (Kauffman) Hotson & D.E. Stuntz	6, 13, 14	[22]
A. tlaxcalensis Callac & G. Mata	23	[21]
A. tollocanensis Callac & G. Mata	8, 23	[20]
A. trinitatensis R.E.D. Baker & W.T. Dale	19	[23]
A. volvatulus Heinem. & Gooss.-Font.	24	[38]
A. xanthodermus Genev.	5, 6, 8, 12, 13, 17, 24	[17, 22, 24, 30,32]

States in which the species has been registered: 1. Aguascalientes, 2. Baja California Sur, 3. Baja California, 4. Chiapas, 5. Chihuahua, 6. Distrito Federal, 7. Durango, 8. Estado de Mexico, 9. Guanajuato, 10. Guerrero, 11. Hidalgo, 12. Jalisco, 13. Michoacan, 14. Morelos, 15. Nuevo Leon, 16. Oaxaca, 17. Puebla, 18. Queretaro, 19. Quintana Roo, 20. San Luis Potosi, 21. Sonora, 22. Tabasco, 23. Tlaxcala, 24. Veracruz, 25. Zacatecas.

* Species cited in the Mexican literature. Numbers in brackets correspond to bibliographic references that were cited.

Although only one published work exists that focused on this genus in Mexico [17], other studies have included collections of this species, but a full taxonomic description of the specimens is lacking (Table 1). Although some reports did not specify if the specimens are deposited in a herbarium or this material is preserved in herbaria that were not reviewed in this paper, the literature recorded 17 species more than reported in Table 1. These species are: *A. albolutescens* [11], *A. bambusigenus* [22], *A. bernardii* [23, 24], *A. comtulus* [17], *A. denisii* [23], *A. dulcidulus* [23], *A. fuscofibrillosus* [17], *A. impudicus* [17], *A. pampeanus* [10], *A. phaeolepidotus* [17], *A. porphyrizon* [25], *A. robustisimus* [11], *A. semotus* [17], *A. volvatus* [23], *A. xantholepis* [17], *A. xuchilensis* [26] and *A. yucatanensis* [26]. This means that 49 species of the *Agaricus* genus had been registered from Mexico, representing 25% of the diversity reported by Kirk et al. [15]. However, most of this material has been identified mainly using European and American keys so that the identification of many specimens is probably wrong. The articles of Guzmán [27, 28] and Gutierrez Ruiz and Cifuentes [17] contain keys to the identification of the species collected in Mexico. The maintenance of the majority of specimens deposited in the herbaria is poor, as well as the lack of data of fresh material, particularly chemical reactions; hinder the corroboration of the identifications.

According to Guzman [29], the case of *Agaricus* as and other genera in Mexico must be revised and monographed and this work aims to achieve this goal. Undoubtedly the study of *Agaricus* genus in Mexico will allow, in addition adding new records to the national micoflora, the description of new species to science especially those that inhabit rarely visited placed in the tropic and subtropics. Studies should be primarily based on molecular analysis of the species and it would be desirable that they be made in parallel with genetic and yield researches of the most prized edible species.

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TAXONOMIC SIGNIFICANCE OF ANAMORPHIC CHARACTERISTICS IN THE LIFE CYCLE OF COPRINOID MUSHROOMS

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ABSTRACT

Ink cap fungi (coprinoid mushrooms) are not monophyletic and divide into *Coprinellus*, *Coprinopsis* and *Parasola* (all Psathyrellaceae) and *Coprinus* (Agaricaceae). Knowledge on morphological mycelial features and asexual reproduction modes of coprini is restricted, with *Coprinopsis cinerea* being the best described species. This species produces constitutively on monokaryons and light-induced on dikaryons unicellular uninucleate haploid arthroconidia (oidia) on specific aerial structures (oidiophores). The anamorphic name *Hormographiella aspergillata* was coined for oidia production on monokaryons. Two other *Hormographiella* species are described in the literature, one unknown (*candelabrata*) and one (*verticillata*) identified as *Coprinellus domesticus*. Another, yet sterile anamorph associated with some coprini is called *Ozonium* which describes the incidence of tawny-rust mycelial mats of pigmented, well septated and clampless hyphal strands as a specialized vegetative mycelium. In nature, this can be associated with mushroom formation. Few reports on occurrence of chlamydospores or blastospores and of sclerotia are also found. Here, mycelial characters of 15 coprini species from the clades *Coprinellus* (*curtus*, *disseminatus*, *domesticus*, *ellisii*, *flocculosus*, *micaceus*, *radians* and relatives, *xanthothrix*), *Coprinopsis* (*cinerea*, *gonophylla*, *scobicola*, *strossmayeri*), and *Coprinus* (*comatus*) were evaluated. *Ozonium*-type of mycelium was specific to dikaryons of all species of the genus *Coprinellus* (often in connection with fruiting bodies) but *C. flocculosus* where it exists at least rudimental. Such phenotype is new for *C. curtus* and *C. disseminatus*. *Hormographiella*-type conidiogenesis was observed on dikaryons of five *Coprinellus* species [*domesticus, ellisii, xanthrothrix, radians,* aff. *radians* (II)] and for *C. cinerea* (dikaryon) and *C. scobicola* (monokaryon). Terminaly and intercalarly formed chlamydospores were detected in *C. cinerea, C. gonophylla, C. strossmayeri,* and *C. comatus.* Dikaryons of *Coprinopsis* species and *C. comatus* have oval-round shaped clamp cells at hyphal septa. Dikaryons of *Coprinellus* species were in contrast mainly clampless, with in some instances rare fused clamps (*disseminatus*, *micaceus*) or pseudoclamps (*curtus*, *flocculosus*, *radians*, aff. *radians* (I) and (II), *xanthothrix*).

Key words: coprini, life cycle, anamorph, *Ozonium, Hormographiella*.

INTRODUCTION

The concept of the "Whole Fungus" considers all possible sexual and asexual stages of reproduction of a fungal species [1]. Teleomorphs and anamorphs might be distinguished with specific genus names for the sexual and the asexual forms of reproduction, respectively. Per species, there may be only one mode of sexual reproduction but there can be more than one mode of asexual reproduction (synanamorphs). The holomorph then summarises all developmental stages of a fungus. The teleomorph name usually is assigned to the holomorph when the biological relations between sexual and asexual stages have unambiguously been established [2]. Exceptionally also an anamorphic name may be chosen for the holomorph [3]. Defining anamorphs on genus and species levels can be important for fungal systematics when no sexual stage of a fungus is known, or when an asexual mode of proliferation and reproduction is not yet recognized to belong to a known teleomorph, or when it is difficult or even impossible to induce sexual reproduction in the laboratory.

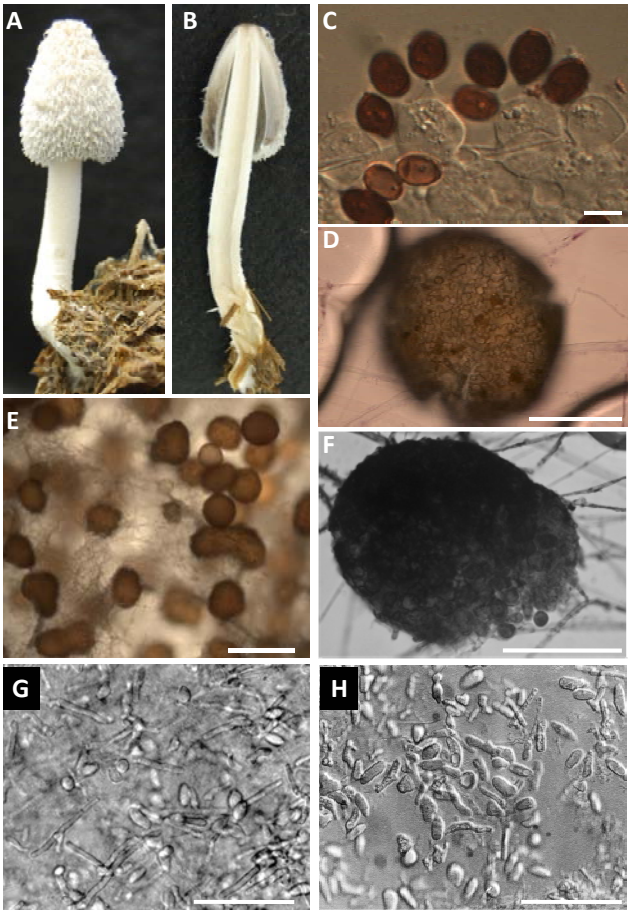

Figure 1: Selection of developmental structures of *Coprinopsis cinerea*. A young fruiting body (A,B) and basidia with basidiospores undergoing spore staining (C) of homokaryon AmutBmut [12,15] at the stage of rapid stipe elongation, cap expansion and basidiospore maturation – the dark colour at the lower ends of the lamella is due to spore staining (B). Sclerotium of dikaryon C344 revealing the structure of densely arranged small cells of the melanised rind (D), sclerotia in overview within the aerial mycelium grown on MEA (E), and a squashed sclerotium releasing individual thick-walled cells (here stained with 0.1 % methylene-blue) of the inner medulla (E), (this study). Chlamydospores within aged mycelium in the substrate of agar-cultures of dikaryon LN118 x 218 (G) and monokaryon 218 (H) [19].
Sizes bars: 10 µm (C), 50 µm (D,F-H)

In the ascomycetes, the concept of the „Whole Fungus“ is largely established [1,2] and many anamorphic genera of ascomycetes and related deuteromycetes are defined [4,5]. In contrast, in the basidiomycetes, especially in the Agaricomycetes, there is overall only limited knowledge on forms of asexual development [1,6,7]. Modes of asexual reproduction might have been mostly ignored or understudied or, also plausible, it might be that many species do not provide any asexual developmental stages. On the other hand, asexual stages might be seen but it might not be possible to verify the connection to a sexual stage due to lack of suitable culturing conditions for fruiting body development. In the advent of molecular typing of species, this problem has been reduced by establishing species-specific sequences such as from the ribosomal RNA gene clusters, specifically also the ITS-regions within [8,9,10].

The developmentally best understood species of the Agaricomycetes is *Coprinopsis cinerea* (previously called *Coprinus cinereus*) that forms typical mushrooms with the sexual basidiospores on the dikaryon (Fig. 1A-C; [11,12]). Oidia, small unicellular and uninucleate haploid mitotic spores (also known as arthroconidia) are constitutively produced in abundant numbers in the aerial mycelium of monokaryons by splitting short oidial hyphae produced at the tips of specialized hyphae (oidiophores) into usually two equally sized spores (Fig. 2; [13,14]). Oidia also form in low numbers upon light induction on the dikaryon (Fig. 3; [15]). Moreover, on mono- and dikaryons, there might be sclerotia, multicellular round bodies with melanised rinds of about 10-100 µm in diameter (Fig. 1D-F; [16-19]). There are two different varieties of sclerotia described which distinguish by the tissue layers of the rind [20]. Mycelial strands of aggregated hyphae (Fig. 4) have been reported on strains of Japanese origin in addition to pseudorhizas (the rhizomorphs in [12]) that function as anchors of the fruiting bodies in substrate [11], which originally lead to the differentiation of two distinct species with (*Coprinus macrorhizus*) and without strand and pseudorhiza formation (*Coprinus lagopus* sensu Buller, respectively *C. cinereus*). Genetics proved them however to be morphological varieties of the same species [21,22]. Oidiophores can also differ very much in structure. There are four main types (six in total) distinguished by the presence or absence of an oidiophore stipe, by presence or lack of a septum that separates oidiophores from their foot cells, by the length of the oidiophores, and by presence or absence of sidebranches at the oidiophores. These different types do not necessarily occur in same percentages on different *C. cinerea* monokaryons. Some strains have a high preference for one specific type of oidiophore (Fig. 2, [13]). An anamorph *Hormographiella aspergillata* had been defined by describing sporulation on conidiophores (~ oidiophores) on mycelial isolates from clinical sources [23] and proven by sexual crosses and molecular data to represent monokaryons of *C. cinerea* [18]. The large variability of oidiophore morphologies on different *C. cinerea* monokaryons (Fig. 2) challenges the *Hormographiella* anamorph - species concept. *H. aspergillata* conidiophore morphologies in the literature [23] match the types 1, 2B and 3 oidiophores defined by Polak et al. (Fig. 2; [13]). Two other *Hormographiella* species (*verticillata* and *candelabrata*) were distinguished by morphological descriptions of the conidiophores [23]. The *H. verticillata* conidiophores in the papers of Guarro et al. [23] and Cáceres et al. [24] resemble in morphologies the types 3, 4A and 4B oidiophores of *C. cinerea* (Fig. 2; [13]). *C. cinerea* strains that produce abundantly these types of oidiophores are thus in possible danger to be mistaken as *H. verticillata*. However, what appears to be a most reliable phenotype is thus not necessarily the structure of the conidiophore (oidiophore), but the number of oidia produced per oidial hypha and likely also the size of spores. *H. verticillata* produces two spores of per conidiogenous hyphae of relative variable sizes [(3)4 - 15(17) x (1.2)1.5 - 2(3) µm] and *H. candelabrata* up to seven spores per conidiogenous hyphae of 2(3) -

7(12) x (0.8)1 - 1.8 (2.5) µm in size. The conidiogenous hyphae of *H. aspergillata* divide into two spores. The *H. aspergillata* spores are more regular in size and measure (2.5)3.5 - 6(6.5) x (1.5)2 - 2.5(3) µm [23]. Numbers of spores per conidiogenous hypha (oidial hypha) and spore measures correlate very well with the data on oidia of >20 strains of *C. cinerea* [13]. A further difference between conidiophores of *H. aspergillata* and *H. verticillata* is that the spores of the first species assemble in liquid in slimy conidial heads at the tips of the sporophores (wet oidia) whereas the spores of the second species are dry [13,14,23-26]. Discrepancies exist in the literature on the mode of spore release – schizolytic by splitting the septum in between two oidia or oidium and oidiophore (see cytological evidence for *C. cinerea* in [13]) or rhexolytic by rupturing periclinal cell walls in plasma free hyphal segments in between two living cells [6,13].

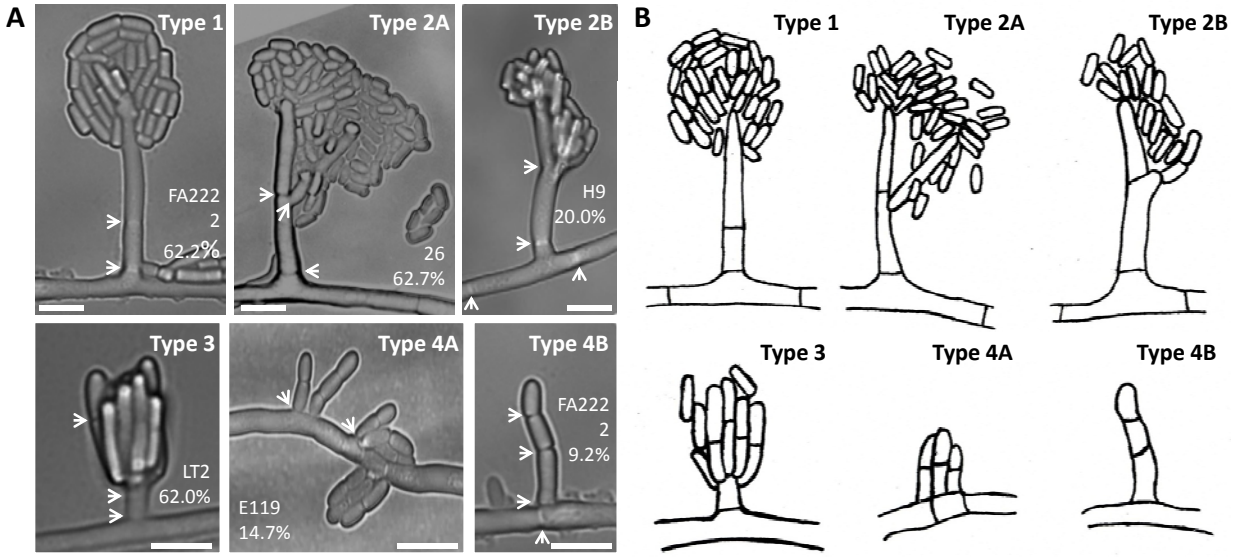

Figure 2: Different types of oidiophores on monokaryons of *Coprinopsis cinerea* [13, 19]. Prominent types of oidiophores within a strain, names of monokaryons and the percentage of occurrence of an oidiophore type [13] are indicated (A).
Arrows point to septa within oidiophores and hyphal footcells. Size bars: 10 µm. For better differentiation of individual features, outlines of oidiophore types are shown in B. Photos by courtesy of E. Polak

Recent definitions of anamorphic forms were presented for species of the Bolbitiaceae and Strophariaceae [27,28], adding to the descriptions of other Agaricales summarized in a recent review by Walther et al. [6]. There is a huge range of varieties in the Agaricales in morphologies of conidiophores as well as of the produced spores and in mechanisms of spore production and release. However, there are also noticeable similarities in species from different families or different genera which indicates that it will not be as easy to define distinct anamorph types to distinct teleomorph genera. For example, *H. verticillata* has been verified to be *Coprinellus domesticus* – which points out another problem to the morphological *Hormographiella* anamorph concept: species with comparable anamorphic morphology belong to distinct sexual genera [24]. Other anamorphic genera describe even species that are mixed from ascomycetes and from basidiomycetes – a prominent example is the anamorphic genus *Sclerotium* (not linked to any coprini) defined by sclerotia formation on sterile mycelium [29]. Moreover, in basidiomycetes there is a novel problem not existent in the ascomycetes: finding in

C. cinerea oidia production on both mycelial growth stages of the life cycle (Fig. 2 and 3), how will the *Hormographiella* anamorph concept be applied to oidia production at monokaryotic and dikaryotic mycelial growth stages of the fungus? Furthermore, in other processes of asexual reproduction, *C. cinerea* also can develop thick-walled chlamydospores within the hyphal cells of the vegetative mycelium of monokaryons and dikaryons (Fig. 1G,H). Formation of blastospores (blastocysts) as another pathway of producing large thick-walled spores has also occasionally been observed [11,19,30,31]. Would these needed to be considered as synanamorphs to the *H. aspergillata* anamorph? Walther et al. [6] recognized previously the unresolved problem of the poorly understood chlamydospores in the Agaricales. Strikingly, the chlamydospores in the mycelia of *C. cinerea* resemble in sizes and forms the inner thick-walled cells of the sclerotia of the fungus but the possible relationship between these structures has not firmly been established (Fig. 1F-H; [19,31,32]). Notably, also the feature of sclerotia production may define specific (non-sporulating) anamorphic forms, as documented by the example of the anamorphic genus *Sclerotium* [29].

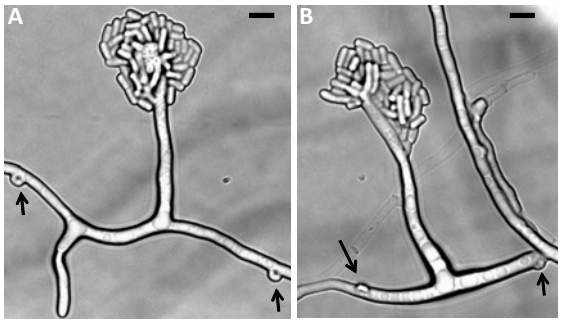

Figure 3: Oidiophores of *Coprinopsis cinerea* dikaryon FA2222 x 306 [19] formed in illuminated microslide cultures [13].
Arrows point to fused clamp cells at hyphal septa. Size bar: 10 µm. Photos by courtesy of M. Hollenstein

Another *mycelium sterilium* morphotype is described by the genus *Ozonium* and is characterized by the formation of colored hyphal strands in sterile vegetative mycelium. The *Ozonium*-type mycelial strands are not whitish as those in *C. cinerea* (Fig. 4) but are yellow-brownish cords of parallel-growing hyphae [24,33].The literature concerning this anamorphic genus is confusing with fungi of the basidiomycetes and of the ascomycetes being variously named *Ozonium* [24,33-35]. In nature, fruiting bodies of several different coprini (*Coprinus* s.l.) have been observed to arise from rust mycelial *Ozonium* mats of fiber-like structures. As a vegetative phase that may anticipate the formation of fruiting bodies, it may develop on wooden substrate or in soil in the vicinity of a substrate [33,36-39]. Associated with specific coprini, the generic name *Ozonium* has restricted priorability [37]. As a stage of development without the necessity to distinguish species of an *Ozonium* genus, it has however its position for the "Whole Fungus" description within the coprini in nature [33,36-39] as in culture (this study; [24]).

Rhacophyllus (*lilacinus*) is another anamorphic name of limited priority reported in the coprini literature [37]. *Rhacophyllus* forms sterile mushroom-like structures that lack basidiospores and have sheets of sac-like structures (lysomeres) in place of lamellae [40-42]. Studies revealed that an own *Rhacophyllus*-like isolate may carry bulbous bodies or basidiospores or mixtures of both underneath the caps (Fig. 5; [43,44]), reinforcing that the

structures are not necessarily fully anamorphic but present developmental variations of fruiting bodies with more or less blocked basidiospore formation [40,41]. *Rhacophyllus* structures develop on hard compact stromata, sclerotium-like aggregated mycelial bodies serving also as a taxonomic criterion [40,44]. In conclusion, the multitude of possible asexual structures that may be formed in coprini is impressive.

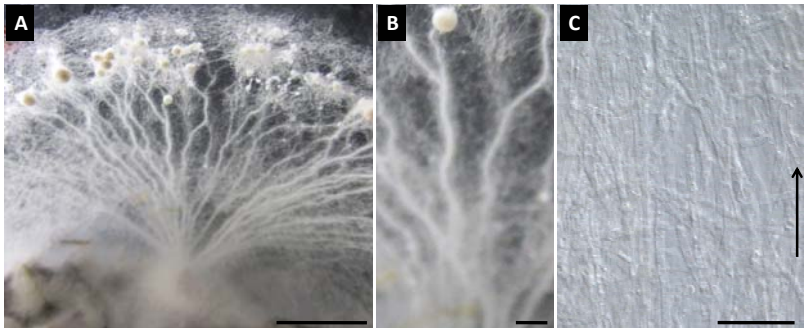

Figure 4: Mycelial strands in horsedung cultures [12] of *Coprinopsis cinerea* AmutBmut (A,B) and microscope view of a mycelial strand revealing parallel growth of aggregated hyphae with perpendicularly interlacing hyphae for stabilization (C).
Note the formation of primary and secondary hyphal knots and fruiting body primordia up to stage 3 [12] at the edges of the fan of mycelial strands. Arrow: growth direction. Size bars: 2 cm (A), 2 mm (B), 50 µm (C).

The genus *Coprinus* Pers. (*Coprinus* s.l.) is the largest group of the former family of Coprinaceae Overeem (Homobasidiomycetes) with more than 200 species. The traditional genus *Coprinus* is however not monophyletic. Therefore, it was divided into the three new clades *Coprinopsis*, *Coprinellus* and *Parasola* within the family of Psathyrellaceae whereas the genus *Coprinus* with just three species was assigned to the Agaricaceae [8,37,38,45]. Beside the descriptions above, data related to anamorphs in coprini are rare. However, comparative morphological observations of anamorphs from different clades of coprini and description of taxonomically relevant characters will assist further understanding of phylogenetic relationships within this group of fungi. In this paper, characteristics of in total 15 species from the clades *Coprinus*, *Coprinopsis* and *Coprinellus* and their taxonomic significance are evaluated.

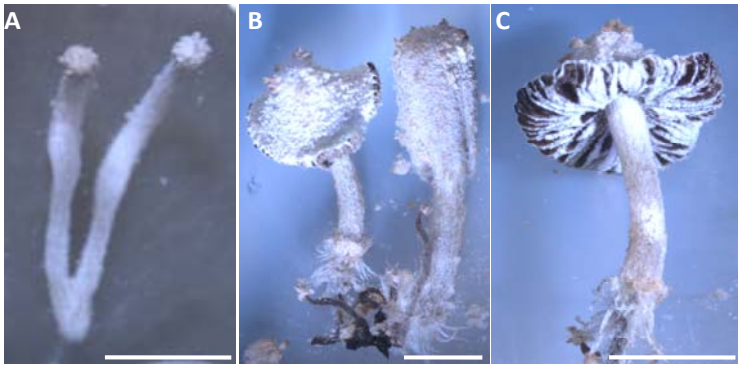

Figure 5: Views of *Rhacophyllus*-like structures of a strain isolated in Germany [43,44] (A-C).
White areas within the cap contain bulbous bodies and on black areas of the lamellae basidiospores can be found (C). Size bars: 2 cm

MATERIALS AND METHODS

Strains, culture conditions and microscopy. Strains used in this study are listed in Table 1. They are cataloged in the Culture Collection of the Laboratory of Fungal Biology and Biotechnology (FBBL), Yerevan State University [46] and most are also stored in the fungal strain collection of the Büsgen-Institut in Göttingen. Strains were grown on malt-extract agar medium (MEA; 1.5% malt-extract, Oxoid, Basingstoke, England; 1.5% agar, Serva, Heidelberg, Germany; pH 6.0) in Ø 90 mm Petri dishes in darkness at 25 °C for up to 10 days. Growth of cultures were checked every second day during which the colonies shortly received light. After growth, plates were stored at room temperature in the laboratory in a normal day/light regime. Fruiting body development and sclerotia production was observed in some of the cultures after 3 to 6 weeks (see results).

For micro-morphological observations, strains were inoculated at the edges of MEA plates, 2 to 4 coverslips were placed onto the agar surface and strains were cultivated at 25° in the dark until mycelium grew over the surfaces of the coverslips. Coverslips were cautiously lifted from the agar, turned around and placed with the mycelial side onto a drop of 0.1% methylene-blue or of water present on a microscope slide. After about 30 sec, samples were slightly pressed prior to inspection with an Axioplan-2 imaging microscope (Zeiss, Göttingen, Germany) [47]. Photos were taken with a Colour View II Mega Pixel digital camera for picture imaging using analySIS® software (Soft Imaging System, Münster, Germany).

Table 1: Strains used in this study

Species	Strains	Origin/reference*
Coprinellus curtus	C71, C311	Duke University˜; [44]
Coprinellus disseminatus	C50	Duke University˜; [10,44]
	Dikaryon 30	Armenia; [46]
Coprinellus domesticus	C72	Duke University˜; [44]
Coprinellus ellisii	C140	Duke University˜; unpubl.
Coprinellus flocculosus	C65	Duke University˜; [10]
	Dikaryon 1C	Armenia; [46]
Coprinellus micaceus	Dikaryons 15C, 15-2C, 15-2S, 15-3C, 15-3S, 15-4C, 15-4S, 9-2C, 9-1C, 10, 10-1, S-II, I	Armenia; [44,48]
	Dikaryons 1-OH, 2-OH	Germany; Büsgen-Institut
Coprinellus radians±	C22	Duke University˜; [10]
Coprinellus aff. *radians* (I)±	1-2PS, 1-1C, L1C, L2C, L2S, L3C, L4C, L5C	Armenia; [44,46,48]
Coprinellus aff. *radians* (II)±	C35	Duke University˜; unpubl.
Coprinellus xanthothrix	C144, C398, C482	Duke University˜; [10]
Coprinopsis cinerea	C344	Duke University˜; [10]
Coprinopsis gonophylla	C399	Duke University˜; unpubl.
Coprinopsis scobicola	Monokaryon Cb1	HRI, Wellesbourne¶; [10,50]
Coprinopsis strossmayeri	Dikaryons 1-1S, 1-2S, 1-3S, 1-4S, 1-5S, 1-5C, 1-6S, 1-6C, 15-1S, 15-1C, 15-2C, 15-3C, 16S, 16C	Armenia; [44,46,48]
Coprinus comatus	C53, C108	Duke University˜; [10]
	Dikaryons IV, 1-C, 1C, 2C, 3S, 6S, 8S, 8C, 9S, 9PS	Armenia; [46,48]

* Species identity determined by ITS sequencing; ± ITS sequences are 95-97% identical suggesting that these belong to three closely related species ([10,48]; MNG unpublished); ˜,¶ kindly received via T.Y. James and M.P. Challen, respectively; ˜ no further information on mycelial status was available

Strains of a same species grow on MEA at 25°C in the dark comparably well with similar growth speeds (data not further shown; [48]) and comparable colony morphologies (Fig. 6; not further shown). Most species had a fluffy white or off-white colored mycelium. However, mycelia of *C. domesticus* were at places (Fig. 6F) and mycelia of *C. ellisii* and *C.* aff. *radians* (I) and (II), throughout yellowish-orange, *C. radians* C22 rust and *C. xanthothrix* light yellow to brownish colored, respectively (Fig. 6L,M). In colonies of *C. disseminatus*, patches of young mycelium were slightly yellow colored and later brownish (not shown). As exceptions, *C. curtus* strains and *C. gonophylla* had little whitish aerial mycelium but *C. curtus* C71 formed concentric thin lines of originally white dense mycelium at the edges of the Petri-dishes in which stained hyphal knots and primordia (definitions in [12]) arose (Fig. 6A-C). Older mycelium of *C. curtus* strains however were slightly yellowish colored. In contrast, older cultures of *C. micaceus* and *C. comatus* strains stained the agar reddish-brown with aerial mycelium in the centers of *C. micaceus* colonies partially collapsing (not shown). *C. comatus* colonies grew slower than other species and formed loose mycelial strands in the whitish aerial mycelium. *C. strossmayeri* produced vigorous cottony, silky-shining hyphal strands in white aerial mycelium ([48]; not further shown). Production of primordia in a culture of *C. strossmayeri* 15-3C [48] and fruiting bodies in cultures of *C. curtus* C71, *C. domesticus* C72, *C. ellisii* C140, *C. micaceus* I and SII, *C.* aff. *radians* (II) C35, *C. xanthothrix* C144 (Fig. 6), and *C. cinerea* C344 (not shown) identified the strains as dikaryons. *C. cinerea* C344 furthermore produced masses of sclerotia (Fig. 1D-F) in aging cultures after fruiting body formation.

Further in this study, micro-morphological characters of cultures (clamps; mycelial strands/*Ozonium* production; asexual spores: *Hormographiella*-type conidiophores with spores, chlamydospores) were observed as documented in the following.

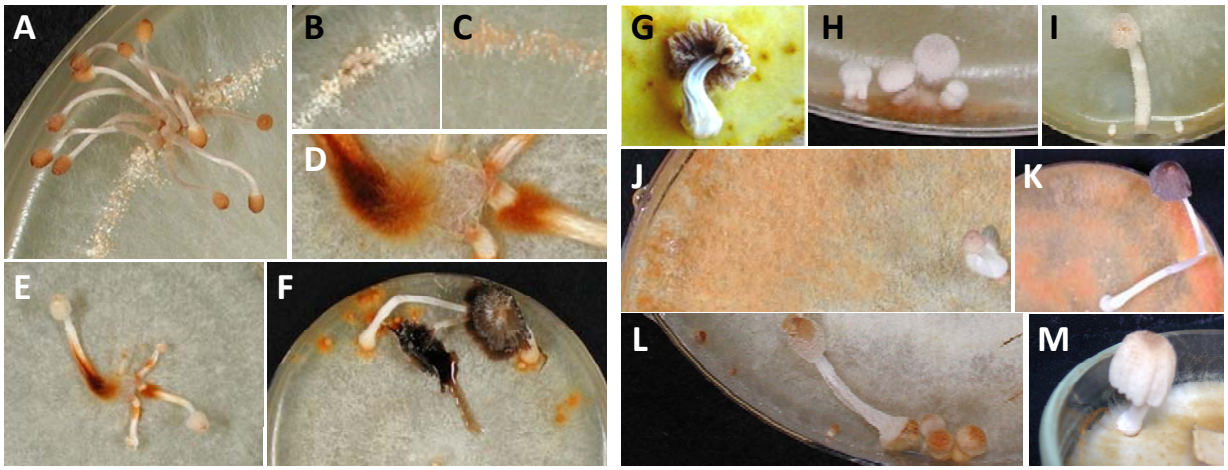

Figure 6: Cultures of *Coprinellus* species with *Ozonium*-type mycelia, primordia and fruiting bodies. *C. curtus* C71 fruiting bodies with rust-stained caps (A), and stained hyphal knots and primordia (B,C). *C. domesticus* C72 fruiting bodies with *Ozonium*-type mycelium formed beneath and at bases of their stipes (D,E,F). *C. ellisii* C140 fruiting body in a yellow-stained *Ozonium*-type mycelium (G). *C. micaceus* fruiting bodies growing within (strain SII; H) and without (strain I; I) an *Ozonium*-type mycelium. Fruiting bodies of *C. radians* C35 (J,K) in a largely extended *Ozonium*-type mycelium. *C. xanthothrix* C144 fruiting bodies growing in sparse (L) and extended (M) *Ozonium*-type mycelium.

***Coprinellus* species.** As indicated above, a striking feature of many *Coprinellus* species was the yellowish-brown-rust coloration of whole colonies or of patches of them (Fig. 6). Only *C. curtus*,

C. micaceus and *C. flocculosus* strains did not obviously stain their mycelia. The results support the conclusions in the literature [24,33,36-38] that a tawny-rust mycelial color can indicate an affiliation to the genus *Coprinellus*. In accordance, species of other coprinoid genera than *Coprinellus* did not show such coloration. Mycelium coloration occurring in our cultures correlated with reports in the literature of *Ozonium* formation in nature for *C. disseminatus*, *C. domesticus*, *C. ellisii*, *C. radians*, and *C. xanthothrix* [33,36-39]. Although we did not register obvious staining of mycelium in *C. curtus* cultures, hyphal knots and primordia that appear to prepare for fruiting body maturation stained orange-brownish (Fig. 6B,C). Cells on the surface of the fruiting body caps had a strong orange-brown color typical for *C. curtus* [36,38,39]. Buller [33] described *Ozonium* formation in *C. micaceus* but noted that not all individuals of the species do so. Our observations on lack of mycelium coloration in *C. micaceus* I and restriction in *C. micaceus* SII to bases of fruiting body stipes and beneath (Fig. 6H,I) thus do not contrast literature reports. To our best knowledge, no *Ozonium* formation has ever been reported for *C. flocculosus* which might indicate that, consistent with our results, this species probably shows no tawny-rust *Ozonium* formation.

On the micro-morphological level, *Ozonium* stages are characterized by tight parallel growth of well septated brownish hyphae as part of mycelial strand formation [33]. Therefore, we observed vegetative mycelium of our strains under the microscope (Fig. 7).

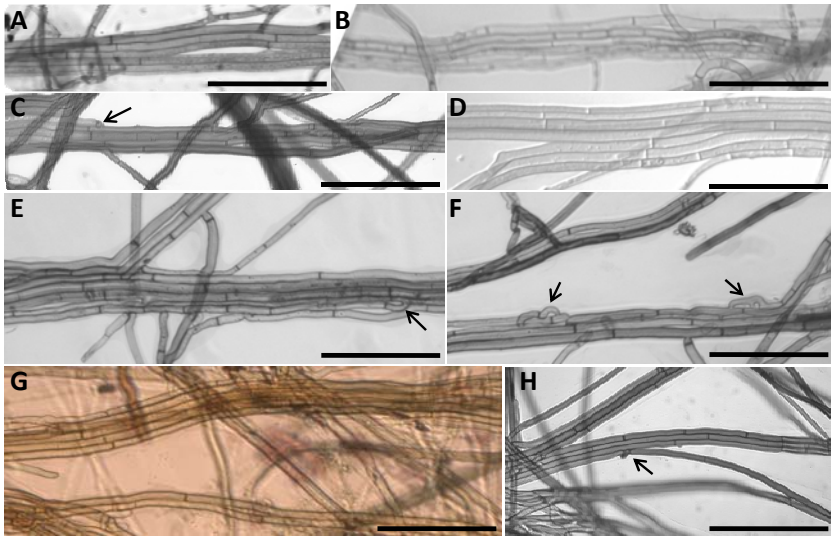

Figure 7: *Ozonium*-type of mycelium in *Coprinellus* species. *C. curtus* C311 (A), *C. domesticus* C72 (B), *C. ellisii* C140 (C), *C. micaceus* 1-OH (D), *C. radians* C22 (E-G), *C. xanthothrix* C482 (H). Samples stained with methylene-blue (A-F,H). Natural coloration of *C. radians* C22 hyphae (G). Arrows point to unfused clamps and clamp-like structures. Size bars = 100 µm.

Strains of *Coprinellus* species of which we observed in colonies degrees of colored *Ozonium* formation were found on microslides to form strands of highly septated parallel growing hyphae with homogenous cell content (Fig. 7). Since methylene-blue was used for better contrasting, the original color of hyphae was however concealed. Though, the cellular contents of *C. domesticus* and *C. micaceus* and hyphal strands were more translucent than those of other species (Fig. 7C,D). In unstained samples of *C. radians* C22, it became obvious that the natural cellular color was brown (Fig. 7G). For the other strains, such more differentiated

investigation remains to be done. Although colored *Ozonium* formation was not observed in cultures of *C. flocculosus* strains, it should be pointed out that rudiments of parallel *Ozonium*-typical hyphal growth (strands of just 2 or 3 hyphae) were detected on mycelium grown on microslides (not shown).

Septa in the hyphal strands of all *Coprinellus* strains were typically clampless (Fig. 7). Occasionally at an outer hypha of a younger strand of yet few hyphae there was an unfused cell (pseudoclamp; Fig. 7H) or a clamp-like structure from which outgrowth of another hypha may start (*Ozonium* specific branching; see also [33]) for strengthening the strand size by parallel growth of a further hypha that attach closely over the length to the mother hypha (Fig. 7C,E,F) and may undergo anastomosis with different hyphae within a strand (Fig. 7F).

Clamp cells are formed in filamentous basidiomycetes at septa of dikaryotic mycelia as part of a mechanism ensuring that each cell in the dikaryon obtains two genetically distinct haploid nuclei [11,19,49]. At freely growing hyphae of all tested strains of *C. disseminatus* and *C. micaceus*, we saw clamp cell formation, albeit at only some septa (Fig. 8A,B). Particularly in *C. micaceus* strains, clamp cells occurred at very low frequencies. *C. curtus*, *C. flocculosus*, *C. radians*, *C.* aff. *radians* (I) and (II) and *C. xanthothrix* strains in contrast gave only rise to rare pseudoclamps (Fig. 8C,D,E; [49] and not shown), whereas we did not detect any clamps on hyphae of *C. domesticus* C72 and *C. ellisii* C140. While the way of strain isolation from inner cap (C strains) or stipe tissues (S strains) or from dense plating of basidiospores (PS strains) specifically targeted at the isolation of dikaryons from own mushrooms collected in Armenia or Germany ([44,46,48] and unpublished), for strains from Duke University (not included in the Duke coprini molecular identification project [8,37]) no such information was available. Occurrence of either fruiting bodies, clamp cells or pseudoclamps argue for a dikaryotic status for all tested *Coprinellus* strains from Duke. Nuclear staining should confirm this in the future.

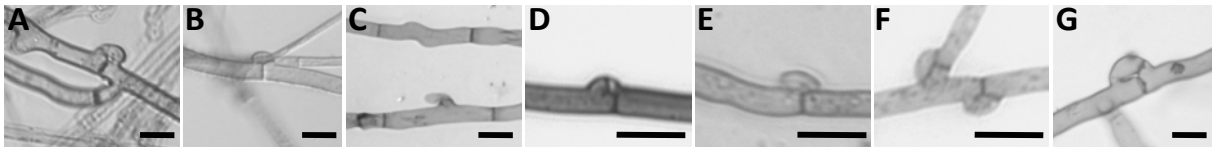

Figure 8: Fused (A,B,F,G) and unfused clamp cells (C,D,E) in *C. disseminatus* C50 (A), *C. micaceus* 1-OH (B), *C. curtus* C71 (C), *C. radians* 1-2PS (D) modified from [48], *C. xanthothrix* C398 (E), *C. cinerea* C344 (F), *C. comatus* 3S (G). Size bars = 10 µm.

Vegetative forms of sporulation were only in some species observed in our current test system. *Hormographiella*-like structures occurred in *C. domesticus* (Fig. 9A,B), *C. ellisii* C140 (Fig. 9D), *C. radians* C22 (rare; not shown), *C.* aff. *radians* (II) C35 (abundant; Fig. 9C) *C. xanthothrix* (Fig. 9E). The structures clearly differed between the species, with strongly staining rod-like spores in *C. domesticus* and *C. xanthothrix*. In *C. domesticus*, spores assemble on tips of short conidiophores (Fig. 9A,B), similarly as it has been described for the species in the literature [23,24], whereas in *C. xanthothrix*, spores appear to arise next to each other in comb-shaped manner (Fig. 9E). The chamfered spores of *C. ellisii* and the rounded, often slightly falciform spores of *C.* aff. *radians* (II) C35 are more variable in sizes (Fig. 9C,D). These two species appear to distinguish mainly by their conidiophore structures. In *C. ellisii*, spores seem to

arise mainly along one side of unbranched and branched conidiophores, directly on conidiophore stem cells or on short conidiogeneous hyphae (Fig. 9D). In *C.* aff. *radians* (II) C35, spores apparently arise on tips of slightly swollen branches of conidiophores (Fig. 9C). None of the conidiophore structures observed in this study resembled the *H. candelabrata* anamorph [23].

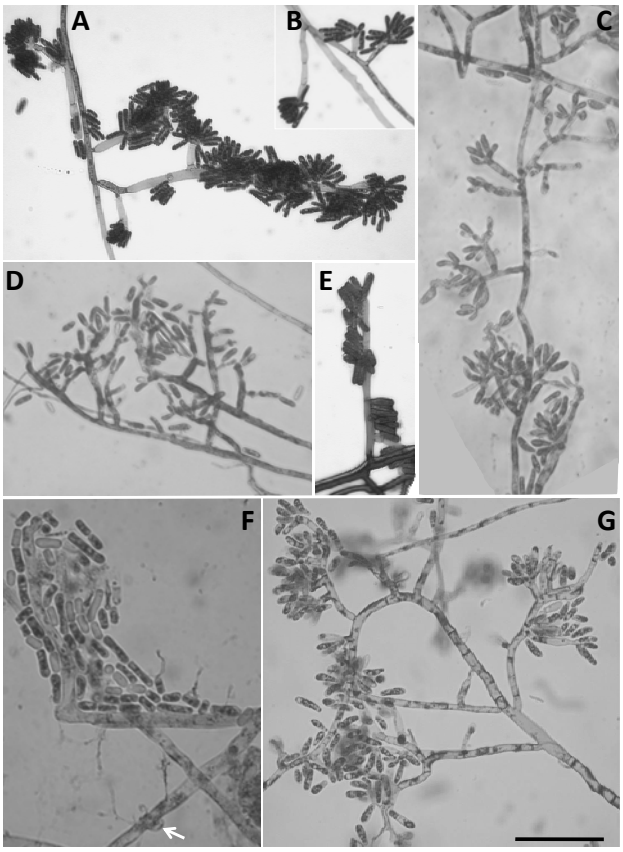

Figure 9: *Hormographiella*-type sporulation in *C. domesticus* C72 (A,B), *C.* aff. *radians* (II) C35 (C), *C. ellisii* C140 (D), *C. xanthothrix* C482 (E), *C. cinerea* C344 (F), and *C. scobicola* Cb1 (G). The arrow points to a clamp cell at a *C. cinerea* hypha (G). Size bars = 50 µm.

***Coprinopsis* species.** Of the analysed *Coprinopsis* species under the selected test conditions, the dikaryotic *C. cinerea* strain C344 (note fused clamp cells in Fig. 8F and Fig. 9F) produced oidia in rare events (Fig. 9F) in contrast to the clampless monokaryon Cb1 of *C. scobicola* that formed complex structured oidiophores throughout the aerial mycelium (Fig. 9G). *C. scobicola* has previously been reported not to produce oidia [26] and *C. cinerea* wet oidia [13,14,25,26]. Our current test system observing structures in a liquid does not allow us to distinguish between these two types of sporulation. Neither the dikaryotic *C. gonophylla* C399 (fused clamps present at all septa; not shown) nor the *C. strossmayeri* strains (for fused clamps see [48]) yielded in our cultures any *Hormographiella*-type of conidiophores. These species however produced large swollen thick-walled chlamydospores on the microslides, intercallarly or terminally at hyphal tips (Fig. 10A-C). Chlamydospores in *C. cinerea* are typical structures of aging cultures (Fig. 1G,H; [11,19,30,31]) and absence in other cultures of a few days growth does not exclude

the existing possibility of chlamydospore formation with growing age. Mycelial strands should be mentioned for *C. gonophylla* (not shown) and *C. strossmayeri* [48]. These strands clearly distinguish from the *Ozonium*-type of growth of species of the genus *Coprinellus*. Mycelial strands are whitish and hyphae are only loosely entangled and usually not as compact aggregated than the strands shown for *C. cinerea* in Fig. 4A-C. At points of hyphal attachments, there often are incidents of anastomoses (not shown).

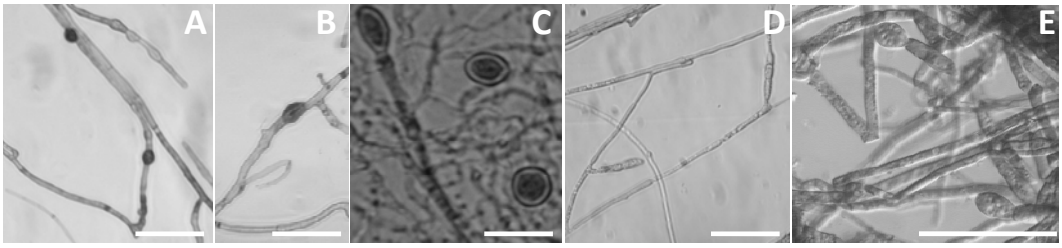

Figure 10: Chlamydospore formation in *C. gonophylla* (A, B), *C. strossmayeri* 15-2C (C), and *C. comatus* 1-C (D,E).
Size bars = 50 µm**.**

***Coprinus comatus*.** Fused clamp cells at hyphal septa were present in *C. comatus* but at low frequency (Fig. 8), consistent with the strains being dikaryons. *Hormographiella*-type sporulation was not observed in *C. comatus* cultures. In hyphae of *C. comatus* strains 1C and 3S, evidence for chlamydospore production was found by observation of swelling intercalar and terminal hyphal cells and large, rounded, thick-walled cells within mycelium (Fig. 10D,E). Spherical spores in *C. comatus* have been reported before [26].

CONCLUSION

In an initiative study on morphological anamorphic features (sporulating and non-sporulating) of coprini, we presented our current observations on a selection of 15 different species from the clades *Coprinellus*, *Coprinopsis* and *Coprinus*. Mostly, dikaryons were analyzed and only exceptionally a monokaryon. For definition of anamorphic characters it is of interest to know whether they are typical for the monokaryon, for the dikaryon or both. Eventually therefore both types of mycelia will have to be evaluated under selected environmental conditions for entire descriptions of holomorphs. For instance, light is crucial for both asexual and sexual reproduction in *C. cinerea* dikaryons [15]. The way of culturing in this study and the time of occurrence may for example suggest that induction of stained hyphal knots and primordia in firstly dense white mycelium in *C. curtus* (Fig. 6A-C) is stimulated by short illumination. Larger culturing series will need to confirm this. Monokaryons and dikaryons within a species might differ in vegetative sporulation – monokaryons of *C. micaceus* fort instance produce arthrospores by septation of more or less normal hyphae [26] what was not observed here with the dikaryons.

Asexual conidiogenesis in the life cycle of coprini and evaluation of their taxonomic significance provide valuable information for fungal systematics [1,2,4-7]. *Hormographiella*-type sporulation was found in species of the genera *Coprinellus* and *Coprinospsis*, on some dikaryons (*C. domesticus*, *C. ellisii*, *C.* aff. *radians* (II), *C. xanthothrix*, and *C. cinerea*; Fig. 9A-H) and on monokaryons (*C. cinerea*, *C. scobicola*; Fig. 2, Fig. 9G). Further studies will need to define the specific morphological features (structures, sizes) of conidiophores and spores and to

reveal potential regulation factors (such as light) and whether these feature can lead to definition of species-, mycelium- and strain-specific *Hormographiella*-stages with significance for fungal taxonomy. In this study, also some observations on chlamydospore production in the four species *C. cinerea* (Fig. 1G,H), *C. gonophylla*, *C. strossmayeri*, and *C. comatus* (Fig. 10) were presented. Particularly also these need deeper analysis generally in the Agaricomycetes to understand the potential taxonomic value of this type of spores [6].

As an important result for taxonomy, the sterile *Ozonium*-stage was confirmed to be a good morphological character for dikaryons of *Coprinellus* species. Nine out of ten species had clearly an *Ozonium*-stage, which was reported here for the first time in *C. disseminatus* (not shown) and *C. xanthothrix* (Fig. 6M,L and Fig. 7H) and there are at least rudiments of *Ozonium* formation in *C. flocculosus* (not shown). Linkage to fruiting body development (Fig. 6) may indicate a dikaryon-specific stage but currently it is unsolved whether monokaryons also form *Ozonium*-like mycelia.

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BIODIVERSITY, CONSERVATION AND UTILISATION OF MUSHROOM FLORA FROM THE WESTERN GHATS REGION OF INDIA

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ABSTRACT

The Western Ghats region of Indian subcontinent is one of the globally recognized biodiversity hotspots that has an unestimated wealth of biodiversity. A survey was conducted in the Anaikatti, Attapadi, Palghat, Siruvani, Nilgiris and Kallar regions of the Western Ghats of India during 2008 -10 and about 68 mushroom flora belonging to 19 genera were recorded. The seasonal occurrence of *Volvariella* from June-September; *Calocybe* from February-September; *Pleurotus* from June-July and November-January; *Auricularia, Lentinus, Agaricus* from October-January; *Tricholoma* during June; *Ganoderma*, *Polyporus*, *Trametes* from September-January and June-August; *Lycoperdon* during October; *Termitomyces* from July-October; *Tramella*, *Mycena* and *Rusulla* from January; *Ramaria* from October-November; *Schizophyllum* from June-October and January; *Amanita* from November-December and *Macrolepiota* during December were observed.
Among the mushroom flora a wild strain of *Pleurotus djamor roseus* was found suitable for commercial cultivation with bioefficiency of 132 percent and cost benefit ratio of 1:2.9. The mushroom contained all essential nutrients and could be stored under room temperature for one day and under refrigerated storage for 2 days. The ITS 1 and 2 regions of the mushroom was sequenced and submitted in NCBI (Gen Bank accession No. HM107001). The diethyl ether fraction (10 percent concentration) of *G.lucidum* and *L. edodes* showed the inhibition of mycelial growth by up to 70 percent and 68.2 percent, respectively against *Collectotrichum gloeosporioides*, the fruit rot pathogen of Mango.

Keywords: Biodiversity,Western ghats, Mushroom flora

INTRODUCTION

The Indian sub continent is blessed with diverse agroclimatic zones that harbour a treasure trove of fungal diversity. Though the occurrence of mushrooms is of diverse nature in India, they are not well known. The collections of mushrooms began in India four decades ago [1,2,3]. To date, about 1,200 species of fungi belonging to the order Agaricales, Russulales and Boletales are described in comparison to about 14,000 species of mushrooms reported worldwide that contributes 10 percent of the global mushroom flora. So far, about 1,105 to 1,208 species of mushrooms belonging to 128-130 genera have been documented and among these, 300-315 species belonging to 75-80 genera are considered edible. The Western Ghats region, one of the

four globally recognised biodiversity hotspots forming a long mountainous region along the west coast of India harbor to date consists of 750 species of mushrooms. It has an unestimated wealth of mushroom biodiversity that needs to be tapped properly as there are still several undescribed species yet to be identified. Efforts need to be made to identify and exploit these mushroom flora for utility as their biodiversity and conservation strengthen the food security of a country [4].

Out of 2000 edible species available in the world, about 283 are reported from India. Nearly 6,500 collections of mushroom flora belonging to 223 species, 59 genera and 15 families of Agaricales have been reported from North West Himalayas by Lakhan Pal and his students since 1976 [5]. These data on the occurrence of the mushrooms reveals the richness of mycoprotein in the country. Not only in terms of edibility, there lies enormous applications of these mushrooms for bioremediation, biodegradation, biopesticidal and pharmacological values that could be exploited.

The Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, India has contributed to the development and domestication of six species of *Pleurotus*, two isolates of *Agaricus bisporus*, and one species of *Calocybe* for commercial utilization. However, due to increased awareness of the pharmacological values and nutritional values of mushrooms, there is more demand and consumer preference for different varieties of mushrooms among the people and farmers that are urged to exploit the wild mushrooms for utlisation. The mushroom genome stands out as a virtually untapped resource for novel antimicrobials. Recently many antibacterial antifungal and insecticidal properties have been identified from mushrooms. Scientists worldwide are now focusing on the exploitation of biomolecules from mushrooms for pest and disease management which is a challenging field of study. With this aim, surveys were conducted in Western Ghats region so as to exploit the mushrooms for utilization and also to identify their antimicrobial nature.

MATERIALS AND METHODS

Collection and identification of wild mushrooms. Collections of wild mushroom flora were made from the Anaikatti, Attaipadi, Kallar, Nilgiris, Palakkad and Siruvani regions of the Western Ghats of India during the period 2008-2010. The mushrooms collected were identified morphologically as per the keys provided by Directorate of Mushroom Research, Solan, pure cultured and maintained in potato dextrose agar slants and used for further studies.

Molecular characterization

Isolation of DNA from *Pleurotus djamor*. Among the wild mushrooms collected, a new isolate of oyster mushroom, *Pleurotus djamor roseus* was selected. The mushroom was grown in malt extract broth and the mycelial mat was collected and ground with lysis buffer. After maceration the tube was kept in room temperature for 30 min and 150µl of potassium acetate was added, vortexed for 2-3 seconds and kept in freezer for 30 min. The tubes were centrifuged at 15,000 rpm for 5 min. The supernatant was transferred to another tube and equal volume of isopropyl alcohol was added. The tube was mixed by inversion and centrifuged at 15,000 rpm for 2 min and the supernatant was discarded. The DNA pellet was washed in 300 µl of 70 percent ethanol, centrifuged at 10,000 for one min and then the supernatant was discarded. The pellet was air dried and dissolved in 50 µl of 1X TE buffer and used as genomic DNA for PCR reaction [6].

Amplification of the ITS regions with ITS-1 and ITS- 4 primers. The genomic DNA extracted from the pure culture of *Pleurotus djamor* was used for PCR studies. The polymerase chain reaction primers, ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used to amplify the ITS of ribosomal DNA [7]. The PCR reaction was performed in a total reaction mixture of 40 µl containing 20 µl of master mix , 4 µl each of ITS-1 and ITS-2 , 4µl genomic DNA and 8 µl of distilled water. The PCR conditions consisted of 34 cycles of 1 min denaturation at 95°, 30 s annealing at 50˚C, 1 min 20s elongation at 72˚C and ending by 10 min final elongation step at 72˚C with lid heating option at 110˚C [6]. Amplified products were run on 2 percent agarose gel, stained with ethidium bromide and visualized under UV illumination. The sequencing was done using ITS-1 and ITS-4 primers and the nucleotide sequence comparisons were performed using Blast Multiple Alignment Tool (BLAST) network sequences from the National Centre for Biotechnology Information (NCBI) database.

Performance testing of the selected oyster mushroom *Pleurotus djamor roseus.* The yield performance of *P.djamor roseus* was tested at the Mushroom Research and Training Centre, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. The new oyster mushroom, *Pleurotus djamor roseus* with pale pinkish white sporophores was selected and tested for its yield performance for commercial cultivation. The spawn of *P. djamor roseus* was prepared in sorghum substrate and used for bed prepaparation. For this, purpose using paddy straw as substrate, cylindrical beds were prepared with sorghum based spawn of *P. djamor roseus* (500 g substrate/bed; 300 g of spawn/bed). The beds were placed in cropping rooms made of thatched shed (using coconut leaves) where temperatures of 23-30˚C with relative humidity of 80 percent was maintained thorough out the cropping period. The beds were held in place with a hanging rope system in the cropping rooms. Five beds were prepared monthly from June, 2009 to May, 2010. The yield characters were days for spawn run (DFSR), days for pinhead formation (DFPF), days for first harvest (DFFH), Total yield (g per kg substrate) and pest and disease levels were recorded. The bioefficiency percentage was calculated. Harvested mushrooms were cleaned and packed (250 g) separately in perforated polythene bags and placed under natural conditions (room temperature). Under refrigerated condition they were placed in non-perforated polythene bags and the observations on keeping quality were recorded.

Comparison of *Pleurotus djamor roseus* with other oyster mushrooms. The mushroom *P. djamor roseus* was tested for its yield performance in comparison with the mushroom varieties that are cultivated on a commercial scale in the state. The mushroom varieties *viz.*, *Pleurotus florida* (var. PF), *P. eous* (var. APK1), *P. djamor* (var. MDU1-white), *Hypsizygus ulmarius* (var. CO2) were used for comparison. The beds were prepared with paddy straw as substrate as mentioned earlier and the yield characters were recorded as mentioned earlier with four replications. All experiments were laid out based on completely randomized block design (CRD). Statistical software (AGRES) was used for the analysis of the data.

Studies on the nutritive value of *Pleurotus djamor roseus.* Moisture content of the mushroom was estimated by drying 50 g of fresh mushrooms in an oven at 80^{o}C for three consecutive days. It was later cooled in a desiccator and reweighed. The moisture content was arrived from the differences in the weight [8]. The crude protein content of the mushroom was estimated by Micro Kjeldahl method [9]. The total carbohydrate content was determined by following

Anthrone method [10]. Estimation of ascorbic acid [11], crude fat [8], total phenolic content using the Folin-Ciocalteu method [12], crude fibre [13], total ash content (14), total nitrogen analysed by Diacid extract method-semiautomatic Kjeldhal distillation, total phosphorous by Triacid extractmethod-vanodamolybdate calorimetric method, total potassium and total calcium [15] as described previously. Antioxidant activity was measured using Ferric reducing antioxidant power (FRAP) assay [16]. All nutrients and ingredients were analyzed on fresh weight basis. The experiments on the estimation of nutritive values were performed at the Post harvest Technology Centre, Tamil Nadu Agricultural University, Coimbatore-3.

***In vitro* testing of mushroom flora for antimicrobial properties.** Among the mushroom flora collected *viz., Ganoderma lucidum, Lentinus edodes, Trametes versicolor, Pleurotus djamor roseus* and *Auricularia polytricha* were selected and screened for their antifungal activity against *Colletotrichum gloeopsporioides*, the mango fruit rot pathogen by a dual culture technique [17]. Based on the results obtained from dual culture technique, both *Ganoderma lucidum, Lentinus edodes* were selected for *in vitro* testing of antimicrobial activity against *Colletotrichum gloeosporioides* by a poisoned food technique [18]. For this purpose, the discs (9 mm) of both *Ganoderma lucidum* and *Lentinus edodes* were inoculated in potato dextrose broth and the culture filtrate was collected after seven days and the antimicrobial substances were separated using diethyl ether. The diethyl ether fractions *ie.,* the aqueous phase at different concentrations *viz.,* 1, 5 and 10 percent were prepared in PDA medium separately. The medium containing diethyl ether fractions of *Ganoderma lucidum* and *Lentinus edodes* was plated separately and a mycelial disc of the pathogen *Colletotrichum gloeosporioides* was placed at the center. Three replications were maintained for each treatment. After five days incubation, the mycelial growth of the pathogen was recorded in both treatments as well as in respective controls and the percent inhibition was calculated [19].

RESULTS AND DISCUSSION

Collection of wild mushroom flora. About sixty eight mushroom flora belonging to 19 genera *viz., Agaricus sp., Amanita sp., Auricularia polytricha, Calocybe, Ganoderma lucidum, Lentinus edodes, Lycoperdon esculentum, Mycena sp., Macrolepiota sp., Pleurotus cystidiosus, P.djamor, P. pulmonarius, Polyporusversicolor, Ramaria sp., Russula sp., Ramella sp., Schizophyllum commune, Termitomyces, Trametes versicolor, Tricholoma giganteum,* and *Volvariella volvacea* collected from Anaikatti, Siruvani, Palghat, Kallar and Nilgiris regions of Western ghats during 2008-10 were identified morphologically based on the keys provided by the Directorate of Mushroom Research, Solan, India.

Collections revealed the occurrence of *Volvariella* from June-September; *Calocybe* from February-September; *Pleurotus* from June-July and November-January; *Auricularia, Lentinus, Agaricus* from October-January; *Tricholoma* during June; *Ganoderma, Polyporus, Trametes* from September-January and June-August; *Lycoperdon* during October; *Termitomyces* from July-October; *Tramella, Mycena* and *Rusulla* from January; *Ramaria* from October-November; *Schizophyllum* from June-October and January; *Amanita* from November-December *Macrolepiota* during December (Table 1).

Table1. Wild mushroom flora of the Western Ghats region of India

S. No.	Mushroom flora	Place	2008-09	2009-10
1.	*Volvariella volvacea*	Palghat, Anaikatti	June August July	September August -
2.	*Pleurotus djamor* *P. cystidiosus* *P. pulmonarius* *Pleurotus* sp.	Kallar Anaikatti Nilgiris Siruvani	June November January -	- July - June
3.	*Lentinus edodes* *L. crinitus*	Nilgiris Anaikatti	October November	January -
4.	*Ganoderma lucidum*	Anaikatti Coimbatore	September, October September	January September
5.	*Calocybe indica*	Anaikatti Coimbatore	February May, July	June, July September
6.	*Agaricus sp.*	Nilgiris	October,December	January
7.	*Polyporus versicolor*	Siruvani	January	November
8.	*Trametes versicolor*	Siruvani Kallar Attaipadi	September October, December November	September October November
9.	*Auricularia polytricha*	Attaipadi Kallar	November	November
10.	*Termitomyces sp.*	Anaikatti Attaipadi	July, September September, August October	July , August October
11.	*Tricholoma giganteum*	Coimbatore	June	-
12.	*Schizophyllum commune*	Coimbatore	June, August , September	July, October, January
13.	*Lycoperdon esculentum*	Anaikatti Siruvani Kallar	October October, November November	October
14.	*Ramaria sp.*	Anaikatti	November	October November
15.	*Russula sp.*	Nilgiris	-	January
16.	*Tramella sp.*	Nilgiris	January	January
17.	*Amanita sp.*	Nilgiris	November	December
18.	*Mycena sp.*	Nilgiris	January	January
19.	*Macrolepiota sp.*	Nilgiris	December	-

Hot season: March–May/June
Rainy seasons: June–October /November
Cool weather conditions: November–February

It was observed that the climatic conditions prevailing in the areas of Anaikatti, Attaipadi, Kallar, Nilgiris, Palakkad and Siruvani regions of the Western Ghats favored the occurrence of diverse mushrooms. The mushrooms occurred through out the year and collections were grouped into three seasons: hot season from March–May/June, rainy seasons from June–October/November and cool weather conditions from November-February. The *V. volavcea* was collected during periods coinciding with rainy seasons followed by a hot and humid climate. *Pleurotus* sp. was mostly observed during rainy seasons. *Lentinus edodes* and *L. crinitus.*, *Agaricus* sp., *Amanita* sp., *Mycena* sp., *Tramella* sp., *Ramaria sp.,Russula sp., Macrolepiota sp., A.polytricha*, *P.versicolor* during cool seasons prevailing after rains. The occurrence of *L. esculentum* coincided with the initiation of rains from North West monsoon during that season . However, *T.versicolor, Termitomyces sp*., and S. *commune* were collected from both rainy and cool periods. Collections of *T. giganteum* were made during the period when the weather is warm just before the onset of southwest monsoon. *C. indica* and *G. lucidum* was collected during the period before and after the southwest monsoon. Distribution of 134 species of mushrooms representing 45 genera was recorded in Kerala during monsoon seasons. Best collection of fleshy agarics especially the popular edible mushrooms, the species of the termitophilic genera *Termitomyces, Podabrella*, the wood decomposer *Pleurotus*, and the ectomycorrhizhal fungi belonging to Boletaceae was dominant in the Western Ghats [20]. About, 90 mushroom accessions in 35 genera were documented in the Nilgiris biosphere reserve (21).

Molecular characterization. Amplification of the ITS regions of *Pleurotus djamor* with ITS-1 and ITS-4 primers shared 99 per cent homology with *P.djamor* . The sequences were submitted to NCBI and given Gen bank accession number HM107001.

Performance of the oyster mushroom *P. djamor roseus.* The mushroom required a maximum of 8.6 days for complete spawn run, 10.2 days for pin head formation, and 12.6 days for first harvest. The pin heads were pale pink in color and mature mushrooms were pale pinkish white in color with pileus measuring 5 cm and stipe length of 2 cm (Figure 1). Maximum yield of 660 g of mushroom/500 g substrate (132 percent bioefficiency) was recorded during the month of June. However, the bioefficiency ranged from 124 to 132 percent through out the year (Table 2). This mushroom can be best cultivated in thatched sheds with temperature range of 23-30°C and relative humidity of 70-80 percent through out the year.

Figure1. Morphology of *P.djamor roseus*

Table 2. Performance of the oyster mushroom *P.djamor roseus*

Month	DFSR	DFPF	DFFH	Total yield-g/ bed)	Bioefficiency (%)
June,2009	7.4	8.0	10.0	660.0	132.0
July	7.8	8.8	10.6	610.0	122.0
August	7.4	8.4	10.4	620.0	124.0
September	7.8	8.8	10.0	632.0	126.4
October	8.0	9.4	11.6	650.0	130.0
November	7.4	8.4	11.4	634.0	126.8
December	7.6	8.2	10.6	620.0	124.0
January. 2010	7.2	8.0	11.0	620.0	124.0
February.	7.0	8.0	10.6	635.0	127.0
March.	7.0	8.0	11.2	655.0	131.0
April	7.6	8.6	10.6	630.0	126.0
May	8.6	10.2	12.6	645.0	129.0
CD(P=0.05)				29.2	

Mean of five replications . g: Grams; % : Per cent

The mushroom when compared with the existing commercially grown oyster mushroom species revealed that *P. djamor roseus* performed on par with *P. eous* (var. APK-1) with bioefficiency of 132 percent (Table 3). Among the oyster mushrooms, *P. djamor roseus* recorded an earlier harvest and the cropping period was completed by 17-20 days which is very short when compared to *P. eous* (var. APK1) and other mushrooms. The mushrooms could be stored under room temperature for one day and under refrigerated storage for 2 days without any microbial spoilage, color change and liquefaction.

Table 3. Comparison of *P. djamor roseus* with commercially grown oyster mushroom

Mushrooms	DFSR	DFPF	DFFH	Yield g/500 g substrate			Yield g/ bed	BE (%)	C: B ratio
				I H	II H	III H			
P. florida var. PF	17.3	20.8	24.8	400	250	100	750	150.0	1:3.6
P. sajorcaju var. M2	19.3	22.5	25.0	350	250	100	700	140.0	1:3.4
P. eous var. APK-1	12.0	13.2	14.8	350	220	105	675	135.0	1:3.2
P. djamor var. MDU1	20.0	23.0	26.3	350	250	100	700	140.0	1:3.5
H. ulmarius var. CO2	22.0	23.0	24.6	400	200	100	705	141.0	1:3.5
P. djamor roseus	8.3	10.3	11.3	360	250.0	50.0	660	132.0	1:3.2
CD (P=0.05)							42.23		

Mean of four replications; H : Harvest; C:B: Cost: Benefit Ratio

The nutritive values analyzed for 100 g fresh mushroom (*P. djamor roseus*) showed the presence of all essential nutrients viz., carbohydrates (3.8%), protein (2.6%), fat (0.2%), crude fibre (0.94%), total ash (1.12%), ascorbic acid (15.43 mg/g), total nitrogen (1.28%), total phosphorous (0.445 %), total potassium (2.56 %), calcium (1.2 %), sodium (0.38 %) with total antioxidant

activity of 186.3 (µg/g) and a calorific value of 19.8. No pest and disease was recorded during the cropping period.

Antimicrobial activity of mushroom fungi against plant pathogen. Among the mushrooms tested by dual culture technique, *G. lucidum* and *L. edodes* inhibited mycelial growth of *Colletotrichum gloeosoprioides* to 68 percent and 65.3 percent, respectively (Table 4). The diethyl ether extract (10 percent) of *L. edodes* and *G. lucidum* was tested by a poisoned food technique and the results showed that the mycelial growth was inhibited by 70 percent and 68.2 percent, respectively (Figure 2). Perusal of reports show that the shiitake mycelial leachate contained an antibiotic substance that exhibited clear zones of inhibition against plant pathogenic bacteria *Pseudomonas syringae pv. glycinea, P. syringae pv. tabaci, X. campestris pv. campestris* and *Ralstonia solanacearum* [22]. In addition, we found that *Ganoderma lucidum* exhibited a broad spectrum of antifungal, antibacterial and antiviral activities [23]. The ethanolic and methanolic extracts of *Agaricus bisoprus* and *P. sajor-caju* exhibited antimicrobial activity against human pathogens *viz*., *Escherichia coli, Enterobacter aerogenes*, *Pseudomonas aeruginosa* and *Klebsiella pseudomonaie* [24]. Though there are several reports on the novel biomolecules from higher basidiomycetes, the scientific community is now focusing on the exploitation of the antimicrobial compounds against plant pathogens as well.

Table 4 .*Invitro* testing of mushroom fungi against *C.gloeosporioides* by dual culture technique

S. No.	Mushroom Species	Mycelial growth of *C. gloeosporioides* (mm)	Percent inhibition
1.	*Ganoderma lucidum*	24	68.0
2.	*Trametes versicolor*	50	33.3
3.	*Lentinus edodes*	26	65.3
4.	*Pleurotus djamor roseus*	59	21.3
5.	*Auricularia polytricha*	44	41.3
6.	Pathogen control	75	-

Means of three replications

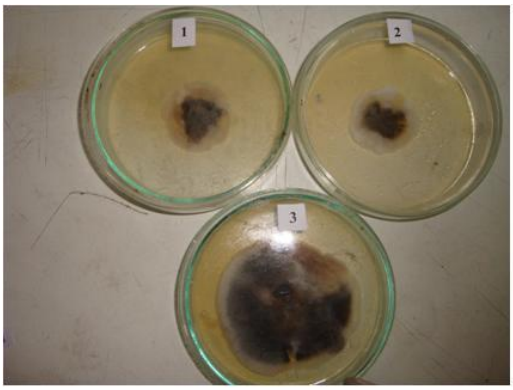

Figure 2. Antimicrobial activity of diethyl ether extract of *G. lucidum (1)* and *L. edodes (2)* against *C. gloeosporioides; 3= pathogen control*.

CONCLUSION

Perusal of literature shows there is no doubt that the diverse climatic condition in India made this country a natural habitat for many mushrooms. Currently due to deforestation activities several mushroom species are endangered and hence it is crucial to conserve the mushroom flora to strengthen the food security of a country. In that way, this paper clearly depicts the biodiversity of mushroom flora occurring in the Western Ghats region of India and their conservation in order to identify the potential mushroom species for domestication also to exploit the biomolecules. The oyster mushroom *Pleurotus djamor roseus* is highly suitable for commercial cultivation in subtropical regions of the world. This study on the antimicrobial nature of *L.edodes and G. lucidum* paves way for developing biopesticidal molecules from mushrooms against plant pathogens.

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DIVERSITY OF FRUITING PATTERNS OF WILD BLACK MOREL MUSHROOM

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ABSTRACT

Morels (*Morchella* spp., Pezizales) are commercially important edible mushrooms, known for their delicate taste and aroma, which mostly reach the market from growth in the wild. However, despite their high market value, knowledge of morel behavior in its natural habitat and of the fruiting patterns exhibited by the various species in different habitats is scarce. Morels are found worldwide in a range of natural habitats, and many reports have linked morel fruiting with extreme changes in the environment, including deforestation, soil dryness, heavy rains, pesticide application, etc. Knowledge of morels' fruiting patterns in nature is needed for both conservation practice and to increase the value of non-timber forests. We located several distinct populations of black morels growing in different habitats, and monitored their population dynamics to define their fruiting patterns. Based on molecular identification, the mushrooms of the different black ecotypes belonged to *Morchella* species *conica* and *elata*. Each of these species exhibited more than one fruiting pattern, affected by both spatial heterogeneity of the growing site and climatic conditions, as will be discussed in the presentation.

Keywords: *Morchella*; Morel; Fruiting pattern; Mushrooms; Forest management

INTRODUCTION

Morel mushrooms (*Morchella* spp., Pezizales, Ascomycetes) include a range of species growing in temperate zones throughout the world—the Asian and Himalayan mountains, European and Mediterranean countries, and the Americas, from Alaska to Mexico. Due to high market demand for these delicate mushrooms, they are harvested in commercial quantities from certain locations for global supply, highlighting the need for a better understanding of factors affecting morel fruiting in the wild. Moreover, overharvesting of mushrooms in general has raised concern about forest health, as well as about the decline in morels [1]. Although in recent years, there has been an increase in studies focusing on morel biological and ecological features [1-4], knowledge of its behavior in natural habitats is still scant. Understanding phenology and spatial distribution of the mushroom population in forests or other habitats is important for both conservation policies and for increasing the value of non-timber forest products.

The morel's general life cycle was tentatively described by Volk and Leonard [5]. However, there is still much to learn with respect to the complexity of the morels' distribution due to the high diversity of habitats in which they grow, high polymorphism in head shape and color [6,7], and high genetic variation [8]. The issue is further complicated by their undefined trophic state: some are presumably mycorrhizal, while others are free-living [9]. Confusing reports in the literature make it difficult to follow the morels' natural population dynamics or growth patterns in natural heterogeneous environments, and to relate certain ecological behaviors to particular species. To study the fruiting pattern of wild morels, it is necessary to locate morel

patches as soon as they emerge. This is a difficult task, as in many cases morels appear in unexpected locations, and then only for short periods.

In recent years, we have studied the diversity of morels in Israel, where they are considered rare. As in other places in the Mediterranean region, in Israel, both yellow and black morel mushrooms have been observed [3, 4 10, 11]. We have observed morels in a range of habitats, including a natural Mediterranean grove, a forest after a fire, residential yards, waste-disposal sites and more. A similar range of habitats is known for morels in other parts of the world where they are found near healthy vegetation or in disturbed soil. In most cases, morels are detected only in their mature state, and there are only a few of them. This has made it difficult to study fruiting patterns of the different populations. Nevertheless, we were able to spot a few patches of morel populations in their early stages of emergence, and to follow their growth over a season or more [3].

Here we describe the different fruiting patterns of two distinct populations of black morels located in their early stage of emergence. While both fruited in early spring, they grew in different habitats, and exhibited different fruiting behavior.

MATERIALS AND METHODS

Morel populations and field characterization. Fruiting bodies of the different populations of black morels were located in five different habitats in Israel in the year 2006: in a post-fire forest, a residential backyard, along asphalt pavement, and at two sites in a healthy, undisturbed Mediterranean forest. The post-fire population and the Mediterranean forest populations were spotted at first fruiting body emergence, and their spatial and temporal fruiting patterns were monitored. The fire at the post-fire forest site occurred in the summer of 2006, and the burnt forest was under heavy forest management. All habitats were characterized according to dominant plantations, general soil type and environment (Table 1). Fruiting was monitored throughout the 2006-2007 season and in the following 2 years.

Molecular identification of morel species. Molecular identification of the black morels was performed on mycelial culture isolated from each of the defined (according to growing site) populations [4]. DNA was extracted by phenol/chloroform procedure from the freeze-dried mycelial biomass of each isolated strain. The internal transcribed spacer (ITS) region of its nuclear ribosomal DNA (nrDNA) was analyzed according to Wipf et al. [12] by PCR amplification using ITS1/ITS4 primer pairs and sequencing. The sequences of the local black morels were compared to those of reference *Morchella* strains obtained from DSMZ (The German microbial collections).

Climate data. Data on rain events were obtained from a meteorological station located 5 km from the burnt forest site.

RESULTS AND DISCUSSION

Black morel species and habitats. Distinct populations of black-headed morels were observed in 2006 in a range of sites across Israel. The sites varied in nearby vegetation, especially in dominant tree species, the soil surface on which the mushrooms appeared and the environment (disturbed or pristine) (Table 1). Head polymorphism was observed within each site's population with a variety of color nuances, ridge arrangements, head and strip ratios and head thicknesses.

The black morel species of all distinct populations were identified using a molecular, rather than morphological approach. In recent years, there has been an increase in studies using molecular tools for morel classification [12]. Here, molecular identification of the different populations was based on use of the ITS1/IT4 primer set to obtain the ITS region sequence (nrDNA). A comparison of the sequences from the different black morel populations to reference *Morchella* strains obtained from DSMZ (German) revealed two species, *M. conica* and *M. elata*. Molecular identification showed that all black morels found in the disturbed sites (i.e. post-fire forest, pavement and disturbed residential soil) were *M. conica* Pers (=*M. Vulgaris* (Pres) Boud). In general, these sites were near pine trees. It is assumed that these sites enjoyed a nutrient boost during the soil-disruption event, enabling the morel mycelium to gain biomass and fruit. *M. elata*, on the other hand, grew in the non-disturbed oak forest, on litter-rich soil beneath the trees, or on the surface of chalk rocks in a shaded area. It is assumed that this ecotype grew in association with the plantation, i.e. was dependent on nutrients released from the vegetation, mainly the oaks.

Table 1: Characterization of morel species and their habitats

Ecotype	Morel species	Habitat	Dominant plants	Growing surface
MS790	*M. conica*	Post-fire forest	Pine (*Pinus halepensis*)	Soil with burnt debris
MS782	*M. conica*	Disturbed soil in residential backyard	Grass, pine (*Pinus halepensis*)	Mixture of gravel and sand
MS765	*M. conica*	Roadside	Healthy pine (*Pinus halepensis*)	Asphalt pavement
MS807	*M. elata*	Mediterranean grove	Oak (*Quercus calliprinos*)	Chalk rock covered with moss
MS794	*M. elata*	Mediterranean grove	Oak (*Quercus calliprinos*)	Heavy organic soil with high litter content

Spatial fruiting patterns. Spatial distribution of the mushrooms at each site was influenced by the spatial heterogeneity of disruptive factors and food sources. In the post-fire forest, forest-management activity affected the distribution of the morel ascocarps [3], with two main fruiting patterns observed. In one type of fruiting pattern, the morels emerged in a straight line. In the second, ascocarps were distributed over a wide area of the burnt site with no defined order. While the straight-line pattern was found along piles of burnt tree debris which had been lined up by forest personnel, the undefined distribution pattern was found in a wide, open area in which the burnt tree debris had been dispersed, with mushrooms appearing all across this area. In the post-fire forest, morels appeared in both sunny and shaded open spaces.

Morchella elata mushrooms appeared near healthy oak trees in undisturbed soil. Two distinct fruiting patterns were observed here as well. In one, morel mushrooms were distributed across a wide forest soil surface area at an average of 1 fruiting body per 10 m^2, mostly singly. The second pattern was emergence of several patches of dense morel populations on the surface of chalk rocks at 2 to 5 morels per 0.5 m^2. In all *M. elata* cases, while morels grew in open spaces 2 to 5 m from the tree trunks, most of the time they grew in shaded areas.

Temporal fruiting patterns. In the burnt forest, high quantities of morels appeared in the first year after the fire, with amounts declining over the next 2 years of field inspection. Two waves of mushroom emergence were detected for this ecotype in the first year: the first peaked in early March (on 2 March only a few mushrooms were observed, on 7 March, there was a high peak, followed by a decline observed on 20 March); on 20 March, a new wave emerged in another area of the same burnt forest. No re-growth of black morels was seen in the soil that supported the

first growth wave. The second wave peaked at the end of March/early April. Fruiting events of *M. conica* in the post-fire forest were correlated with rain events (Fig. 1). The first wave of mushrooms emerged after heavy rains in February and disappeared within 3 weeks. The second wave appeared after a second wave of heavy rain in early March followed by a few dry days.

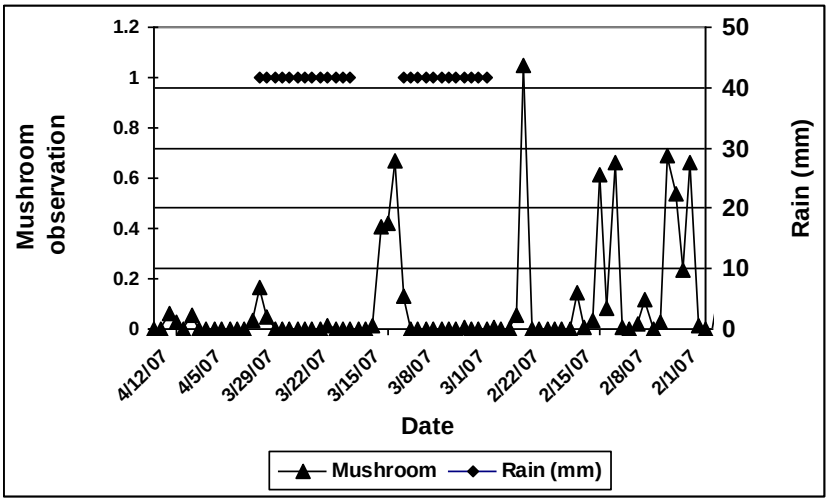

Figure 1: Rain profile during the winter of 2006-2007 and *M. conica* fruiting in the post-fire forest

For *M. elata*, only one major fruiting wave was detected, but the number of fruiting bodies increased gradually, such that they were observed at the site for almost 6 weeks. Unlike the *M. conica* ecotype, the *M. elata* population was stable over the years and was spotted each year at the same site, in the same soil.

The other *M. conica* ecotypes, found in the residential yard and along the pavement, were spotted only in their mature state, with only a few in each habitat. Therefore, it was not possible to study their fruiting patterns. Similar to the post-fire ecotype, these mushrooms disappeared from the site in the following year. We concluded that the *M. conica* morels that fruited in disrupted soil have no nutrient reservoirs to support their fruiting in a second or third season at the disturbed site. They are opportunistic and appear for only one season. In contrast, the *M. elata* ecotype probably has some relationship with the surrounding live vegetation, which affords it a continuous supply of nutrients, enabling it to fruit at the same site for many years.

Temporal fruiting pattern in morels has been shown to be controlled by climate or other seasonal conditions. Buscot [9] reported that *Morchella rodundra* fruiting-body emergence in France is controlled by soil temperature degree-days. Rain has been found to be a key factor in the emergence of morel fruiting bodies [1]. In the post-fire site, while fire enhanced fruiting-body emergence, the timing of their emergence was related to precipitation.

CONCLUSIONS

Morels show a variety of fruiting patterns. While these are dependent on the morel species or its trophic state, they are much more dependent on the heterogeneity of the habitat. In the post-fire forest, management by forestry authorities affected the dispersal of the mushrooms, which were either distributed over a wide area where the burnt wood debris was spread over the forest soil, or concentrated near piles of burnt debris. Here we present a few ecotypes of *M. conica* and *M. elata*. More work is needed to define the temporal and spatial fruiting patterns of the different

species and their ecotypes on a global scale, to gain a better understanding of the morel's complex life cycle.

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EXPRESSION OF GENES FOR THE GLUCOAMYLASES (GLYCOSIDE HYDROLASE FAMILY 15, GH15) IN EDIBLE MUSHROOMS

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ABSTRACT

The glycoside hydrolase family 15 (glucoamylases) comprises exohydrolases that catalyze the release of β-glucose units from the non-reducing ends of polymersaccharides. A glucoamylase gene (*PnGlu1*) from *Pholiota nameko* was cloned and characterized. The 1743 bp coding region of *PnGlu1* encoded a 581-amino acid polypeptide with a signal peptide comprising 17 amino acids at the N-terminal end. In addition to showing high homology with the glucoamylase from *Laccaria bicolor*, the *PnGlu1* gene encodes a protein with an N-terminal catalytic domain and a C-terminal starch-binding domain. Quantitative reverse transcription-PCR (qRT-PCR) was used to examine the role of the glucoamylase gene in the growth of mycelia when the fungus was cultured in minimal medium containing glucose or soluble starch, as well as the growth of mycelia and tissue development in fruit bodies at different stages. The findings suggested that expression of *PnGlu1* in the dikaryon strain was higher than observed in the monokaryon strain, with *PnGlu1* expression induced by soluble starch. However, during cultivation on sawdust medium, the expression of *PnGlu1* decreased drastically in dikaryotic mycelia after mycelial stimulation (*kinkaki*). Conversely, although *PnGlu1* expression was not observed during fruit body development, the glucoamylase enzyme activity was stable and the glucose content increased dramatically. These results suggested that at least two glucoamylases are produced by *P. nameko*; the one cloned in this study, which only catalyzed the conversion of soluble starch into glucose and which, in turn, increased mycelial growth, and another glucoamylase that may be involved in fruit body formation.

Keywords: Fruit body formation; Glucoamylase; Quantitative reverse transcription-PCR; Southern hybridization

INTRODUCTION

In saprophytic fungi, such as *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys & Moncalvo and *Lentinus edodes* (Berk.) Singer, three glycoside hydrolase families have been found related with the degradation of cellulose. The GH6 and GH7 families are specially for degradation of cellulose, and the ability of GH 15 (glucoamylase) to cleave terminal α-1, 4 glucosidic bonds and α-1, 6 branch bonds is important for the complete degradation of starch to glucose, and thus, for

the provision of a simple, soluble carbon source for nutrition [1-3]. However, in mycorrhizal fungi, such as *Laccaria bicolor* (Maire) P.D. Orton and *Tricholoma matsutake* (S. Ito & S. Imai) Singer, only the GH15 family has been identified [4, 5]. The observation that GH15 is common to both saprophytic and mycorrhizal fungi suggests that the glucoamylase gene may play an important role in the morphogenesis of basidiomycetous fungi.

Starch, an abundant glucose polymer in nature, has been shown to strongly induce glucoamylase gene expression during fruit production in basidiomycetous fungi [2, 6]. GH15 catalyzes starch hydrolysis and thus, the release of β-glucose units from the nonreducing ends of starch molecules. *L. edodes*, *Flammulina velutipes* (Curtis) Singer, and *Pholiota nameko* (T. Ito) S. Ito & S. Imai are economically important edible mushrooms in Japan. Thus, in order to study the genetic processes involved in the fruiting of *P. nameko*, we cloned and characterized *PnGlu1* in mono- and dikaryotic *P. nameko* cultured on different starch media using quantitative reverse transcription-PCR.

MATERIALS AND METHODS

Amplification of GH15 genes. Initially, fragments of genomic DNA encoding the putative glucoamylase gene (*PnGlu1*) were amplified by PCR with degenerate oligonucleotide primer pairs F-15-GP2-AF and F-15-GP2-BR (Table 1). The F15-GP2-AF and F15-GP2-BR primers were designed based on the amino acid sequences GLGEPKF and FDLWEEI, respectively, which have been reported to be conserved in the glucoamylase protein of *L. edodes* [3]. PCR reactions were conducted in 50 µl reaction volumes containing 1 x Ex *Taq* buffer (Takara Bio Co., Shiga, Japan), 50 ng of genomic DNA, 50 pmol of each primer, 0.2 mM of each dNTP, and 1.25 U of Ex *Taq* polymerase (Takara Bio Co.). PCR reactions were performed on a Takara PCR Personal Thermal Cycler (Takara Bio Co.) using an initial denaturation of 1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 2 min at 50 °C, and 30 s at 72 °C. Finally, the temperature of the reaction mixture was maintained at 72 °C for 10 min. The PCR fragments, which were approximately 400 bp in length, were cloned into the pMD20 T-vector (Takara Bio Co.) and sequenced. The partial *PnGlu1* sequences thus obtained were used to identify the *PnGlu1* flanking regions by cassette amplification using several sets of PCR primers. These primer sets were designed based on the partial nucleotide sequences of *PnGlu1* obtained from the initial PCR. All of the fragments were subcloned into pMD20 T-vector (Takara Bio Co.) and directly sequenced to determine the sequence of the flanking region.

The nucleotide sequences of *PnGlu1* from monokaryon NGW19-6 were deposited into the DNA Data Bank of the Japan database under the accession no. AB639794.

Real-time PCR assay. Total RNA from monokaryon NGW19-6 and dikaryon NGW19-6/12-163 was used as template material for all RT-PCR reactions performed in this study. The *actinI* gene was used as the housekeeping gene. A partial *actinI* gene fragment in monokaryon NGW19-6 was cloned using the degenerate PCR primers, UnivActF1 and UnivActR1 [7]. Primers for *PnGlu1* and actin used for the real-time PCR assay were designed according to their cDNA sequences using GENETYX (ver. 10.0.3, Genetyx). Primers were designed based on the principles of primer design, with 3 to 6 bases of the 3' splice site designed to cross the intron in the primer spanning the intron. All primers were tested to ensure that they amplified a single band with no primer-dimers. Plasmid extraction was performed according to a modification of

Birnboim [8] and five plasmid dilutions (10^4 to 10^8) were used to construct standard curves. Real-time PCR was conducted using the one step RNA-direct SYBR green Real-time PCR master mix (Toyobo) and Linegene (BioFlux, Hangzhou, China). Each reaction was performed in duplicate. The PCR cycle conditions consisted of 90 °C for 30 s to activate thermostable DNA polymerase, 61 °C for 20 min for reverse transcription, 95 °C for 30 s for predenaturation, followed by 35 cycles of 95 °C for 15 s, 60 °C for 15 s, and 74 °C for 30s. Melting curves were determined according to the manufacturer's instructions. To confirm amplification specificity, the real-time RT-PCR samples were then run on a 1.5% agarose gel and analysis was performed according to manufacturer's instructions. Relative gene expression was expressed as the ratio of the target gene (*PnGlu1*) concentration to the housekeeping gene concentration, with the obtained values representing mean gene expression obtained from at least two separate PCR experiments using the same RNA preparation.

Table 1: Primers used in this study

Primer	Sequence	Remark
F-15-GP2-AF	5'- GGNYTNGGNGARCCNAARTT -3'	Used for initial amplification
F-15-GP2-BR	5'- ATYTCYTCCCANARRTCRAA -3'	
Realtime Gulco F	5'-GCAACAAGTATCTAACCCCAGTG-3'	Used for real-time RT-PCR of *PnGlu1*
Realtime Gulco R	5'-GTAAGGCAGGTCCATCCCGT-3'	
Actin forward	5'-TCGGTCTTGAGGCTGCTGGT-3'	Used for real-time RT-PCR of actin
Actin reverse	5'-AGTCAACTCCTTCTGCATACGGTC-3'	

RESULTS AND DISCUSSION

Characterization of glucoamylase gene from *P. nameko.* In *P. nameko*, the nucleotide sequence from the initial ATG to the stop codon of the coding region consisted of 1743 bp and encoded 581 amino acids. The locations of the exons and introns of the gene were determined from the nucleotide sequences of RT-PCR products. All of the introns started with GT and ended with AG, and the coding region was interrupted by ten introns varying in size from 52 to 66 bp (Fig. 1). The deduced amino acid sequence from *P. nameko* was 32-70% similar to glucoamylase genes from other fungi, with the highest similarity obtained between the sequences from *P. nameko* and *L. bicolor* S238N-H82 [4]. Predictions obtained using the PSORT II program revealed that the sequence coded an extracellular protein of the cell wall, and that the first 17 amino acids coded a putative signal peptide found in the N terminal region of the protein. The SOSUI program predicted that the coded protein was soluble. Comparisons of the amino acid sequence of the putative glucoamylase with other fungal glucoamylases suggested that the structure of the glucoamylase protein from *P. nameko* comprises an N-terminal catalytic domain that was related to enzyme activity and a C-terminal region containing the starch binding domain, linked by a glycosylated hinge region.

Real-time PCR analysis of *PnGlu1* gene expression at different fungal developmental stages. Expression of the *PnGlu1* gene at different developmental stages (vegetative mycelial growth, appearance of primordia and formation of fruiting bodies) was examined when *P. nameko* was cultured on sawdust medium. *PnGlu1* gene expression was highest immediately before mycelial stimulation and decreased gradually thereafter. Thirty days after mycelium

stimulation, *PnGlu1* gene expression in both the mycelia of primordia and in the fruit bodies of different sizes decreased to very low levels.

Figure 1: Nucleotide and amino acid sequences of the *Pn-Glu1* gene from *P. nameko.*
Capital and lower case letters indicate the exons and introns, respectively. The amino acid sequences corresponding to the putative signal peptide and glucoamylase active site are underlined. The nucleotide consensus sequence of yeast MAT a and α homeodomain protein binding site are boxed. Italic case letters indicate the putative poly A(+) signal.

Quantitative reverse transcription-PCR assays of glucoamylase expression in the mono- and dikaryon of *P. nameko* grown on glucose and on soluble starch showed that soluble starch is the optimum carbon source for supporting the growth of *P. nameko*; starch induced glucoamylase expression in both the mono- and dikaryon. In order to study the regulation of the glucoamylase gene, we also cloned the upstream sequence of the open reading frame. In doing so, we found consensus sequences of mating type protein binding sites upstream of the start codon (Fig.1), which are necessary for homeodomain protein formation. Mating type protein binding sites have also been reported in the mycorrhizal fungus *T. matsutake* [5]. The observation that glucoamylase expression levels were higher in the dikaryon and transformant strains compared with the monokaryon strain implies that the glucoamylase gene is regulated by mating-type loci. In this study, we attempted to elucidate the function of glucoamylase in fruit body formation. However, the finding that the putative glucoamylase gene cloned in this study was expressed only during the mycelial growth stage indicated a need for further research aimed at identifying a new glucoamylase gene expressed specifically during fruiting body formation.

CONCLUSIONS

The nucleotide sequence of a *PnGlu1* gene was identified and characterized. The consensus nucleotide sequence of yeast MAT a and α homeodomain protein binding sites were observed in an untranslated region upstream of the ATG codon of *PnGlu*. *PnGlu1* gene expression in the dikaryon and transformants with the *hox* gene was higher than in the monokaryon. *PnGlu1* was expressed in the fungal mycelium but not in the fruiting body.

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REGULATION OF FRUITING BODY FORMATION IN *COPRINOPSIS CINEREA*

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ABSTRACT

Fruiting body formation in the edible dung fungus *Coprinopsis cinerea* is regulated by environmental cues (nutrients, temperature, light, humidity), physiological conditions (mycelial status, age) and genetic control elements (e.g. *A* and *B* mating type genes, *dst1* and *dst2* genes for light regulators). Fruiting body development consists of a series of defined steps occurring in a concerted process over seven days. Following hyphal aggregation, stipe and cap tissues differentiate under the control of light and dark phases. Once light-induced karyogamy takes place in basidia present at the surface of the gills in the cap of mature primordia, stipe elongation and cap expansion start parallel to meiosis in the basidia and subsequent basidiospore formation. All these processes are expected to appoint numerous intracellular as well as extracellular protein functions, many of which might be specific to steps in fruiting. Since the genome of the fungus is available, identification of proteins can now be addressed by large scale proteomic techniques. In order to gain an insight into participating proteins from a first analysed developmental step, we collected immature fruiting bodies after meiosis II. Stipe and caps were separated from each other, and intra- and extracellular proteins from both types of samples were isolated and compared by 1D- and 2D-gel electrophoresis. Whilst there might be contaminating intracellular proteins in the extracellular fractions, there are many proteins not present in the intracellular fractions. Stipe and cap fractions clearly differed from each other, less so in the intracellular and strongly in the extracellular proteome.

Keywords: Secretome; Intracellular proteome, Meiosis II, Rapid stipe elongation, Cap expansion

INTRODUCTION

Coprinopsis cinerea is a saprotrophic fungus, which in nature grows on dung [1, 2]. It can also easily grow on simple artificial media based on e.g. malt extract [3]. *C. cinerea* can readily fructify under laboratory conditions and thus serves as a model basidiomycete for fruiting body development [4]. Although it is quite easy to obtain *C. cinerea* fruiting bodies, this fungus in general is considered to be of little edible value because of the fast autolysis of the cap at maturity. However, *C. cinerea* is cultivated as a speciality in small farming businesses in Thailand and it is consumed by workers on sisal and sugar cane farms in certain African countries and Sri Lanka, where the species fruits abundantly on plant waste heaps [5].

Fruiting body development occurs on the dikaryon, or on specific self-compatible mutants with defects in the mating type loci (Fig. 1), under the control of environmental signals (depletion of nutrients, temperature of 25-28 °C, a light/dark regime following the typical day/night rhythm, humidity >85%), physiological conditions (dikaryotic mycelial status, young

age) and endogenous regulators (master regulators, i.e. the *A* mating type transcription factors and the *B* mating type pheromone-pheromone receptor system, and also putative light regulators encoded by *dst1* and *dst2*). On fully established fresh vegetative mycelium growing on artificial medium (YMG/T; [6]), fruiting body development starts in the dark in the form of intense localized hyphal branching resulting in loose hyphal aggregates about 0.03 mm in size (primary hyphal knots). On receipt of a light signal, the primary hyphal knots aggregate firmly into small round bodies called secondary hyphal knots [7]. After another light signal and a 12 h dark period, i.e. on day 2 of development, differentiation of cap and stipe tissues becomes evident within the now approximately 0.5-1 mm oblong-shaped structures, the outer appearance of which is still relatively homogenous (primordia stage 1). Primordia maturation occurs during the subsequent three days under the control of alternating light and dark conditions (stages 2 to 4). On day 3, the stage 2 primordia are egg-shaped, 1-2 mm in size and clearly demarcated into cap and stipe. At this time, gills develop. With progressing development, the growing primordia reach 2-3 mm on day 4 (stage 3) and contain primary as well as secondary gills with specific hymenial cells defined (probasidia, cystidia). One day later, primordia are 3-6 mm in size with the gills and hymenia fully developed (mature primordia). These structures correspond to the stage when, according to Lu [8], karyogamy and DNA replication in the pre-meiotic S-phase is induced. At this stage, the shaggy outer veil on the cap starts to loosen from the lowest part of the stipe. The bulging cap is slightly pink in colour. After another dark period, the primordia (now 5-7 mm) extend during the following 12 h of day 6 by further cap expansion and the commencement of slow stipe elongation. Early in the morning of that day, synchronized karyogamy occurs, followed some hours later by meiosis, spore formation and eventually pigmentation which will darken the colour of the cap. The spores mature and stain dark brown during the first 6 h in the dark. In parallel, stipes elongate rapidly and the further expanding caps of the maturing fruiting body open promptly like an umbrella in order to release the ripened spores. In the first hours after fruiting body maturation, spores are discharged from the basidia freely into the air. Auto-digestion of the fruiting body pileus starts simultaneously with this spore release. In the early hours of the next light period, the cap autolyses completely, releasing all the remaining spores in droplets of auto-digested fruiting body fluid [4].

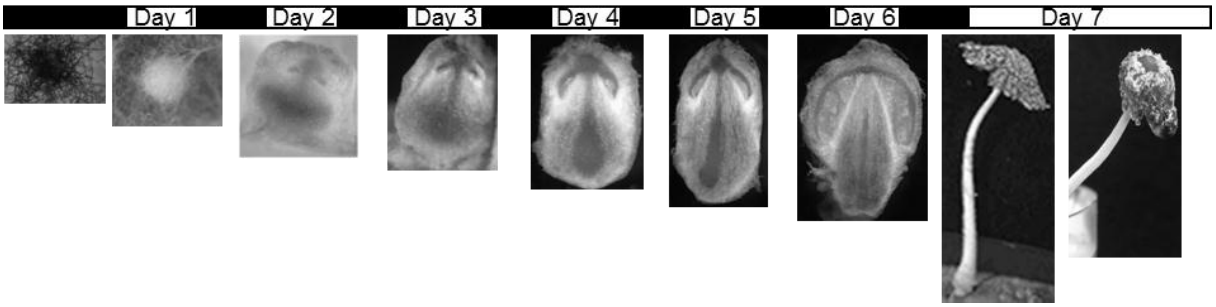

Figure 1: Fruiting body development of the self-compatible *C. cinerea* homokaryon AmutBmut on YMG/T complete medium under standard fruiting conditions
Standard fruiting conditions are defined in [6]. Photos from left to right: primary and secondary hyphal knots, primordium differentiation (stages 1 to 4), mature primordium at karyogamy, mature (~5 cm tall) and autolysing fruiting bodies. Dark blocks in the horizontal bar indicate night phases (12 h darkness), white blocks indicate daylight phases (12 h light); actual days are defined according to the start of the corresponding light period (modified from [5]).

The complexity and rapidity of the fruiting process suggest the occurrence of massive physiological and metabolic changes in the different tissues of the developing structures. To give some examples, in the first stages of the process, new combinations of cellular activities appear.

Correlated with karyogamy, there is massive synchronized DNA replication, while structural reorganisation of the chromosomes, DNA recombination and chromosome migration processes are connected to the two nuclear divisions in meiosis. Also, during cap expansion and stipe elongation, substantial cell wall stretching and restructuring takes place [4]. Multiple sets of proteins are expected to contribute to the different events.

Proteomics providing detailed descriptions of the structure, function and control of complex mixtures of proteins in a determined biological system [9] is a very useful tool for understanding complex molecular processes. The objective of this work is to characterize for the first time the proteome linked to *C. cinerea* fruiting body development. In this study, we chose to analyse the developmental phase directly after meiosis II at the beginning of spore formation, rapid stipe elongation and opening of the cap as a stage at which we expect manifold changes in the overall protein compositions. Thereby, we consider cap and stipe and their intra- and extracellular proteomes separately.

MATERIALS AND METHODS

Strain and culture conditions. *Coprinopsis cinerea* strain AmutBmut (*A43mut, B43mut, pab-1*) is a self-compatible homokaryon that, due to mutations in the *A* and *B* mating type genes, produces fruiting bodies without mating to another compatible monokaryon [10,11]. Stocks were cultivated on YMG/T agar plates (4 g yeast extract, 10 g malt extract, 4 g glucose, 10 mg tryptophan, and 10 g agar per l) for 5 days at 37 ^{O}C in black boxes with wet tissues to create a humid atmosphere [6,12]). About 10 small agar pieces with fresh mycelium were used to inoculate sterile fresh horse dung (about 80 g) in 1 l jars. Cultures were incubated at 37 ^{O}C for 4-5 days until the mycelium fully covered the substrate. For fruiting, cultures were transferred into a climate chamber at a chamber temperature of 25 ^{O}C (i.e. 28 ^{O}C directly on the cultures due to temperature effects caused by the light sources [6]) and 80-90% humidity in a 12 h light/12 h dark cycle. Cultures and development of fruiting structures were observed daily.

Primordia dissection. Primordia were dissected with the help of a razor blade and a needle under a stereo microscope (Stemi 200-C, Zeiss, Göttingen, Germany). For protein analysis, rhizomorphs (basal tissues for anchoring the stipes in the substrate), cap and stipe tissues were separated and directly frozen in liquid nitrogen and stored at 20 ^{O}C until further use. For microscopy, gills were taken from the caps with fine forceps and squashed on glass slides and nuclei in the basidia were stained with DAPI (4′,6-diamidine-2-phenylindole dihydrochloride; Boehringer Mannheim, Germany) following the procedure of Polak et al. [13]. Microscopy of tissues was performed with a Zeiss Axiophot microscope. A computer-linked Soft Imaging ColorView II Mega Pixel digital camera was used for photography and images were analyzed with the AnalySIS® software program (Soft Imaging System, Germany).

Protein isolation. Frozen tissue samples were freeze-dried and mildly ground to a powder using a mortar and pestle. Sample powders in portions of 0.5 g in 10 ml of Tris-HCl buffer, pH 7.5, containing 2% Tween 80 and 1 mM PMSF (phenylmethanesulfonyl fluoride) were incubated at room temperature for 30-40 min with gentle shaking and then centrifuged at 4,000 g at 4 ^{O}C for 20 min. Supernatants with extracellular proteins (and possible intracellular contaminations) from two successive extractions were collected and stored at -20 ^{O}C for later analysis. The remaining mycelial debris were frozen overnight at -20 ^{O}C and freeze-dried. To break all the cells, the dried material was ground three times for 3 min in a horizontally oscillating mill under liquid nitrogen cooling (Mixer Mill 2000, Retsch, Haan, Germany) at an amplitude setting of 80. The broken cells were washed twice with cell breaking buffer (CBB; 20 mM Tris-base [pH 7.5], 200 mM

NaCl, 2 mM EDTA, 0.01 % Tween 80, 1 mM PMSF), centrifuged for 20 min at 650 g at 4 ^{O}C, stored overnight at -20 ^{O}C and then suspended in 10 ml CBB buffer. To separate the cell wall fraction from the intracellular material, the solution was bedded onto 15 ml of 30% sorbitol and centrifuged at 650 g for 20 min. The supernatant with the intracellular proteins was stored at -20 ^{O}C until further use. The remaining pellet was washed with CBB and subsequently with distilled water, frozen at -20 ^{O}C and freeze-dried. Cell wall proteins were extracted by boiling 4x for 30 min 10 mg samples with 1 ml SDS-extraction buffer (60 mM Tris-base, pH 7.5, 50 mM EDTA, 2% SDS, 0.5% 2-mercaptoethanol, 1 mM PMSF) and centrifugation for 10 min at 3,200 g and 4 ^{O}C. The four extracts were pooled together and stored for subsequent gel electrophoresis.

Proteins in solution were precipitated by adding 10% TCA (v/v) from a TCA stock solution (100 g TCA in 45.4 ml water), incubation overnight at 4 ^{O}C on ice and centrifugation at 1,700 g for 20 min at 4 ^{O}C. Pellets were washed 5x with each 10 ml 80% acetone/ 20% 50 mM Tris-buffer, pH 7.5 and centrifuged 20 min at 4,000 g at 4 ^{O}C. Finally, the samples were washed with 10 ml acetone for 30 min at -20 ^{O}C, air dried, and stored at -20 ^{O}C. For gel electrophoresis, samples were re-suspended in 200-300 µl of rehydration buffer [8 M urea, 2% CHAPS, 18 mM DTT, 0.5% buffer for IPG (immobilized pH gradient) (pH 3-10) (Amersham Biosciences, Freiburg, Germany) and centrifuged for 15 min at 4 ^{O}C and 4,000 g. Protein extraction was repeated 3x and protein concentrations of the combined supernatants were determined using a Coomassie Plus-Bradford assay kit (Pierce, Rockford, USA) with bovine serum albumin (BSA) as the standard. All assays were performed in triplicate.

Protein gel electrophoresis. SDS-PAGE was performed with 12% resolving gel and 4% stacking gel as described previously [14]. A protein aliquot of 10-20 µg per sample well was mixed with 30 µl of the loading buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 18 mM DTT and traces of bromophenol blue) and heated at 95 ^{O}C for 5 min. Upon loading, gel separation was performed at a constant current of 15 mA until the samples reached the resolving gel and continued at 30 mA for further migration of proteins. After the run, gels were fixed in 40% ethanol/10% acetic acid/50% H_2O overnight and subsequently stained with RuBP [ruthenium (II) Tris (bathophenanthroline disulfate)] as described previously [15]. Stained gels were scanned using an FLA-5100 fluorescent reader (Fujifilm, Düsseldorf, Germany). The gels were further stained in colloidal Coomassie solution (10% phosphoric acid, 10% $(NH_4)_2SO_4$ and 0.12% Coomassie Brilliant Blue G250; Serva Electrophoresis GmbH, Heidelberg, Germany) in 20% methanol. The gels were washed several times with water until an optimal contrast between bands and background was reached and then scanned (TMA 1600 scanner, Microtek, Willich, Germany).

For 2D-gel electrophoresis, protein samples were dissolved in loading buffer [8 M urea, 2% CHAPS, 18 mM DTT, 0.5% IPG buffer (pH 3-10), and traces of bromophenol blue]. Aliquots (350 µl) of sample solution containing approximately 100 µg protein were loaded onto a 18 cm IPG-strip, pH range 3-10 (Amersham Biosciences) in ceramic holders, rehydrated and focused using an Ettan IPGphor (Amersham Biosciences) with the following settings: 20 V for 10 h; 150 V for 1 h; 200 V for 1 h, 500 V for 2 h, 1000 V for 1 Vh; gradient 4000 V for 2 h; gradient 8000 V for 1 h; 8000 V for 60,000 Vh). Afterwards, the strips were incubated twice for 10 min in 4 ml of equilibration buffer (6 M urea, 2% SDS, 30% glycerol, 50 mM Tris-base, pH 8.8 and traces of bromophenol blue) containing 20 mM DTT in the first step and 200 mM iodoacetamide in the second. The proteins were then separated in the second dimension on a 12% SDS-PAGE using an Ettan DALTsix electrophoresis chamber (Amersham Biosciences) together with a protein marker 14-116 kDa (Fermentas, St. Leon-Rot, Germany). After separation of the proteins, first for 30 min at 120-130 V and then for 5 h at 300 V, the gels were fixed in 40% ethanol/10% acetic acid/50% dH_2O and stained with RuBP and Coomassie

Brilliant Blue G250 as described above. Prominent protein bands of Coomassie-stained gels were cut with a glass Pasteur pipette and gel pieces were stored at -20 ^{O}C for future analysis.

RESULTS AND DISCUSSION

Material requirements. For 1D- and 2D-gel electrophoresis about 10-20 µg and 100 µg protein, respectively is required for loading. From vegetative mycelium of *C. cinerea* cultures, our laboratory found that the fungus may secrete up to 0.2 µg protein/mg dry biomass and produces up to 0.7 µg intracellular protein/mg dry biomass [16]. In order to estimate how many immature fruiting bodies were needed to be collected, we first separately collected caps and stipes of 60 immature fruiting bodies from YMG/T plates [from day 6 of development (Fig. 1) after 10 h of illumination]. On average, caps and stipes had wet weights of 16 mg and 32 mg, respectively. The corresponding dry weight was about 7-11%. Based on our experience with vegetative mycelium, this corresponded to between 1.5-2.4 µg intracellular protein. We observed that structures of the same age on horse dung are 2-3x times larger and 9-18x heavier than on YMG/T plates, which helped to collect enough material (~10x less mushrooms) for the isolation of protein in amounts sufficient for electrophoretic analysis.

Fruiting body development on horse dung. Fruiting body development on horse dung was observed over time. The process on the natural substrate followed the same time schedule as established previously on YMG/T medium (shown in Fig. 1) but the developing structures were generally larger. Stage 4 primordia (day 5 of development) were 4-6 mm in size, and 9-11 mm at karyogamy on the morning of the next day (Fig. 2). The course of further events over day 6 for fruiting body maturation were defined for the target to best identify the wanted stage directly after meiosis II. Immature fruiting bodies were harvested on day 6 at different times after the light was switched on. Over this time, structures grew into sizes up to about 4 cm in length by both stipe elongation and cap expansion. The colour of the caps changed from pinkish in the first 8 h of illumination to light grey at later times (9 and 12 h of illumination).

When karyogamy was completed after one hour on day 6, the veil and the gills in the now 9-11 mm sized primordia were still connected throughout to the stipes although all the connections were already somewhat loosened which made it easy to separate caps and stipes from each other. Already at this time, a hollow lumen developed throughout the stipes. At hour 3 of day 6, veil connections of the outer edge of the caps separated from the lowest part of the slowly stretching stipes but the lamella were still connected to the stipes over their whole length. At this time, the length of the caps was still longer than the length of free part of the stipes. Separation of gills from the stipes began after about 4 h of illumination at the outer end of the gills and moved up with time to the tip of the stipes. At 10 h of illumination, about one half to two thirds of the length of the still extending gills was free from the hollow stipes. The lamellae were white throughout without any brown colouration typical of spore maturation and staining (Fig. 2). Nuclear staining of basidia was performed at one hour intervals between hours 7 and 10 of day 6. At hour 9, >60% of basidia had completed meiosis II and 100% at hour 10 (Fig. 3). The first sterigmata were seen on some basidia in the 9 h samples whereas, in 10 h samples, most basidia had sterigmata and, in some cases, budding

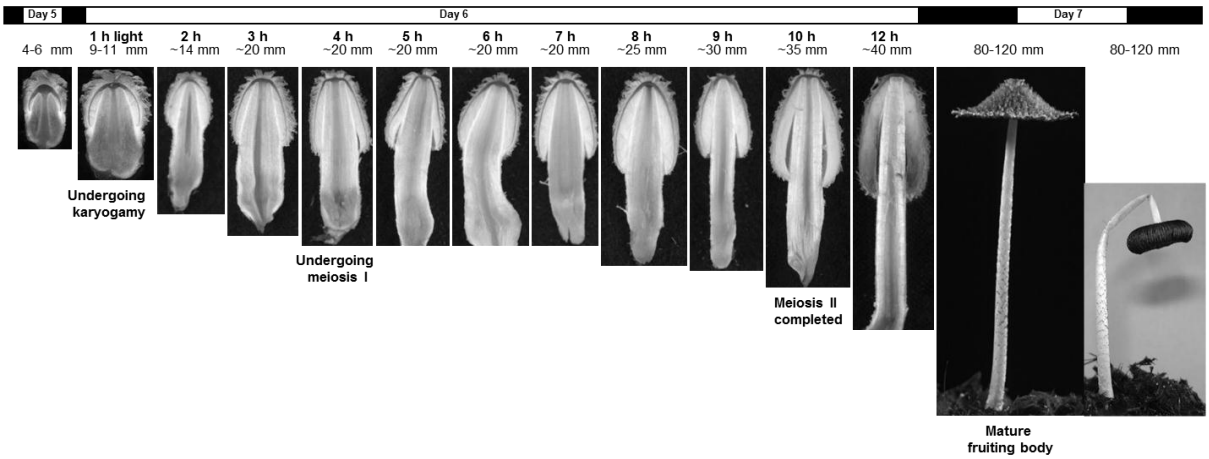

Figure 2: Later stages of primordia and fruiting body development of *C. cinerea* homokaryon AmutBmut on horse dung.

of basidiospores had begun at the tips. Basidiospore maturation started at the outer edges of the gills and moved quickly up to the top of the gills in the center of the fruiting bodies. After 12 h of illumination, gills were dark-stained over half of their length. Stipes rapidly elongated and the expanding caps opened. After 6 h in the dark, the mushrooms were fully developed and the black caps fully opened. Next morning, the mushrooms collapsed due to autolysis.

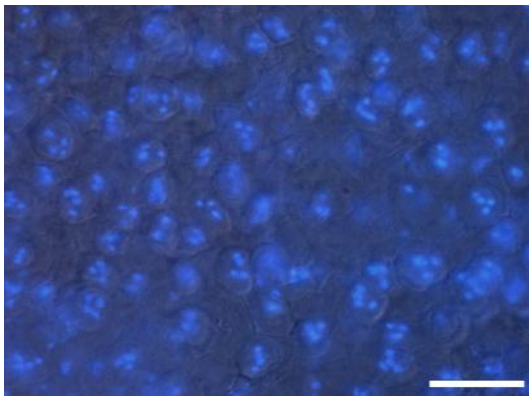

Figure 3: Nuclear staining of basidia in immature fruiting bodies of *C. cinerea* homokaryon AmutBmut at hour 10 of illumination on day 6 of development. Basidia finalised meiosis II as documented by the four nuclei in each basidium. A photo from DAPI stained basidia was overlaid with a differential interference contrast photo from the same basidia set at 30% transparency using the corelDRAW 12 program (Corel Corporation, Unterschleißheim, Germany). Scale bar 20 µm.

Protein characterisation of cap and stipes from immature fruiting bodies after meiosis II. Immature fruiting bodies were collected from horse dung on day 6 of development after 10 h of illumination, representing the stage after meiosis II with sterigmata formation, the onset of basidiospore formation, and the onset of rapid stipe elongation and cap expansion (Figs. 2 and 3). All harvested structures were checked for gill colour. Any immature fruiting bodies showing the first shades of staining at the outermost parts of the lamellae (suggesting onset of basidiospore pigmentation) were not considered. Caps and stipes were separated. Of 60 structures, the average weights of caps and stipes were 293 mg and 290 mg, respectively. Accordingly, caps and stipes from structures grown on horse dung had an 18x higher weight than structures of the same age grown on YMG/T medium.

For protein isolation, caps and stipes of structures were separated, frozen directly upon harvest, and intra-, extracellular and cell wall proteins were isolated as described above. First, the different samples of protein were separated by SDS-PAGE (Fig. 4). The 1D-resolution of the intracellular proteins and the fractions of the cell-wall-bound proteins resembled each other, suggesting a high portion of contamination of intracellular proteins in the cell wall samples. However, the patterns of freely secreted extracellular proteins and of the intracellular proteins differed very much from each other. Patterns from intracellular proteins from caps and stipes looked similar to each other as might be expected from intercellular functions, many of which might cover the normal cellular housekeeping functions. In contrast, the patterns of the extracellular proteins of caps and stipes were very different to each other, although there were also some overlapping banding patterns between the samples. A number of larger and smaller prominent bands were present in both extracellular samples but these did not correspond to each other in size.

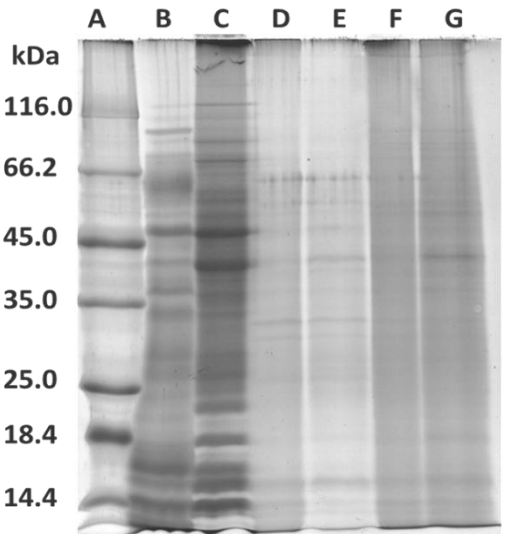

Figure 4: SDS-PAGE of proteins from immature fruiting bodies of *C. cinerea* after meiosis II. **A.** Protein-marker; extracellular proteins from **B.** cap and **C.** stipe; intracellular proteins from **D.** cap and **E.** stipe; proteins from cell walls of **F.** cap and **G.** stipe.

The extracellular proteome, i.e. the free secretome from fruiting body tissues may contain proteins that affect cell wall structure and cell wall formation [17], proteins that help in cell-cell-communication and aggregation, proteins that change hyphal surface properties, proteins required for pigmentation, and proteins that act in defence [18-20]. Obvious differences between the secretomes from cap and stipes suggests that organ-specific, or even tissue-specific, extracellular processes take place during development. This idea was further promoted by the protein patterns obtained after separation of the samples by 2D-gel electrophoresis (Fig. 5).

More than 270 protein spots were visible in the 2D-gel of the free secretome of the caps, and >200 spots were recorded in the 2D-gel of the free secretome of the stipes. Very few strong spots appeared to overlap between the two samples (see white circles in Fig. 5) whilst others were obviously unique to either one or the other of the samples (see black circles in Fig. 5). Due to the preparation technique of grinding frozen tissues prior to washing out the free proteins, intracellular protein contamination was very likely present in the washing buffer. However, the entire protein patterns from the intracellular samples were clearly distinguishable from their extracellular counterparts (compare Figs. 5 and 6), suggesting that the majority of the protein spots seen in the 2D-gels in Fig. 5 were actual secreted proteins.

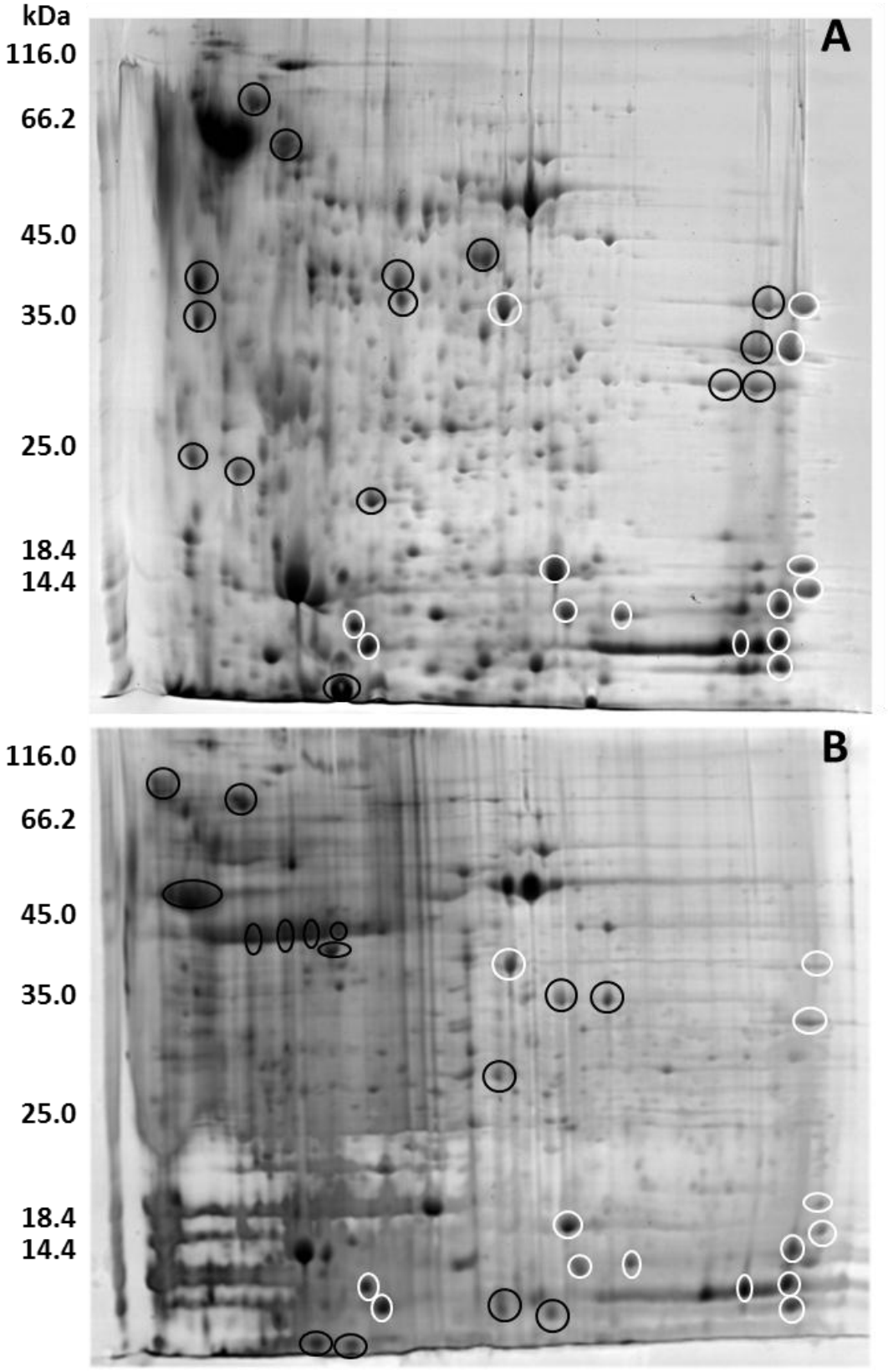

Figure 5: 2D-Gel of the extracellular secreted proteins from immature fruiting bodies of *C. cinerea* after meiosis II. **A.** Caps and **B.** stipes. Gels were stained with RuBP. Examples of protein spots shared between the two samples are encircled in white and unique protein spots in black. Note that the background smear was due to polysaccharides that cannot easily be separated from the proteins [20].

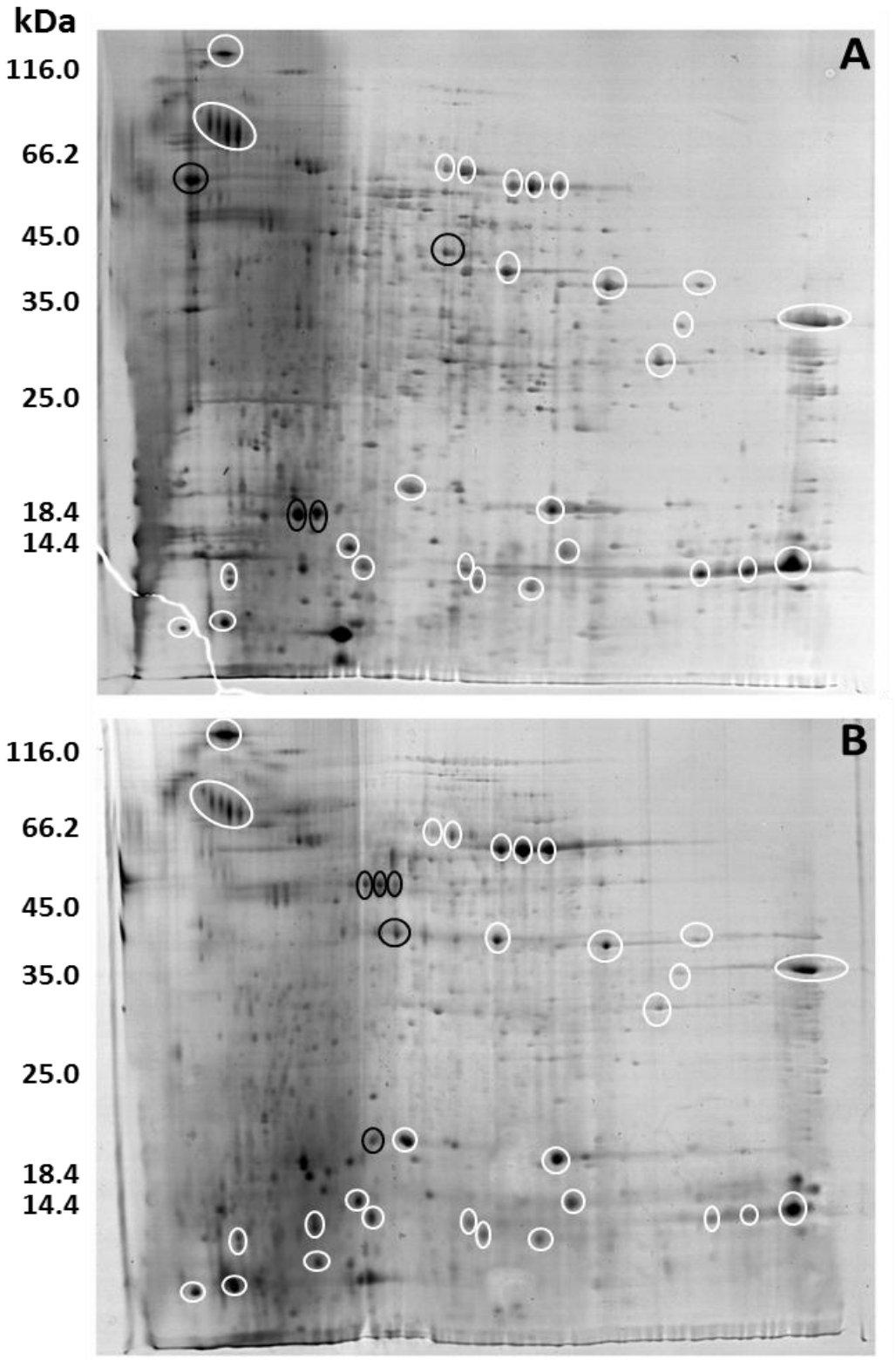

Figure 6: 2D-Gel of the intracellular proteins from immature fruiting bodies of *C. cinerea* after meiosis II. **A.** Caps and **B.** stipes. The gels were stained with RuBP. Examples of protein spots shared between the two samples are encircled in white and unique protein spots in black.

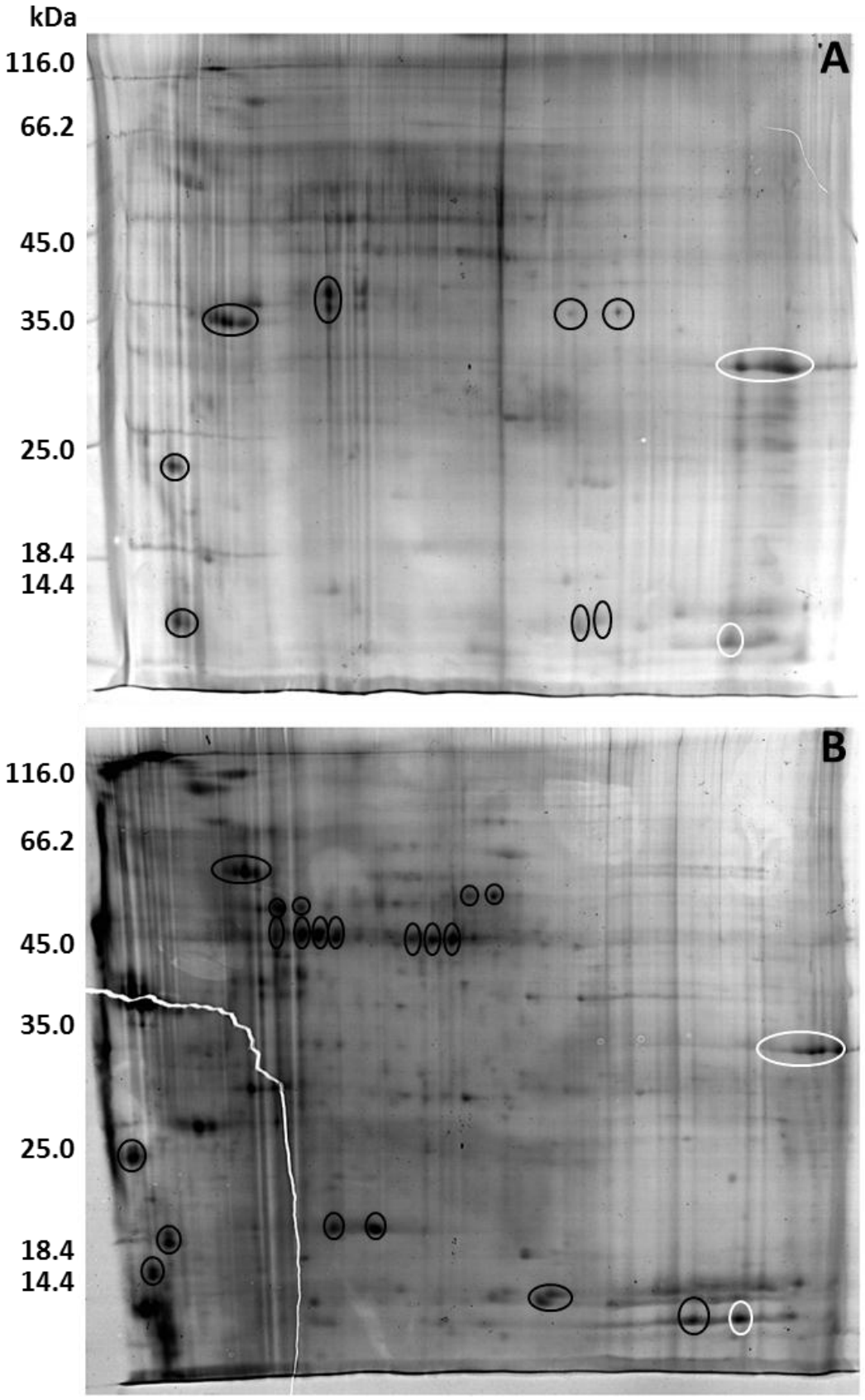

Figure 7: 2D-Gel of the cell wall proteins from immature fruiting bodies of *C. cinerea* after meiosis II. **A.** Caps and **B.** stipes. The gels were stained with RuBP. Examples of protein spots shared between the two samples are white and unique protein spots black encircled. Note that the very strong background and vertical smear were due to high polysaccharides content in protein samples.

In the SDS-soluble protein fraction of the cell walls, despite the likelihood of background spots arising from intracellular contamination (compare Fig. 6), there were lower numbers of strong spots (Fig. 7). Importantly, however, other prominent spots were visible than in the free secretome (compare Fig. 5) indicating a functional separation of the free secretome from the cell-wall-bound proteins. As in the case of the free secretome, the cell-wall-bound secretome of the cap and stipe also differed from each other considerably (Fig. 7).

In contrast to the extracellular samples, the spot patterns of the intracellular proteins of caps and stipes in 2D-gels are much more similar to each other, both in spot positions and in spot densities (see examples of white marked spots in the gel; Fig. 6). Nevertheless, there are also a number of proteins unique to the caps and to the stipes, respectively (see black encircled spots marked in the 2D-gels in Fig. 6). Overall, there were >400 spots visible on the 2D-gels loaded with intracellular proteins.

CONCLUSIONS

Our pioneering protein analysis of extra- and intracellular proteins from cap and stipe tissues of immature fruiting bodies of *C. cinerea* revealed interesting perspectives to be uncovered by proteomic identification of the whole sets of proteins. The annotated genome of *C. cinerea* has been published [22] and has already been used successfully to identify proteins of the secretome of vegetative mycelial cultures of the fungus [16]. The results of this study suggest that the differences in the free and cell-wall-bound secretomes of cap and stipe will be particularly important for the morphology of the fruiting body organs and the formation of their different tissues within the fruiting bodies. Prominent spots from 2D-gels have been picked for future analysis for protein identification.

Our presentation of the whole process of fruiting body development (Fig. 1) indicates that many different developmental stages have to be harvested and fractions of proteins extracted for further proteome definition. Moreover, similar approaches can be taken with characterized or newly developed mutants such as those with mutations in the *dst* (dark stipe) genes. Such mutants are defective in light perception and thus form elongated stipes with underdeveloped caps instead of normal primordia with gills in the cap (Fig. 8A). Also, other mutants that are likewise defective in morphology such as stunted stipe growth (shown in Fig. 8B) or mutants with white caps due to defects in basidiospore formation or pigmentation (Fig. 8B,C).

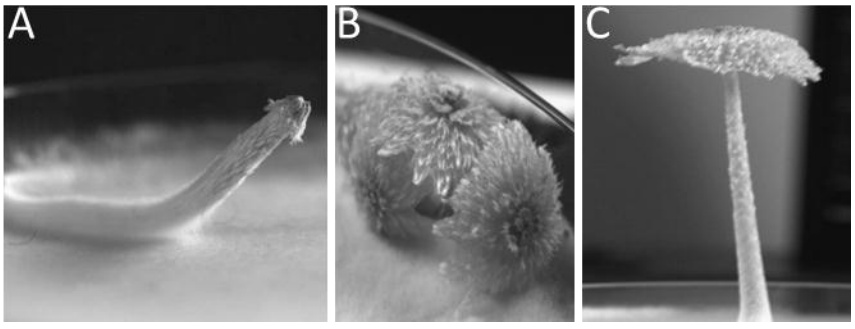

Figure 8: Mutants in fruiting body development of self-compatible homokaryons. **A.** Strain PUK1 with a *dst1* defect forms etiolated stipes in light. **B.** Mutant UFO is a mutant with a defect in stipe elongation (*eln*) forms dumpy mushrooms. The cap is white due to an additional mutation in basidiospore formation (*bad*). **C.** Mutant OU2 with a yet uncharacterized *bad* mutation has normal-sized white mushrooms without spores (after [5]).

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HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF MANNITOL-1-PHOSPHATE DEHYDROGENASE FROM THE BASIODIOMYCETE *PHOLIOTA NAMEKO*

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ABSTRACT

The gene encoding mannitol-1-phosphate dehydrogenase, *mpd*, which has similar function with alcohol dehydrogenase, has been sequenced and characterized from the basiodiomycete *Pholiota nameko*. The coding region of *mpd* was composed of 2992 bp and found to encode a polypeptide of 359 amino acids that has similarity with *Laccaria bicolor*. To evaluate the expression level of mannitol-1-phosphate from *P. nameko*, *mpd* cDNA was inserted into pCold shock vector and expressed in host BL21 (DE3) by 24 h induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 15 ^{O}C after 2 h growth at 37 ^{O}C. The purified protein was detected by SDS-PAGE and western blot.

Keywords: Mannitol-1-phosphate dehydrogenase; Alcohol dehydrogenase activity

INTRODUCTION

Ethanol as a petroleum substitute has many desirable features that are compatible with sustainable development. Most ethanol is produced in large quantities from natural resources including corn grain and sugarcane juice. However, there is a need to find an inexpensive and widely available source of lignocellulosic biomass. It is well-known that basidiomycete fungi cause white rot decay, and are able to degrade the lignin in wood and convert cellulose into glucose which is the most desirable sugar for fermentation.

According to Kalavati [1], mannitol-1-phophate dehydrogenase from *Crytococcus neoformans* is a zinc-containing long-chain alcohol dehydrogenase. Mannitol is a six carbon polyol which is abundant in plants, fungi and bacteria. It accumulates in some fungi and may be involved in fungal tolerance to environmental stress such as salt stress, and may also function as an osmoregulatory compound in supporting turgor and in fruit body development. Mannitol-1-phosphate dehydrogenase catalyzes the conversion of fructose-6-phosphate to mannitol-1-phosphate, which plays an important role in mannitol biosynthesis. Based on the similarity between mannitol-1-phosphate dehydrogenase and alcohol dehydrogenase, we have attempted to mutate the conserved domain in mannitol-1-phosphate dehydrogenase from *P. nameko*, and have investigated the alcohol-producing potential of the mushroom.

MATERIALS AND METHODS

Fungal strain and plasmids *P. nameko* NGW19-6 (*A4, pdx1*), an auxotrophic mutant for pyridoxine, was used in this study [19, 20]. *Escherichia coli*, strain DH5α, (Takara Bio, Shiga, Japan) was used as the host for plasmid amplification. Plasmid pMD20 vector (Takara Bio) was used to clone polymerase chain reaction (PCR) products. *E. coli*, strain BL21, (DE3) (Novagen, Darmstadt, Germany) was used as the host for recombinant protein gene expression, and pCold (Novagen, Darmstadt, Germany) vector was used to clone and express the recombinant protein gene.

DNA and RNA preparation. *P. nameko* mycelium was grown on potato dextrose agar (PDA) at 25 ^{O}C for 7 d. After harvesting, the mycelium was frozen in liquid nitrogen and ground in a mortar and pestle to a fine power. The genomic DNA extraction kit (Amersham Bioscience, Tokyo, Japan) was used to isolate genomic DNA according to the manufacturer's instructions. RNeasy mini kit (Qiagen, Tokyo, Japan) was used to extract RNA.

Genome walking. Genomic DNA from *P. nameko* NGW19-6 was digested with restriction endonucleases (Bgl, Xho, EcoT221, Sal, Xba, Bam, Pst, Hind, Nhe, EcoRI, Fba, Spe) and fragments of suitable length were ligated with nucleotide linkers and used as templates for PCR. PCR was carried out in 100 µl reaction mixtures containing 1 × Ex *Taq* buffer, 100 ng extracted genomic DNA, 100 pmol of each primer, 0.2 mM dNTPs, and 2.5 U Ex *Taq* polymerase. PCR was carried out using the following cycling parameters: initial denaturation at 94 ^{O}C for 1 min, followed by 30 cycles of 30 s at 94 ^{O}C and 5 min at 68 ^{O}C. All amplified DNA fragments were subcloned into T-vector pMD20 and all plasmids were sequenced. In order to obtain the full sequence of the mpd gene, after genomic walking, four primers were designed for partial sequencing. PCR was carried out in 50 µl reaction mixtures containing 1 × Ex *Taq* buffer; 100 ng extracted genomic DNA, 100 pmol of each primer, dNTPs at a final concentration of 0.2 mM, and 2.5 U Ex *Taq* polymerase. PCR was carried out using the following cycling parameters: initial denaturation at 94 ^{O}C for 5 min, followed by 30 cycles of 30 s at 94 ^{O}C, 30 s at 55 ^{O}C, and 5 min at 72 ^{O}C, followed by 72 ^{O}C for 10 min.

Reverse transcription-PCR (RT-PCR). Amplification of 3'-ends of cDNA by 3'-rapid amplification of cDNA ends (RACE) was performed with a Takara RNA PCR Kit (AMV) Version 3.0 (Takara Bio). The 5'RACE was carried out with the 5'-Full RACE Core Set (Takara Bio). Reverse transcription and PCR were carried out according to the manufacturer's instructions.

DNA sequencing and computer analysis. All the fragments were cloned into T-vector pMD20 and sequenced by ABI PRISM^{TM} 3100 Genetic Analyser (Applied Biosystems, Tokyo, Japan) using the chain-termination procedure with a BigDye Terminator^{TM} Cycle Sequencing Kit (version 3.1; Applied Biosystems) according to the manufacturer's instructions. Sequence data editing and assembling were performed using Seqman (DNAStar, Lasergene software, Madison, USA); the subcellular location of MPD was predicted by the program SOSUI [2]; the motif search program online (http://motif.genome.jp/) was used for motif searching. Amino acid sequences of 26 fungal mannitol-1-phosphate and 12 alcohol dehydrogenase amino acid sequences were downloaded from the NCBI at the NIH (http://www.ncbi.nlm.nih.gov/). Initially, all the amino acid sequences were aligned with the Clustal X program [3]. Then, the PHYLIP 3.69 package [4] was used to construct a Jones-Taylor-Thornton matrix from the raw data

(PRODIST program) [5]. Cluster analysis was performed using the unweighted pair group method with arithmetic averaging (UPGMA) [6]. The robustness of the phylogenetic trees was evaluated by bootstrap analysis with 100 replicates [7].

Expression of mannitol-1-phosphate dehydrogenase cDNA in *E. coli*. For expression of MPD cDNA in *E. coli*, a DNA fragment containing the MPD coding region, framed with restriction enzyme sites at both ends, was obtained by PCR with the sequence specific primers designed based on the restriction enzyme sites *Nde*I and *Eco*RI. The PCR fragment was digested with *Nde*I and *Eco*RI and ligated with *Nde*I/*Eco*RI-digested pCold shock vector; this generated plasmid pCold-MPD, which was used to transform BL21 (DE3). The empty vector pCold was used as control. Both plasmid and control were grown at 37 ^{O}C in LB medium supplemented with 100 mg/ml ampicillin until the OD_{600} reached between 0.4-0.6. The culture was adjusted to 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and further cultivated for 16 h at 15 ^{O}C. The cells were harvested by centrifugation at 2,600g for 10 min, resuspended in Tris-HCl buffer (pH 7.5), and disrupted by ultrasound. Following centrifugation at 2,600 g for 10 min, the supernatant was used for enzyme assays [8-10]. One unit of the enzyme was defined as the amount that catalyzed the formation of 1 μmol of NADH in 1 min under the reaction conditions.

RESULTS AND DISCUSSION

The nucleotide sequence of the mannitol-1-phosphate dehydrogenase gene in *P. nameko* contains 2992 bp, and the coding region (from ATG to the stop codon) is 1363 bp and encodes 359 amino acids. The locations of the exons and introns in the gene were determined from the nucleotide sequences of the RT-PCR products. All the introns started with GT and ended with AG. The coding region contained six exons and five introns. In addition, the SOSUI program predicted that the MPD protein was a soluble protein, which was located in the cytoplasm. The motif search program online revealed that MPD has a zinc-containing alcohol dehydrogenase motif from 1916 bp to 2011 bp. A phylogenetic tree was constructed using the deduced amino acid sequences of MPD and 27 mannitol-1-phosphate dehydrogenase proteins and 11 alcohol dehydrogenase proteins. *P. nameko* MPD protein had a 73% amino acid identity with MPDH1 of *Laccaria bicolor* [11]. The phylogenetic tree separated into two main parts, one consisting entirely of mannitol-1-phosphate dehydrogenases and the other a mixture of alcohol dehydrogenases and mannitol-1-phosphate dehydrogenases. In the mixed group, ten fungal alcohol dehydrogenases were clustered together, while another alcohol dehydrogenase from *Saccharomyces cerevisiae* clustered with ten fungal mannitol-1-phosphate dehydrogenases. MPD was expressed in cultures of engineered *E. coli* BL21 (DE3), and the crude cell extract had alcohol dehydrogenase activity.

There have been many reports on mannitol-1-phosphate dehydrogenase genes, which mainly focused on mannitol metabolism. These include the proposal that mannitol is involved in an NADPH-producing cycle involving the utilization of NADH and ATP [12]. However, mannitol was still produced by mannitol-1-phosphate dehydrogenase and mannitol dehydrogenase double deletion mutants [13] and the existence of a novel mannitol phosphorylation pathway for mannitol biosynthesis and metabolism was deduced. Mannitol is the main storage material in *Agaricus bisporus* [14] and also plays important roles in the sporulation of *Stagonospora nodorum* [15]. Mannitol accumulation in fungi may be involved in fungal tolerance to environmental stress such as salt stress [16], and function as an

osmoregulatory compound to support turgor and fruit body development [17]. However, there are no reports describing the relationship between mannitol-1-phosphate dehydrogenase and alcohol dehydrogenase. Here, we report for the first time a mannitol-1-phosphate dehydrogenase gene from *P. nameko* that encodes a protein with the potential to produce alcohol by fermentation. The long chain alcohol dehydrogenase from horse liver has approximately 350 amino acids per subunit, and the short chain alcohol dehydrogenase from *Drosophila* has approximately 250 amino acids per subunit [18]. According to our data, mannitol-1-phosphate dehydrogenase from *P. nameko* has 359 amino acids and is a zinc-containing long chain alcohol dehydrogenase.

Phylogenetic analysis suggests that mannitol-1-phosphate dehydrogenase and alcohol dehydrogenase are evolutionary homologous, and some mutations affecting mannitol-1-phosphate dehydrogenase may lead to the appearance of alcohol dehydrogenase. This may explain why some fungi can produce alcohol under anaerobic conditions. Yeasts have the capability of alcoholic fermentation but cannot degrade lignocellulose, while basidiomycetes can degrade lignocellulose but cannot produce alcohol by fermentation. Mannitol-1-phosphate dehydrogenase from *P. nameko* expressed in *E. coli* has alcohol dehydrogenase activity and this makes mushroom alcoholic fermentation possible. Compared with *Agaricus blazei*, *Flammulina velutipes* and *Tricholoma matsutake* [8, 9, 10], the alcohol dehydrogenase activity is very low, but genetic engineering methods such as promoter replacement or mutation could be used to improve alcohol dehydrogenase activity in *P. nameko*.

CONCLUSIONS

Mannitol-1-phophate dehydrogenase from *P. nameko* was cloned and characterized. The presence of the zinc-containing long-chain alcohol dehydrogenase suggests that the mushroom has the potential to produce ethanol by fermentation.

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MUTATIONAL ANALYSIS OF A *GPD* PROMOTER SEQUENCE USING TRANSIENT GENE EXPRESSION – A NEW PROMOTER ASSAY SYSTEM FOR BASIDOMYCETOUS FUNGI

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ABSTRACT

During the development of a transformation system in the selective lignin-degrading white rot fungus, *Ceriporiopsis subvermispora*, we observed many unstable transformants showing transient resistance to the selection drug, hygromycin B, in addition to a few stable and integrative transformants. The number of drug resistant transformants changed when distinct promoter sequences were used to drive the gene for hygromycin phosphotransferase (*hph*). We used this phenomenon to estimate promoter activity by using the number of colonies on the first screening plate as a *merkmal*. A series of deletion mutants of *C. subvermispora gpd* (glyceraldehyde-3-phosphate dehydrogenase) promoter fused to *hph* were constructed and transformation experiments showed that a 141-bp sequence was required for the basic promoter function. Subsequent point- and insertion-mutation analyses were conducted to test the functional significance of putative *cis* elements predicted from conserved sequences in ascomycetes. The assay system presented in this report may serve as a new tool to understand the regulation mechanism for gene expression in basidiomycetes, minimizing undesirable effects from multiple and random integration of the reporter construct in the chromosomes of the stable transformants.

Keywords: Promoter assay; Transformation; Transient gene expression

INTRODUCTION

In mushroom-forming fungi, transformations are brought about by the integration of the introduced DNA sequence into the host chromosome. Generally, integration occurs randomly at multiple sites and the copy number of the insert varies among the transformants [1, 2]. The resultant phenotype is stably maintained in mitotic and meiotic cell divisions. This situation has made it very difficult to do promoter assays in these stable transformants since the expression of a gene is affected by its dosage and position in the chromosome. In 2004, in order to overcome this problem, Bertossa et al.[3] used a large number of transformants for each reporter construct containing deletion or insertion mutant derivatives of the *Coprinopsis cinerea cgl2* promoter sequence. They demonstrated that a 627-bp sequence is a minimal promoter and that a 120-bp internal region was required for induction of the gene in darkness by measuring the transcript level in 100-120 different strains gathered from plate cultures [3]. However, it is not always so easy to obtain such a large number of transformants in other mushroom-forming fungi. It is also

very difficult to collect RNA samples from so many transformants in liquid culture. In this context, a precise, simple and rapid promoter assay method is required.

In addition to stable and integrative transformation, unstable transformants have occasionally been reported in some mushroom-forming fungi [4-7]. They were sometimes described as "false" or "abortive" transformants, although it was often difficult to say if they were all background unless similar phenomenon was also observed equally in the control experiment with no DNA introduction. These unstable transformants might be useless for isolating desirable strains by molecular breeding, but it is possible that some could be used as strains in a temporal stage with transient expression of the introduced gene.

Transcription is often controlled in its initial efficiency, and *cis*-acting regulation elements have been found near to or within the promoter region of a gene in various organisms. In eukaryotes, they are recognized by specific *trans*-acting factors including general transcription factors and activators. General transcription factors are thought to recognize the fundamental structure of the promoter to recruit the pre-initiation complex, which releases RNA polymerase to produce mRNA. Activators interact with general transcription factors and influence the efficiency of several transcriptional steps including initiation. Many kinds of transcriptional factors and their binding sequences have been reported and registered in the database that allow us to find putative regulation elements in a DNA sequence with computer software such as MOTIFS [8]. Also, many reports have described the existence of such consensus sequences in the promoter regions of basidiomycetous genes. However, most of the data relating to the fungal transcription factors and their target sites have come from experimental results in ascomycetes. Confirmation of the physiological function of those reported in basidiomycetes is largely lacking. Previously, it was thought that transcriptional regulation was very similar among fungi, and ascomycetous promoters were used to express recombinant genes in basidiomycetes. However, with a few exceptions, most of these trials were unsuccessful. Moreover, gene expression sequences were often incompatible between different basidiomycete species. However, it is now thought that, in basidiomycetes, there might be special features associated with the fundamental sequence, or conformational requirements for promoter function and its regulation. In other words, the molecular mechanisms underlying transcriptional recognition and regulation in basidiomycetes are mostly unclear.

MATERIALS AND METHODS

Plasmids and strains. Recombinant plasmids, pCsGi-*hph* and pCsbtubi-*hph* contain the gene for hygromycin phosphotransferase (*hph*) driven by *gpd* (glycelaldehyde-3-phosphate dehydrogenase) and *β-tublin* promoters from *Ceriporiopsis subvermispora*, respectively (unpublished). *C. subvermispora* Fp-90031 ATCC 90467 was used as the host strain for all transformation experiments. Plamids for the promoter assay were constructed from pCsGi-*hph* by introducing deletion, base-substitution and insertion of synthesized oligo-DNA.

Promoter assay using transient gene expression. The basic protocol for transformation of *C. subvermispora* was the same as the PEG/$CaCl_2$ method used for *Pleurotus ostreatus* [2]. Hygromycin B (Hyg), 100 μg/ml, was used to screen for drug resistant transformants. The activity of promoter sequences was estimated by counting the number of Hyg-resistant colonies on the first screening plate.

RESULTS AND DISCUSSION

To develop a transformation system in *C. subvermispora*, we observed isolates with drug resistance on the first screening plate containing 100 µg of hygromycin B (HygB). When pCsGi-*hph* and pCsbtubi-*hph* were used as vector plasmids, about 4% of the isolates showed stable drug resistance after four successive subcultures on screening plates containing the same concentration of HygB. Remaining isolates failed to grow after the second or third subcultures. Using Southern hybridization, the introduced marker sequence was shown to exist in stable transformants but not in unstable transformants (data not shown). No HygB-resistant colonies were recorded in the control experiment in which no DNA was introduced into the protoplasts. These results indicated that the unstable transformants were not background colonies but transient transformants showing a part-time drug resistance caused by expression of the introduced constructs, most likely in an extra-chromosomal manner. Furthermore, when plasmid pLG-*hph*, containing *hph* under the control of *Lentinus edodes ras* promoter, was used as a vector plasmid, less drug-resistant isolates were observed and no stable transformants were obtained. This suggested that differences in the promoter sequence affected the number of drug resistant isolates and also the possibility of stable transformation. The emergence of drug resistant colonies on the screening plate depends on the balance between growth inhibition by the drug and expression of the relevant drug marker gene, irrespective of its intra- or extra-chromosomal existence. In the case of the extrachromosomal state especially, this balance can be changed during mycelial growth or transfer to a new culture plate, which leads to unstable maintenance of drug resistance.

Table 1: Deletion analysis of the *gpd* promoter

Promoter length (bp)	Number of HygB-resistant colonies	%
1233	139	100
836	85	61
637	92	66
503	81	58
317	67	48
201	47	34
141	65	47
120	5	4
52	0	0
0	0	0

We used this phenomenon to estimate promoter activity by using the number of the colonies on the first screening plate as a *merkmal*. A series of deletion mutants of *C. subvermispora gpd* promoter fused to the *hph* coding sequence was constructed and introduced into wild-type *C. subvermispora* (Table 1). It was demonstrated that a 141-bp sequence is required for the basic promoter function. A point mutation analysis was then conducted using a 200-bp promoter sequence. A significant decrease in the colony numbers was observed when the mutation was introduced into one of the putative sequences for the GCN4-binding site, the AP-1 binding site and the TATAA box. In contrast, almost no effects were observed for mutants in the

consensus sequence for the GATA factor and Sp-1. Furthermore, introduction of a synthetic GCN4-bonding sequence suppressed the mutation in the original GCN4-binding site. These results strongly suggested that, in *C. subvermispora*, the GCN4-binding site, the AP-1 binding site and the TATAA box play an important role in the function of *gpd* promoter.

CONCLUSIONS

Unstable transient transformants were observed in the selective lignin-degrading fungus, *Ceriporiopsis subvermispora* and used for a new promoter assay system. The number of transformants on the first screening plate changed when distinct promoter sequences were used to drive the marker gene. A deletion mutant analysis was successfully used to show that a 141-bp sequence was required for the basic function of the *C. subvermispora gpd* promoter. The promoter assay system presented in this report may serve as a new tool for understanding the basic organization of promoter sequences and the regulation mechanism(s) of gene transcription in basidiomycetes.

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BIODEGRADATION OF CARBOHYDRATES DURING THE FORMATION OF *AGARICUS BISPORUS* COMPOST

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ABSTRACT

Agaricus bisporus is commonly grown on compost, which consists mainly of straw and horse manure. This means that the majority of the carbon source is present as plant-based polysaccharides, which themselves consist of many different monomeric components. This paper presents an overview of plant biomass degradation by fungi, with a special emphasis on the biochemical and molecular data available for *A. bisporus*.

Keywords: *Agaricus bisporus*, Compost, Polysaccharide degradation, Lignin degradation

INTRODUCTION

Agaricus bisporus is a leaf-litter-degrading basidiomycete fungus that naturally grows in grasslands and forests. It is a nutritious edible fungus cultivated industrially all over the world. *A. bisporus* belongs to the family *Agaricaceae*, order *Agaricales* and subclass *Agaricomycetidae* [1]. Cultivation of *A. bisporus* for human consumption originated in France over 300 years ago. Growth of *A. bisporus* needs a substrate produced by the composting of animal manure (usually horse and chicken), wheat straw, gypsum, water and different additives [2-4]. Composting is an accelerated version of the natural recycling process for plant biomass (decomposition and humification) performed by microorganisms.

The procedure to generate the compost substrate for *A. bisporus* is usually done in two stages. Phase 1 involves regularly mixing, wetting and heating of compost ingredients [2-4]. During phase 2, which is an indoor process, pasteurization is accomplished for 3-6 hours (60 ^{o}C) to relieve the compost of mushroom pests and pathogens that may be present in the compost [2]. After phase 2 the compost is inoculated with millet or rye grains colonized with *A. bisporus* mycelium, a process called spawning [5]. The colonized substrate is covered with casing soil, which is usually a mixture of peat and limestone [4]. *A. bisporus* mycelium colonises the casing soil in about 10 days, after which the temperature is lowered to 16-18 ^{o}C and a relative high humidity with heavy watering promotes sporophore development [6]. The first harvestable mushrooms appear 18 to 21 days after addition of the casing layer and after that in repeating 7- to 8-day cycles known as flushes [7].

As a result of the composting process, the compost consists mainly of lignocellulose components together with microbial biomass [8]. A major part of this microbial biomass consists of the thermophilic fungus *Humicola insolens* var. *thermoidea* (*Scytalidium thermophilum*) which is important for increasing the growth rate of the mushrooms and reducing the ammonia concentration [9, 10].

RESULTS AND DISCUSSION

Composition of plant biomass. The main component of plant biomass is the plant cell wall, which itself consists mainly of polysaccharides, lignin and proteins. Depending on the plant species and tissue, as well as the season, the composition of plant cell walls varies. The different components interact with each other to provide the structural strength for plants and different fractions can be identified. One of these fractions is lignocellulose which consists of lignin, cellulose and hemicellulose (xyloglucan, xylan, galacto(gluco)mannan). The aromatic polymer lignin interacts with cellulose and forms covalent cross-links with hemicelluloses creating an intricate and hard-to-degrade network of polymers, which is a poor substrate for most microorganisms, but not for *A. bisporus* [8].

Cellulose consists of linear β-1,4-linked D-glucopyranose chains that are linked together by hydrogen bonds to form microfibrils. Degradation of this polymer involves endo-β-1,4-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) [11-14]. Endoglucanases hydrolyse cellulose chains randomly to β-1,4-gluco-oligosaccharides. Cellobiohydrolases remove cellobiose units from cellulose chains, which are degraded by β-glucosidases to D-glucose (see Fig. 1).

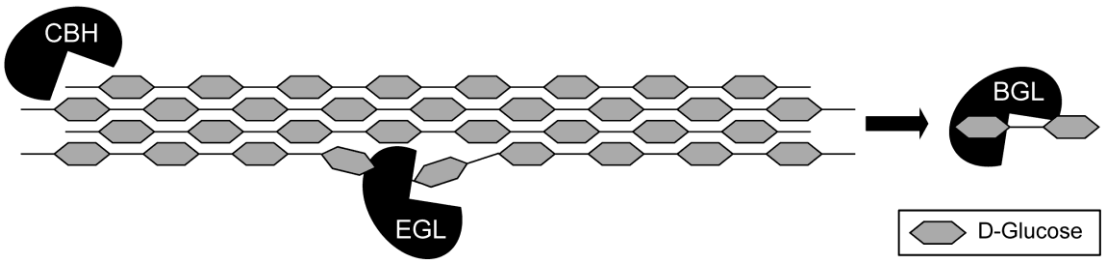

Figure 1: Schematic presentation of cellulose, its degradation and release of D-glucose. Reprinted from [15] with permission from the publisher.

One of the most complex components of hemicellulose present in straw-derived compost is xylan. It consists of a β-1,4-linked D-xylopyranose backbone which can be substituted with a large number of residues (e.g. L-arabinose, D-galactose, D-glucuronic acid, acetyl), depending on the origin [16]. The main enzymes involved in the conversion of xylan to monomeric sugars are endoxylanases (EC 3.2.1.8) and β-xylosidases (EC 3.2.1.37) (see Fig. 2). Endoxylanases cleave the xylan backbone to small xylo-oligosaccharides, which are converted to D-xylose by β-xylosidases [17, 18]. Other classes of enzymes involved in the removal of arabinosyl, glucuronosyl, acetyl and feruloyl residues from the xylan backbone are α-L-arabinofuranosidases (EC 3.2.1.55), arabinoxylan arabinofuranohydrolases, α-glucuronidases (EC 3.2.1.131), acetyl xylan esterases (EC 3.1.1.72) and feruloyl esterases (EC 3.1.1.73) (see Fig. 2) [19-20].

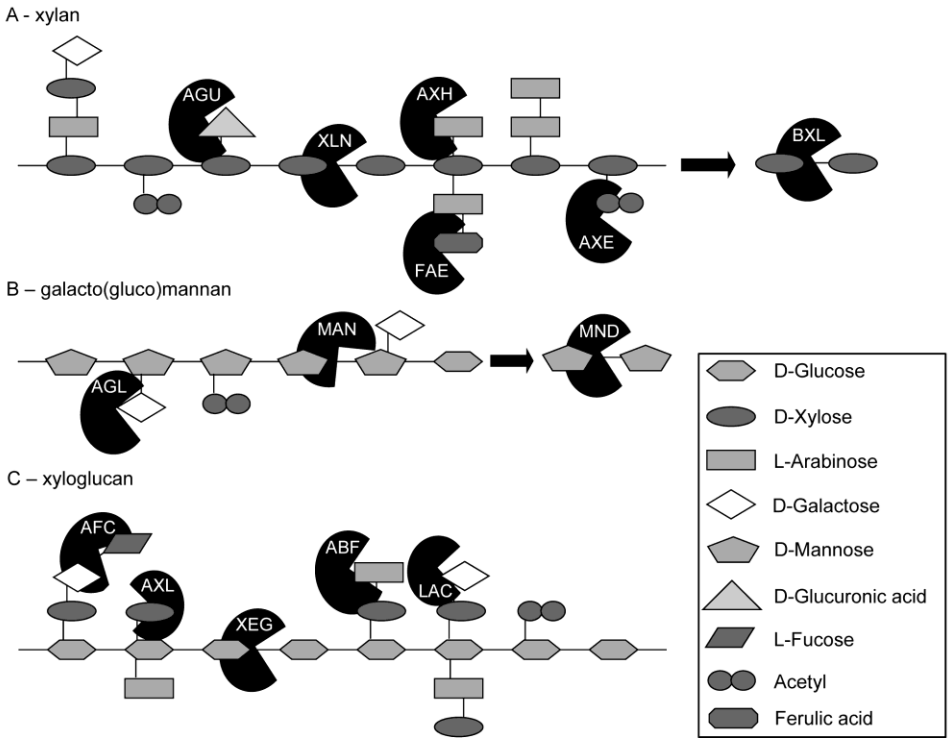

Figure 2: Schematic presentation of hemicellulose components (xylan, galacto(gluco)mannan, xyloglucan) and their degradation.
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Other hemicellulose structures present in plant cell walls include galactoglucomannan and glucomannan. Both consist of a backbone of β-1,4-mannosidic linkages which can have α-1,6-linked D-galactose residues. Endomannanases (EC 3.2.1.78) randomly hydrolyse β-1,4-mannosidic linkages in galacto(gluco)mannan, while β-mannosidases (EC 3.2.1.25) convert the resulting oligosaccharides to mannose monomers (see Fig. 2). The galactosyl residues attached to the mannan are removed by α- and β- galactosidases (EC 3.2.1.21 and EC 3.1.2.23, respectively), while the acetyl residues are removed by galactomannan acetyl esterases [20, 21]. Xyloglucan is a component of hemicelluloses which consists of β-1,4-linked D-glucopyranose chains and is substituted with α-linked xylose residues and other monosaccharides (see Fig. 2). Some enzymes which cleave the cellulose backbone are also involved in the biodegradation of xyloglucan. α-D-xylosidases, α-L-furanosidases, α-L-arabinofuranosidases, α-L-galactosidases, β-D-galacrosidases and xyloglucan acetyl esterases remove xyloglucan side residues (see Fig. 2) [22].

Enzymes involved in the degradation and modification of carbohydrates, including plant polysaccharides, are classified in the Carbohydrate Active enzyme system (CAZy, www.cazy.org) [23].

Lignin is an essential part of plant cell walls. The main extracellular enzymes acting in lignin breakdown are manganese peroxidise (EC 1.11.1.13), heme-containing lignin peroxidise (EC 1.11.1.14) and Cu-containing laccase (EC 1.10.3.2) [24].

Plant biomass degrading enzymes and their encoding genes from *A. bisporus*. Some insight into the molecular basis of *A. bisporus* related to plant biomass degradation was obtained through the isolation of genes encoding the related enzymes and the detection of their

developmental regulation. Screening of a cDNA library, made using RNA isolated from cellulose-grown mycelium, with an endoglucanase antibody identified four cDNA clones assigned cel1 to cel4 [14, 25]. Cel2 and cel3 are similar to fungal cellobiohydrolase I and cellobiohydrolase II, and cel4 shows similarity to fungal β-mannanases belonging to glycosyl hydrolase family 5. Cel2 has homology to glycoside hydrolase family 7 (GH7) while cel3 belongs to GH6. However, no homology to cellulose degrading enzymes was detected for cel1 although it did have homology to fungal cellulose binding domains [14, 21, 25]. The transcript of cel3 gene was detected about 13 times lower in glucose grown cultures than in cellulose grown cultures [11]. It was also shown that cel3 expression increased during fruiting body development and the highest level comes at the veil break [26].

An endoxylanase encoding gene, *xlnA*, was isolated to analyze the hemicellulotic activities during cultivation of *A. bisporus* on compost [17]. It was shown that *xlnA* belongs to GH10 which includes fungal and bacterial enzymes hydrolyzing xylan, cellulose or both [17]. Expression of *xlnA* was detected in vegetative mycelium grown on axenic compost. However, no *xlnA* expression was detected in fruiting bodies. Also, no expression of *xlnA* was detected in mycelium of *A. bisporus* that was grown on compost supplemented with glucose, but a high level of *xlnA* expression was detected after transfer of the mycelium to medium containing cellulose, xylan or xylose [17]. An identical pattern of repression and expression was detected for the cellobiohydrolase II encoding gene *cel3* [17]. This suggests that *xlnA* and *cel3* are co-regulated and that their induction occurs by compost-specific factors.

Analysis of two laccase genes, *lcc1* and *lcc2*, demonstrated that high expression levels were detected in compost during colonization. No expression was detected during fruiting body development, but the expression level of laccase genes increased after harvesting and during the second flush [26].

Studies on the genes encoding cellulases, xylanases and laccases showed that they are developmentally regulated. Laccase increased during mycelia growth but declined at the onset of fruiting while cellulase and xylanase accumulated at the onset of fruiting.

Plant biomass degradation during growth of *A. bisporus* on compost. The major components of lignocellulose are cellulose and hemicellulose that are converted into microbial biomass during composting, while lignin is often complexed with proteins. The activity of endoxylanases degrading hemicellulose present in straw-derived compost (xylan) is slightly increased during the development towards fruiting bodies and the highest point in activity is after the veil break stage. However, increase in β-xylosidase activity was only observed after the button stage of the fruiting body development [18]. Increased xylanase activities during fruiting correlates well with the reduction in hemicellulose content of the compost [18].

The microflora of the compost only degraded lignin slightly whereas vegetative mycelium of *A. bisporus* was discovered to degrade lignin more efficiently, and that this degradation was mediated by the activity of manganese peroxidase and laccase [26, 27]. The activity of both laccase and manganese peroxidase has been observed in compost during the pinning stage of fruiting body formation and decreased at the onset of fruiting body formation. No lignin peroxidase activity was detected [28].

It was shown that lignin is degraded during the vegetative growth of *A. bisporus* in compost. In contrast, cellulose and hemicelluloses are already degraded after the addition of the casing soil. Low levels of cellulase activity have been found in the compost colonized mycelium but this level increases with the beginning of fruiting. In contrast, high levels of laccase activity have

been detected in the compost but after the beginning of the pinning stage these levels decline sharply [13, 29].

CONCLUSIONS

Degradation of plant biomass is an important aspect of the cultivation process of edible mushrooms, but the mechanisms behind it are still largely a black box. The availability of genome sequences for *A. bisporus* as well as transcriptome analysis enables us to obtain a deeper understanding of the genes and enzymes involved in this process. This is likely to result in new strategies to improve mushroom cultivation.

ACKNOWLEDGEMENTS

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BROWNING SENSITIVITY OF BUTTON MUSHROOMS

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ABSTRACT

To study the sensitivity of *Agaricus bisporus* mushrooms to bruising, a reproducible method was developed to apply mechanical damage to mushroom caps and quantify the subsequent discoloration. The newly developed bruising device can apply damage to the cap tissue of intact button mushrooms by a slip-shear sliding process in a fast and reproducible way. A protocol has been developed to obtain the most reliable and reproducible method to compare bruising sensitivity of different *A. bisporus* strains. The severity of the bruise is quantified with a computer image analysis system. Pictures of the bruised mushroom caps were taken under controlled lighting conditions and calibrated to a local reference. Image analysis software was developed to calculate the whiteness index (L-(3xb*), as defined by Hunter). This method of bruising and image based quantification was subsequently applied to a collection of wild, commercial and hybrid *A. bisporus* strains. A significant difference was found between bruising sensitive mushrooms and bruising tolerant mushrooms. A correlation was found between discoloration by the bruising device and discoloration caused by transportation of mushrooms on a conveyor belt. Less correlation was found between post-harvest discoloration of undamaged stored mushrooms and the bruising device. This indicates that discoloration caused by bruising or by storage of intact mushrooms might have different mechanisms.

Keywords: Bruising sensitivity; Computer image analysis; Button mushroom, *Agaricus bisporus*

INTRODUCTION

The quality of *Agaricus bisporus* button mushrooms is determined by colour, texture, cleanliness, maturity, flush number, and flavour. Of these, colour is the most important parameter because it is first perceived by consumers and discoloration decreases the commercial value [1]. Due to picking, handling and storage, discoloration reactions are initiated. Contact-based discoloration, or bruising, is caused by a mechanical process known as ‘slip-shear’ [1], a downwards force and a sideways movement, which can occur during picking by hand or by robotic picking equipment. Mechanical harvesting is more cost-efficient than picking by hand, but cannot be applied yet to serve the fresh market, as commercial strains are too sensitive to bruising. It has been shown that enzymatic browning of mushrooms is caused by polyphenol oxidases (PPOs: tyrosinases and laccases) and peroxidases through an enzyme-catalysed oxidation of phenolic substrates into quinones [2]. These products undergo subsequent reactions leading to the formation of the dark pigment melanin.

This project aims to develop a high throughput tool to quantify bruising sensitivity of mushrooms. This can support the selection of bruising resistant strains, suitable for mechanical harvesting of mushrooms for the fresh market. In order to breed for browning-tolerant lines and to study the molecular and biochemical processes in depth it will be necessary to determine the bruise-related browning sensitivity in a reproducible way. Burton [1] has developed a device to apply a slip-shear stress on mushroom slices. However, the damage applied was much stronger than occurs in practise and the method was too laborious to apply to a large number of samples.

Here we describe the use of a newly developed bruising device and image analysis system to quantify bruising sensitivity [3]. In order to develop a reliable and reproducible method, several parameters were studied in previous experiments. The parameters investigated were the influence of flush, the effects of the developmental stage of the mushrooms, the time between harvest and applying the bruise, and the time between bruising and analysing discoloration. A collection of *A. bisporus* strains was screened for their bruising sensitivity in order to analyse the phenotypic variation among strains. This method identified the genetic variation of bruising sensitivity among strains and now can support unravelling the molecular and biochemical basis for this trait. In this paper, we describe the correlation between discoloration by the developed bruising method and discoloration caused by a conveyor belt and discoloration after 7 days storage at 4 °C or 8 °C at 90% RH of the same mushroom varieties.

MATERIAL AND METHODS

Mushroom strains. Based on a previous collection screen of *A. bisporus* strains, several strains were chosen and cultivated. Eleven white mushroom strains were grown in seven replicates and four brown strains in two replicates. In each experiment, the strains were randomly distributed over the growing room. Strains used in this research originated from the Department of Plant Breeding at Wageningen UR and represent old and present day cultivars and wild collected varieties (ARP culture collection; [4]).

Mushroom growth. Spawn was prepared by boiling sorghum grain (*Sorghum bicolor*) for 20 min in water. After draining of water, gypsum (2.4% w/w) and chalk (0.7% w/w) were added before sterilizing. After sterilizing and cooling, grains were transferred to "full-gas microboxes" (Combiness, Gent, Belgium) and inoculated with a pure culture of an *A. bisporus* strain grown on agar (1% w/w malt extract, 0.5% w/w mycological peptone, 5 mM MOPS, pH 7). The colonization was completed in approximately two weeks with occasional shaking of the boxes to distribute colonized grains. Cultivation was performed in boxes (56 x 36 x 20 cm) filled with 16 kg of phase II compost [5]. Each box was inoculated with 110 ml of spawn. After a spawn run period of 14 days (air temperature at 21–23°C; RH 95%; 3500 ppm CO_2), casing soil was applied. After colonisation of the casing soil for 10 days at 21-23 °C, the casing layer was ruffled. Three days after ruffling, the boxes were vented at a rate of 0.075 °C/h towards 18 °C air temperature. At the same time, CO_2 was lowered at 35 ppm/h to a value of 1000 ppm and the RH was set at 90-92%. Depending on the strain, pins appeared between 3 and 10 days after the onset of venting. Harvest of the fastest growing strains started 7 days after venting and the majority of the strains produced 12-13 days after venting.

Bruising device. A new mushroom bruising device was constructed, which is able to apply damage to the cap tissue by a slip-shear sliding process using a spatula on a moving wheel (Figure 1). Ten mushrooms were fixed on a tray and bruised in series. The force with which the spatula presses on the mushroom caps can be adjusted by the weights placed on the horizontal bar on top of the machine. After initial experiments, the total weight of the spatula was set to 40

g. Mushrooms were kept at 20 °C constantly after harvest. After a recorded period of time, the mushrooms were placed into an illuminated cupboard (four Philips Fluotone TLD on each side of the tray) equipped with a photo-camera (JVC KY-F30E colour video camera with a JVC TV UM lens) and pictures were taken.

Computer image analysis. The developed quantitative image analysis system can measure various parameters from colour images of mushrooms and gives a quantitative value of discoloration. All images of a single experiment were calibrated using the standard Gretag-Macbeth colour checker (MSCCC) in order to compensate for small, unintentional variations in colour and illumination during the course of an experiment. A sheet of white paper was used to calibrate for the brightness and to compensate for variations in illumination within the cupboard. The brown discoloration was analysed using specially developed software (based on [6] and [7]) according to the CIE L*a*b* colour system [8].

Pictures taken give RGB colour space values which first need to be transformed to CIE XYZ values, for which the following transformation matrix is used [9],

0.49	0.31	0.20
0.17697	0.81240	0.01063
0.00	0.01	0.99.

The CIE XYZ values can then be transformed to CIE La*b* values [10], with the following formulae,

$L^* = 116 \int (Y/Y_n) - 16$

$a^* = 500 [\int (X/X_n) - \int (Y/Y_n)]$

$b^* = 200 [\int (Y/Y_n) - \int (Z/Z_n)]$.

Parameters were calculated for each mushroom individually and performed in the L, a*, b* colour space [8]. The software is not calibrated to the standard L, a*, b* colour space and consequently the outcome cannot be compared to results of other instruments. Images can only be compared mutually.

The position of each of the 10 mushrooms on the tray is identified and numbered automatically. The measurement area (Figure 2) is established such that 50% of the cap area is selected to exclude shadow effects. The bruising parameters used for the bruising applied by the bruising device are the whiteness index (WI) and the whiteness index difference (WI difference). WI is calculated as L-(3xb*), as defined by Hunter [11]. The WI difference is the difference between the average WI of a representative spot on the bruised area and the average WI of the control, not bruised tissue on the same mushroom (Figure 2). Tissue of mushrooms after the conveyer belt or after storage does not contain a control area. In this case the WI difference of the conveyor belt was calculated as the difference of the WI of the measurement area before and after the conveyer belt bruising at specific time points. The WI difference after storage was calculated as the difference between the WI of the measurement area before storage and the WI after 7 days of storage.

Outliert test. After calculating the bruising parameters from the normalised pictures, the data were analysed with Matlab. This program calculated the most deviant values (outliers) for each 10 mushrooms on a tray. This calculation is based on an analysis of variances of the trays. Mushrooms within a tray with a standardized residual of more than 2.3 were rejected and not used in further analysis.

Statistical analysis. Statistical analysis was performed using Genstat (13th edition). The linear mixed model (REML) was used to calculate significant differences between strains with a level of significance of 0.05. This was done with the least significant difference (LSD).

RESULTS

Developed bruising method. In previous experiments, the optimal method to bruise mushroom with the bruising device was determined [3]. Medium sized (35-55mm) closed or veiled mushrooms from the first and second flush were used to analyse bruise-related browning sensitivity by applying three times a slip shear force with the spatula over the cap tissue (with the weight of the spatula adjusted to 40 g). Mushrooms were bruised within 4 h after harvest. Pictures of the bruised mushrooms are taken 60 min after bruising and analysed with the computer image analysis system. The computer image analysis system can be used to determine the WI of a specific bruised area on the cap of the mushroom or of the whole cap surface, which is set at 50% of the total cap area (circle in Figure 2).

Figure 1: Bruising device. The spatula can be moved by hand over the tray. The weights in the horizontal bar can be changed (set at 40 g in our experiments).

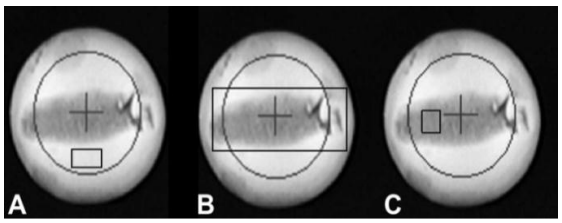

Figure 2: Computer image analysis areas. A control area. B bruise boundary. C bruised area. The circle on each mushroom is the measurement area (set at 50% of the surface).

Bruise-related browning sensitivity of 15 different *A. bisporus* strains. The method described above was used to characterize the genetic variation of bruising sensitivity among a small collection of 11 white and four brown *A. bisporus* strains (Figures 3 and 4). Comparison of the bruising sensitivity of the tested strains indicated that a considerable variation in bruising sensitivity exists among button mushrooms. At this stage of comparing bruising sensitivity of white and brown capped mushrooms, we are not sure whether WI differences can be influenced by large differences in cap background colours. That is why both types of cap coloured mushrooms were analysed separately (Figure 3 for white capped strains and Figure 4 for brown capped strains). Among the white strains, it was found that some of the commercial strains, such as commercial hybrids 1, 2 and 3, were among the least sensitive strains and wild-type white strain 1 also showed low bruising sensitivity (Figure 3). Among the white button mushroom strains, very sensitive strains could also be identified, such as wild-type white strain 7. For the brown cap coloured strains, only wild strains were tested and these showed a significant difference in bruising sensitivity. Within this small selection, it was found that the two tolerant brown strains have a lower WI difference than found for the most tolerant white strain. Several strains were included in more than one experiment and it was shown that the classification of discoloration after bruising into tolerant, moderate and sensitive was reproducible (data not shown).

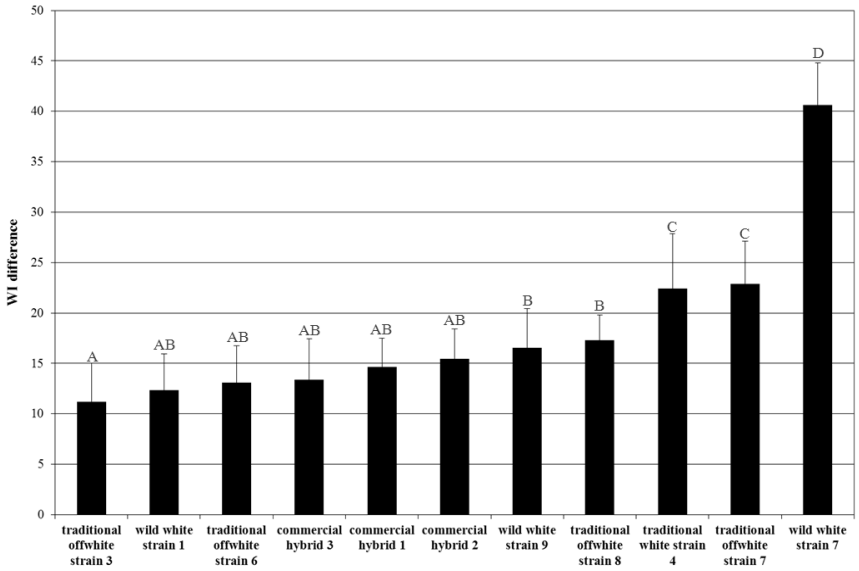

Figure 3: WI difference of 11 white button mushrooms from flush 1, 60 min after bruising. Error bars are from standard deviation. Values with the same letter are not significantly different at the 0.05 level.

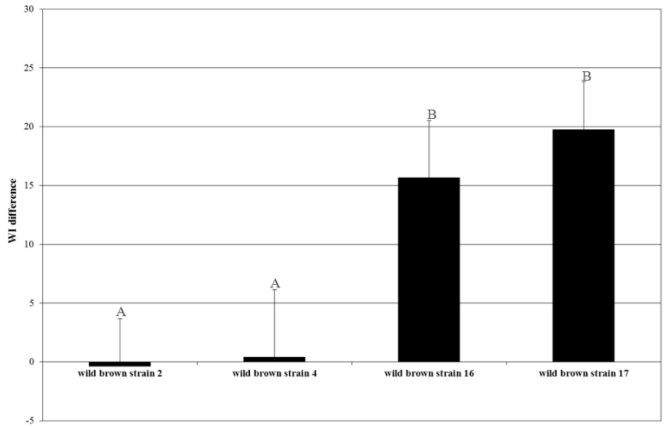

Figure 4: WI difference of 4 brown button mushrooms of flush 1, 60min after bruising. Error bars are from standard deviation. Values with the same letter are not significantly different at the 0.05 level.

Bruising button mushrooms with a conveyor belt. Mushrooms of seven strains were bruised with a conveyor belt to compare the discoloration after bruising with the results of the in-house developed bruising device. A mushroom grower was visited to check the times of bruising during mechanical harvesting and sorting. Approximately 20 falling motions occurred during the sorting process. Mushrooms were applied 5 times in a row on a conveyor belt 5 m in total length (Figure 5). Mushrooms from the second flush were used, and at least 10 mushrooms per time point were tested. The WI of the whole cap was measured and not of a specific bruised part. This was done by using the measurement area (the circle in Figure 2). A comparison was made between control (not bruised) mushrooms at T = 0 min and bruised mushrooms at three time points, 5, 60, and 120 min after bruising.

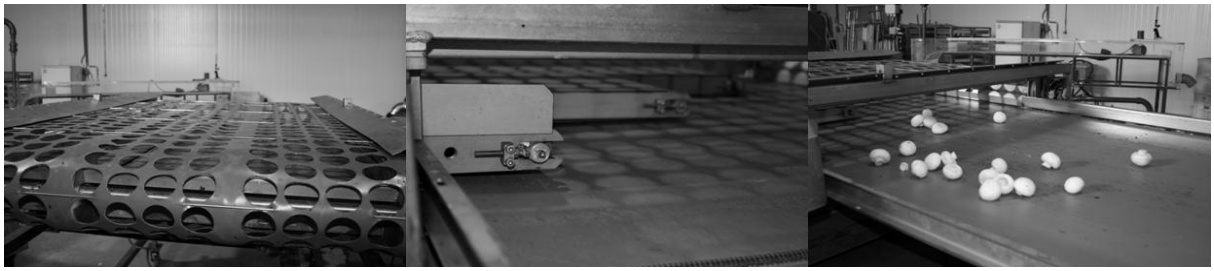

Figure 5: Conveyor belt used for the bruising experiment (from WeBe Engineering)

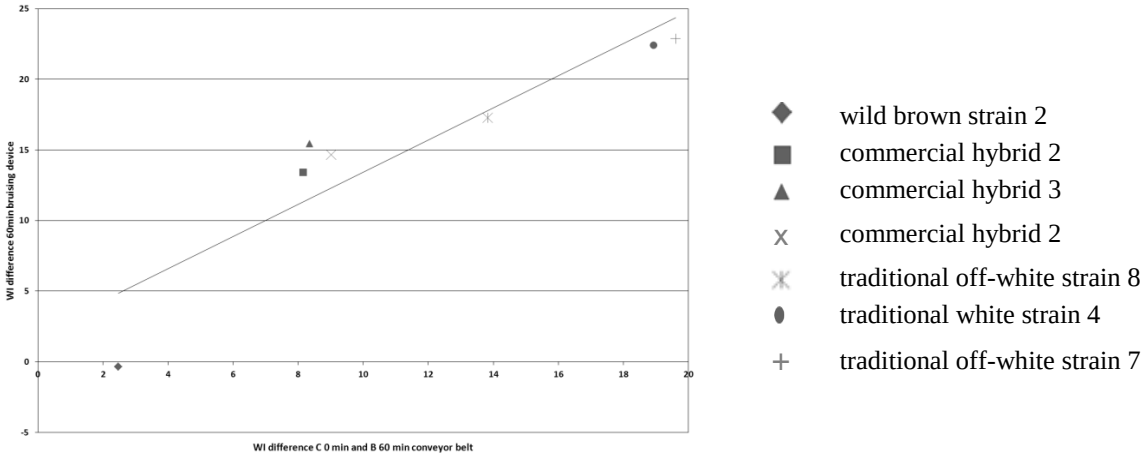

Figure 6: Correlation between conveyor belt bruising and discoloration after bruising with the bruising device. Y = 1.1388x + 2.0361, R^2 = 0.8444 (brown strain left out, gives Y = 0.749x + 7.9264, R^2 = 0.9612). C 0 min = control at T = 0 min, B 60 min = bruised T = 60 min

In figure 6 the correlation is shown between the WI difference 60 min after bruising with the bruising device and the WI difference of the conveyor belt. The WI difference of the conveyor belt is calculated as the difference between WI control 0 min (C 0 min) and WI bruised 60 min (B 60 min) after bruising with the conveyor belt. A correlation of 0.8444 could be found for the 7 strains used. The in-house developed bruising device gives a bruising discoloration comparable to industrially-used machines. When the only brown strain used was left out, the correlation is higher (R^2 = 0.9612).

Shelf Life Performance. Flush 2 mushrooms of 11 different strains were stored for 7 days at 4 ^{O}C or 8 ^{O}C at 90% humidity in boxes with a lid with small holes (as used in Dutch supermarkets). Pictures of the mushrooms were taken at the starting time point and at day 7. Mushrooms were not bruised, so discoloration upon storage was analysed. The WI of the measurement area of the mushroom was analysed (Figure 2). The mushrooms were weighed at day 0 and day 7 to follow the change in weight upon storage, to achieve approximately the same weight in each box and that boxes were filled completely. In general both white and brown button mushrooms discolour more at 8 ^{O}C than at 4 ^{O}C, except for wild white strain 1 and traditional off-white strain 3 (Figure 7 for white capped strains and Figure 8 for brown capped strains). The difference between the discoloration upon storage at 4 ^{O}C and 8 ^{O}C is not the same for every strain. As shown before, the commercial hybrids 1, 2 and 3 show the least discoloration. For the other white and brown strains that were analysed, there is less correlation between the discoloration after bruising compared to the discoloration after storage. This can be due to the fact that mushrooms strains showed a difference in development during this storage period; some strains were open after 7 days and sporulated, while some strains still contained closed or veiled mushrooms. Smith et al. [12] followed mushroom development (cap opening) during

storage at 18 ^{O}C for *A. bisporus* U3 and 2 *Agaricus bitorquis* strains and found a difference in the rate of maturation. One of the *A. bitorquis* strains developed much slower and, even after 5 days of storage, stage 3 mushrooms were found in most cases (based on Hammond and Nichols; [13]). The variation in discoloration after storage is less in the brown mushroom strains than found with the bruising device. The weight loss after 7 days storage was always higher at 8 ^{O}C than 4 ^{O}C, except for traditional off-white strain 3 (Table 1).

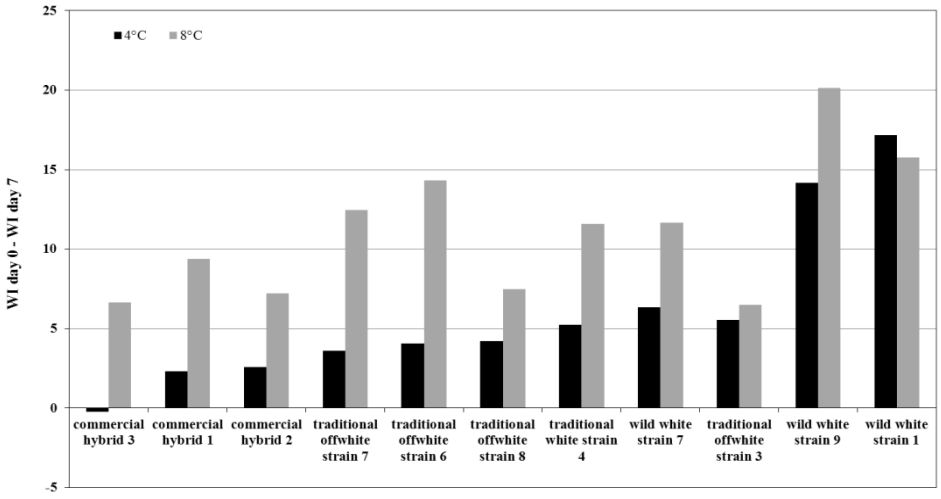

Figure 7: Difference between WI day 0 and WI day 7 after storage at 4 **^{O}C** or 8 **^{O}C** of 11 white button mushroom strains.

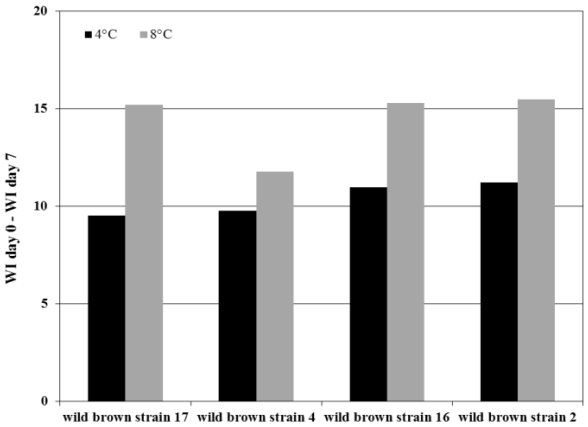

Figure 8: Difference between WI day 0 and WI day 7 after storage at 4 **^{O}C** or 8 **^{O}C** of four brown button mushroom strains.

Table 1: Weight loss in g per mushroom after storage for 7 days.

Strain	at 4 ^{O}C	at 8 ^{O}C
wild white strain 1	0.42	0.85
wild white strain 9	0.33	1.05
commercial hybrid 2	0.54	1.60
commercial hybrid 1	0.50	1.58
commercial hybrid 3	0.36	2.09
traditional off-white strain 3	1.93	1.08
wild white strain 7	0.35	1.11
traditional white strain 4	0.18	1.86
traditional off-white strain 6	0.44	0.79
traditional off-white strain 7	0.31	1.08
traditional off-white strain 8	0.28	0.96
wild brown strain 4	0.05	1.51
wild brown strain 2	0.36	0.69
wild brown strain 17	0.59	1.92
wild brown strain 16	0.33	2.53

DISCUSSION

Bruising-related discoloration of mushrooms is an important loss of quality caused by picking, conveyor belts or storage. In order to unravel the mechanisms behind bruising sensitivity, to compare pre- or post-harvest treatments and to breed for bruising tolerant strains, it will be necessary to have access to a reliable bruising quantification method. In previous experiments,

factors were studied that influence the reproducibility, internal sample variation, and quantification of the bruise-related brown discoloration. This resulted in a standard protocol to determine bruising sensitivity in a quantitative manner [3]. Quantification of the discoloration is based on measuring the WI both of bruised and undamaged tissue of the same cap and subtracting the WI value of the undamaged tissue from the WI value of the bruised tissue.

Here, the bruising method is compared with bruising by a conveyor belt and by discoloration during cold storage using a collection of strains with different degrees of bruising sensitivity. Although the selection was chosen randomly, it can be stated that there was a larger variation in sensitivity within the brown strains than in the white strains upon bruising with the bruising device.

The results indicate that there is a correlation between the bruising device induced discoloration and conveyer belt damaging sensitivity. Including more strains and mushrooms from the same flush in this type of analysis will allow to further detail the correlation between different mushroom strains. Less correlation was found between the discoloration after cold storage of undamaged mushrooms and 60 minutes after bruising with the device. Possibly, different mechanisms are involved in causing storage or bruising-related discoloration, as the stored mushrooms were not bruised. In addition, the loss of water, membrane damage and enzyme activation can cause a different response and result in higher or lower browning sensitivity. The difference in shelf life performance of different strains is also an important factor. For some strains, all mushrooms opened during 7 days of storage and for other strains the mushrooms were closed or veiled. Although not correlating to bruising sensitivity, the possible genetic variation in shelf-life performance and discoloration of the strains tested can be of interest to the industry for increasing shelf life performance by breeding.

The bruise-related browning sensitivity quantification method can be used for in- depth research to unravel the molecular and biochemical pathways behind the trait. These studies can be focused on substrate availability and enzyme activity, as reviewed by Jolivet et al. [2]. Different starting substrates lead to the formation of different types of melanin, of which the intermediate products have different colours. Dedicated transcriptomics, metabolomics and proteomics can be used to unravel the molecular and biochemical pathways behind brown discoloration.

ACKNOWLEDGEMENTS

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ORIGIN OF LACCASE GENE STRUCTURAL DIVERSITY IN EDIBLE MUSHROOMS

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ABSTRACT

Laccase genes have been found in fungi, plants, insects and bacteria. In Basidiomycetes, the number of laccase genes ranges from 0 to 17. The role of these genes is not well known. It seems to be important in fungal interaction, development, melanine synthesis, human and plant pathogenesis, [ectomycorrhizal association and nutrition of the fungi. Their role as lignin-modifying enzymes is controversial. Laccase phylogeny already published is not congruent with species phylogeny. Phylogeny of gene is generally deduced from analyse of aligned sequences of nucleotides or amino acids. Presence of indels, position of introns can give complementary information. To better understand the origin and evolution of Basidiomycete laccase genes, we studied the structure of diverse multi copper oxidase genes in different phylum of Eukaryotes, in basidomycetes and in *Agaricus bisporus*. A mutation has been conserved in all basidiomycete laccases and constitutes a molecular synapomorphy for basidiomycete laccase genes. At least three main laccase subfamilies (A, B and C) are present in Polyporales and Agaricales. In *A. bisporus* the 4 new laccase genes we have sequenced (lcc3-lcc6) belong to a new sub-family for this species. Cladogram of laccase genes from edible mushrooms built with Dollo parsimony of intron position is congruent with dendrograms built with homologous amino acid sequences, but is not identical. Some consequences on the evolution of laccase genes and their diversity are discussed.

Keywords: Laccase phylogeny, laccase sub-families, intron position, *Agaricus bisporus*, basidiomycetes, protein evolution.

INTRODUCTION

Laccase are polyphenol oxydase belonging to two protein families that are involved in various systems of interactions between fungi and their biotic and abiotic environment [1-4]. The multi copper oxidase family (mco) is the large one and contains also other enzymes as ferroxidase, ascorbate oxidase, ferroxidase-laccase [1]. The second family, recently discovered, contains proteins with the DUF152 domain, present in a hypothetical Bacteroides of the bovine rumen and in *Eschericia coli* [2].

Laccases of scomycetes and basidiomycetes form two clades belonging to the mco family. Laccases have the ability to oxidize a wide range of substrates [6]. Some laccases play a role in sclerotization (insects), in lignification process (LMCO of plantes) but there roles in basidiomycetes are not well elucidated (see Kues and Ruhl for review [1]). During a long time, an important role of basidiomycete laccases has been attributed to delignification, but it is also contested [7]. Most of fungal laccases are secreted and some of them seem to play a role in recycling recalcitrant organic matter and in the detoxification of natural environments [8]. Some are involved in ectomycorrhizal association [4], in plant pathogenesis [6] or in fungal-fungal

interaction [2, 9]. For instance, to improve *A. bisporus* strains for their resistance to *Leucanicillium fungicola*, one possible track is to better understand the mechanism of interaction of the two fungi. Thus, laccase genes appeared to be good candidates. At the beginning of this work, two laccase genes, lcc1 and lcc2, were known [10]. We have discovered and annotated 4 new laccase genes in *A. bisporus*.

Intron positions are not suitable information for phylogenetic analysis at long evolution distances [11]. Nevertheless, Kilaru *et al.* [3] on *Coprinopsis cinerea* laccases, Tavares *et al.* [12] and Boulet *et al.* [13] on *Arabidopsis, Caenorhabditis and Drosophila* and Matheny *et al.* [14] for basidiomycota phylogeny, showed that they could be very useful in phylogeny in complement of analyses based on protein or nucleotide sequences. Moreover, the intron positions could help to elucidate general mechanisms of gene family evolution. Indeed intron presence/absence is a relatively very slowly evolving character [15] and selective forces acting on it differ to those acting on protein sequences.

The study of intron positions and protein structure would help to go further in the origin and evolution of basidiomycete laccases. That also may help us to deduce putative laccase functions. We supposed that mco family is a very old family that has experienced several intron gain and loss waves. We also supposed that no parallel intron insertion has occurred in laccase genes. In the present work, we compared the phylogeny of basidiomycetes species using laccase genes, based on protein sequence or on intron positions. We discovered insertion of 5 amino acids present in all basidiomycetes laccases but absent in all other mco. We also discovered a *Lentinula edodes* gene with presence or absence of three introns depending of the strain. Consequences for the evolution of laccase genes and their diversity are discussed.

MATERIALS AND METHODS

Sequences used. Sequences used for phylogeny analyses (Table 1) were obtained from GenBank (http://www.ncbi.nlm.nih.gov/), Joint Genome Institute (http://genome.jgi-psf.org/programs/fungi/index.jsf) and Broad Institute (http://www.broadinstitute.org/scientific-community/data). For phylogenetic analyses, we had chosen all published genomic DNA Laccase sequences to have intron positions. The translated coding sequences of these genes were used for the amino acid phylogeny.

Two Polyporus laccase genes and three different *C. cinerea* laccase genes (two belonging from two different well defined clades of subfamily A and lcc8) were used as controls in the analyses. Three ferroxidase gene sequences have been used as out group.

Identification of new *A. bisporus* genes. *Agaricus bisporus* strain U1-7 (having the same mating type allele than H93) is a homocaryotic strain coming from a protoplast of the cultivar U1 [16, 17]. DNA extraction was made with the Nucleon Phytopure extraction kit (GE Healthcare) from lyophilised mycelium. The *lcc2* cDNA sequence [10] was used to generate primers and to amplify and sequence the gene. Inverse PCR [18] was used to obtain the 3' end of the gene. One (or more) restriction enzyme was chosen for inverse PCR, cutting the known sequence. Primers were constructed with Primer 3 on the 3' side of the restriction site, present on the already known sequence, to orientate inverse PCR. When AK7/AK8 primers from D'Souza *et al.* [19] were used to amplify *lcc1* and *lcc2*, two bands were obtained in electrophoresis. *Lcc1* and *lcc2* corresponded to the same band. So, the second band was expected to be another laccase gene (renamed hereafter as *lcc3*). This bans had been sequenced. To obtain a larger part of the gene, a new degenerated primer (AK9 : TGRCARTGGARGAACCAKGG) was constructed on part of a simplified l4 signature of laccase sequences (P-W-F-(LF)-H-C-H). The totality of the gene was obtained by inverse PCR in both 3' and 5' directions.

Table 1 : Gene list, intron numbers and GenBank entries of fungal laccases and ferroxidases used in the two phylogenetic analyses.

Enzymes	Code	clades	Organism	gDNA accession number and region	Protein accession number	Gene name	Number of introns
Laccases		Agaricales					
	Abi	Agaricoid clade (VI)	*Agaricus bisporus*	L10664	AAC18877	lcc1	14
				FJ838791	ACZ06558	lcc2	14
				EU580106	ACE73659	lcc3	12
				FJ392313	ACR19861	lcc4	12
				FJ617019	ACU52699	lcc5	12
				FJ878811	ACZ57763	lcc6	12
	Lbi		*Laccaria bicolor*	NW_001889910 <486085..>488315	XP_001886681	lcc1	13
				NW_001889940 <72836..>75100	XP_001889567	lcc2	13
				NW_001889873 complement(109817.. 112096)	XP_001874989	lcc3	13
				NW_001889884 194127..196383	ACN49091	lcc4	13
				DS547137 <116239..>118647	EDR01587	lcc5	14
				NW_001889885 complement(429758.. 431860)	XP_001881925	lcc6	10
				NW_001889885 complement(432396.. 434513)	XP_001881926	lcc7	10
				NW_001889885 complement(435301.. 437493)	XP_001881927	lcc8	10
				NW_001889938 <142966..145306	XP_001889429	lcc9	13
	Cci		*Coprinopsis cinerea*	BK004113	DAA04508	lcc3	13
				BK004118	DAA04513	lcc8	8
				BK004117	DAA04512	lcc7	12
				BK004126	DAA04521	lcc16	18
				BK004127	DAA04522	lcc17	19
	Pos	Pluteoid clade (II)	*Pleurotus ostreatus*	AJ344434	CAC69853	poxa3	21
				Z22591	CAA80305	pox1	19
				Z49075	Q12739	pox2	19
				FM202669	CAR48257	pox3	10
				FM202670	CAR48258	pox4	19
				AJ005017	CAA06291	poxA1b	15
				FM202671	PSEUDO: CAR48259	pox5	18
	Led	Marasmioid clade (IV)	*Lentinula edodes*	AB055157	BAB84354	lcc1	27
				AB055158	BAB84355	lcc2=lac1	13
				FJ473386	ACR24356	=lac1 without 3 introns	10
				AB055159	BAB84356	lcc3	11
				AB543788	BAJ12091	lcc5	20
				AB543787	BAJ12090	lcc6	27
				-	BAH80447	lcc4	nd
	Fve		Flammulina velutipes	AY485826	AAR82931	laccase	9
	Pru	Polyporales	*Panus rudis*	AY839935	AAW28932	lacA	12
	Rmi		*Rigidoporus microporus (Fomes lignosus)*	-	CAE81289	lcc1	nd
Ferroxidases	Lbi		*Laccaria bicolor*	NW_001889910 complement(<222411.. >224748)	XP_001886726	lcc11	9
	Apo		*Auricularia polytricha*	AY616035	AAT73205	lac1	13
	sce		*Saccharomyces cerevisiae*	L25090	AAA64929	Fet3	0

Two clones sequenced from a genomic library available in our lab corresponded to partial laccase genes sequences. By inverse PCR we obtained the totality of these new genes (*lcc4* and *lcc5*). *Lcc6* was obtained from an extra band during one of these inverse PCRs.

Amino acid phylogeny. The phylogenetic analysis of laccase sequences was performed on the Phylogeny website platform (www.phylogeny.fr). Sequences were aligned with MUSCLE (v3.7) with default settings. After alignment, ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b) with default settings. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.0 aLRT) with default settings. Reliability for internal branch was assessed using the bootstrapping method (500 bootstrap replicates). Graphical representations of the phylogenetic tree were performed with TreeDyn (v198.3).

Intron position phylogeny. In GenBank, intron positions are indicated on gDNA sequences and protein sequence is deduced by translating CDS. The positions of introns of the encoding gene were determined on aligned protein sequences by an annotation on a BioEdit file [20]. The convention presented in Fig. 1 for schematizing the exact position of the intron on each codon.

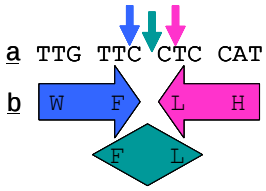


Figure 1: Convention used on BioEdit (version 7.0.5.3) to schematize intron position in a gene on the corresponding protein sequence.
a DNA sequence with three arrows indicating three possible positions of introns in the codons, b corresponding protein sequence with arrows and diamond overlapping two amino acid letters to indicate intron position in the encoding gene.

Dollo phylogeny. A matrix was built with all intron positions in all sequences. Paup was used to perform the Dollo parsimony analysis [21]. One thousand bootstrap replicates were used. We made the approximation that the ancestor gene had no intron at all.

Analysis of insertions in laccase genes. T-Coffee (Phylogeny.fr) was used to align sequences. The alignment were visualised with Jalview tool.

RESULTS AND DISCUSSION

Obtention of *lcc2* gDNA sequence and identification of four new laccase genes in *A. bisporus*. The complete genomic sequences of *lcc2*, *lcc3*, *lcc4* and *lcc5* and the partial sequence of *lcc6* have been obtained (GenBank accession number : FJ838791, EU580106, FJ392313, FJ617019 and FJ878811 respectively). It is not surprising to find at least six laccase genes in this species as seventeen have been found in *C. cinerea* [3] which is in the same Agaricoid clade.

The sequences of the six laccase genes of *A. bisporus* were compared between each other. Intron positions in lcc2 were the same as in lcc1 except for the 3' UTR intron 15 which has not been found in lcc2. The 3' end of lcc4 is still hypothetical, an intron could permit the prolongation of the protein (lcc4bis end: CPIWDSEPNFVKHAATMILDPLINFAFGPIFPVYIL

LRFPTSLICMKPNAFH). Sequencing the mRNA will be necessary to control the end of the protein. The study of intron positions of these genes revealed the presence of a new laccase sub-family in *A. bisporus* (Fig. 2). This has led us to compare the intron positions of numerous laccase genes and especially from *C. cinerea* as two laccase sub-families had been found in this species [3]. To better understand the phylogenetic relationships of these genes we have build and compared a phylogeny based on amino acid homologous sequences and a phylogeny based on the conservation of intron positions.

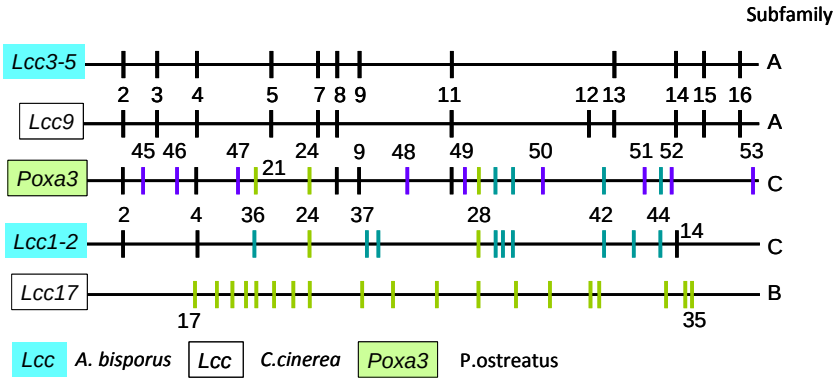


Figure 2: Relative positions of introns in different laccases genes showing the presence of two sub-families in Agaricus bisporus. Nomenclature of introns 2 to 35 [3], 36 to 44 (*A. bisporus lcc1* and *lcc2*), 45 to 53 (*P. ostreatus poxa3*).

Congruence but not identity between protein sequences and intron positions based phylogeny. Fig. 3A shows the maximum likelihood phylogeny of 38 protein sequences of laccases from edible mushrooms. The ferroxidase genes added as outgroup represented a clade. A second clade was composed by laccases of the subfamily B identified by Kilaru [3]. In a third clade (renamed hereafter as "clade C"), there were the large subunit of heterodimeric proteins encoded by *A. bisporus lcc1*, *lcc2* and *P.ostreatus poxa3* cluster. This clade wais confirmed by the analysis of intron position (Fig. 3B). We propose to name the proteins belonging to clade C "laccase subfamily C". In a fourth clade ("clade A"), we found the three control proteins from *C. cinerea* belonging to subfamily A [3]. The proteins contained in clade A were renamed "laccase subfamily A". Clade A corresponds to subfamily 1 in Kues and Ruhl [1], clade B and C to subfamily 2. Here clade A and C clustered, with good bootstrap values. The two most divergent proteins found in this last clade were from Polyporales. Even if the bootstrap values were very low, it was the first time that Polyporales laccases were placed at a basal position of the clade A in a phylogenic tree of laccases belonging to Polyporales and Agaricales. With this positioning, laccase phylogeny is congruent with the phylogeny of species and the origin of laccase subfamily A should be anterior to the divergence of Polyporales and Agaricales.

In this phylogenetic tree *A. bisporus* laccases are divided into two clades, A and C. All the new *A. bisporus* laccases belong to a subfamily unknown before in this species. *Agaricus bisporus* laccases of clade A formed a specific clade. This suggests that these four genes appeared by duplication events after the divergence between the ancestor of *A. bisporus* and the ancestor of the other species of Agaricoid clade VI (ie. *C. cinerea* and *L. bicolor*). These laccases clustered, but with very low bootstrap, with *C. cinerea* lcc7 encoded protein. *Pleurotus ostreatus* laccases were divided into three groups, two in clade A and one in clade C. *Laccaria bicolor* laccases were divided into two groups of clade A as previously shown [4]. *Lentinula edodes* laccases were divided into three groups of clade A.

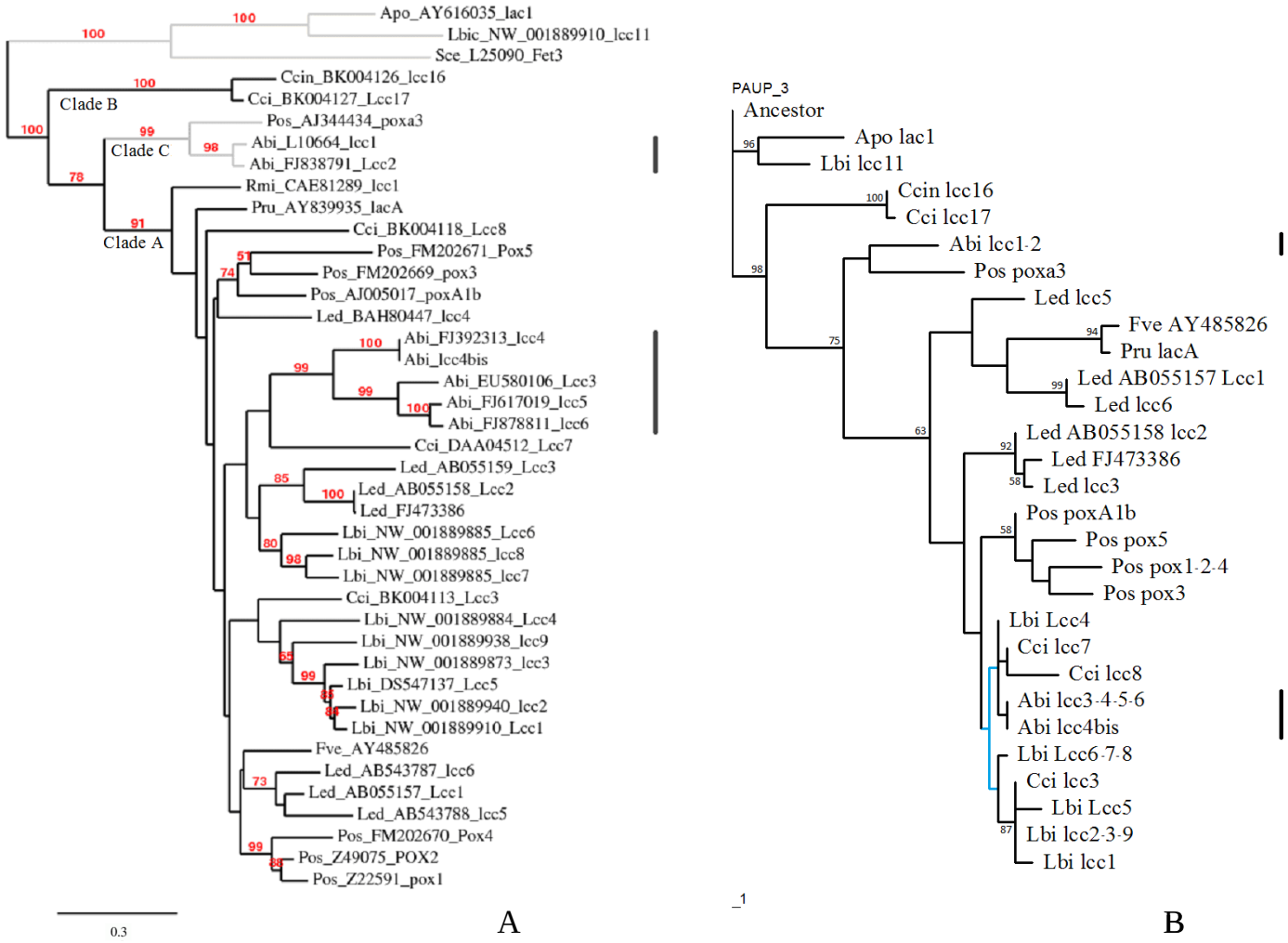


Figure 3: A- Maximum likelihood (ML) tree of edible mushroom laccases. B- One of the eight most parsimonious trees obtained with the Dollo parsimony of intron position of edible mushrooms laccase genes. Blue bare indicate Agaricoid clade VI laccase subfamily A genes.
The scale at the bottom indicates a unique event: insertion or deletion of an intron. Bootstrap values are from 500 replicates in A, 1000 in B. The vertical bars indicate the positions of *A. bisporus* laccases.
Lcc1-2 =lcc1, lcc2; pox1-2-4 = pox1, pox2, pox4. *P. ostreatus* gene nomenclature corresponds to: Pox1(LACC9), Pox2 (LACC10), Pox3 (LACC4), Pox4 (LACC1), Pox5 (LACC11), PoxA1b (LACC6) and Poxa3 (LACC2)

The bootstrap values for the three clades A, B and C wre very high, 91, 99 and 100 respectively. A laccase of clade A had more similarity to any another laccase of this clade that to a laccase of clade B or C. However Polyporales and Agaricales laccases were in clade A and only Agaricales laccases were in clade B and C. As phylogenic studies had demonstrated that Polyporales order was not nested inside Agaricales order [22], we can deduce that the separation between these three clades was anterior to the divergence between Agaricales and Polyporales. In the recent genome sequence of a Russulale, *Heterobasidion annosum* (http://genome.jgi-psf.org/Hetan2/Hetan2.home.html), a putative laccase sequence belonging to subfamily C have been found. This also indicates that the origin of subfamily C might be anterior to the divergence of Russulales and Agaricales. Wahleithner *et al.* [23] demonstrated the presence of large insertions in three of the four *Thanateforus cucumeris* laccases, suggesting the existence of other old sub-families in Cantharellales.

Fig. 3B shows one out the eight most parsimonious trees obtained with the Dollo parsimony of intron positions of the laccase genes used for the phylogenetic tree of protein

presented in Fig. 3A. Two proteins for which gDNA was not available, *R. microsporus* lcc1, *L. edodes* lcc4 and one that has no intron, *S. cereviciae* Fet3, are absent of this second tree. In the 37 genes 112 intron positions were identified. The intron-late theory seems to be more acceptable than the intron-early one supposing that there were 112 different introns in an ancestral laccase gene. Moreover, as we have studied new laccase genes and thus found new intron positions, we may expected to find other new intron positions with the future finding of new laccase genes. Stajich and *al.* [24] stated that an invasion by splicosomal introns took place at the time of fungus-animal ancestor, followed by recurrent intron loss occurring in all fungal clades. In this study we considered that the ancestor of all laccase genes had no intron at all; this constitutes for us the plesiomorphic character. Then introns had invaded laccase genes bit by bit, and then some introns disappeared after gene duplication and after speciation. This phenomenon is well analysed by the Dollo parsimony model in which convergences are excluded but reversions are admitted [25].

The phylogenetic tree of Fig. 3B is globally congruent with the one of Fig. 3A. The ferroxidase clade positioned as outgroup with a bootstrap value of 96%. These two genes share only three intron positions between each other but they share no position with laccase genes. The three principal laccase clades A, B and C, are present, with bootstrap values of 63 and 100% for clade A and B, but a low value for clade C. This was explained by the presence, in others of the eight most parsimonious trees, of clades in which *P. ostreatus poxa3* clustered with the clade A. But two intron positions are common to clade C and absent to clade A: n°42 and 44 (Fig.1). All laccase subfamily A genes from Agaricoid clade VI (*A. bisporus, C. cinerea* and *L. bicolor*) clustered together in Fig. 3A but not in Fig. 3B. Subfamily A laccases of *A. bisporus* had identical intron positions except for *lcc4* where a supplementary intron position was proposed (*lcc4bis*). These *A. bisporus* laccase genes clustered with *C. cinerea* lcc7 as in Fig. 3A but also with Cci_lcc8 which was joined here to Cci_lcc7 and with Lbi_lcc4. All these genes possess intron positions 7 and 11 (see Kilaru *et al.* [3] and Ahlawat and Billette [26] for nomenclature of introns). Here we see an important divergence between the results of the two analyses (Fig. 3) for the position of Cci_lcc8 and Lbi_lcc4, but Lbi_lcc4 clustered with Cci_ lcc7 as previously found in a Neighbour joining analysis done by Courty *et al.* [4]. This similarity reinforces the position of Lbi_lcc4 in the same clade as Cci_lcc7 and Abi_lcc3-4-5-6. Another clade with a bootstrap value of 87% was formed by Cci_lcc3 and five *L. bicolor* genes. This clade was congruent in the two analyses, except for the position of Lbi_lcc4. All the genes of this clade possess intron position 6 and 10. Positions of Lbi_lcc6-7-8 were different in the two analyses. In that case, intron position analysis was not efficient as these genes possess neither intron positions 6,7,10 and 11 which allowed distinguishing the two clades described above. We can name these two clades "the clade of Cci_lcc7" and "the clade of Cci_lcc3". *Volvariella volvacea lcc3*, studied by Ahlawat and Billette [26], also possess intron positions 6 and 10 and belong probably to the Cci_lcc3 clade. So the divergence between these two clades is probably older that Agaricoid clade VI.

Three intron positions are present in two to four *P. ostreatus* laccase subfamily A genes and are absent in all other genes. Consequently, in Fig. 3B, contrary to Fig. 3A, *P. ostreatus* laccase subfamily A genes clustered together (bootstraps value 58%) and outside the cluster formed by Agaricoid clade VI laccase subfamily A genes. This is more congruent with the phylogenetic tree of the species proposed by Matheny [27] or Binder [28] than all already published laccase phylogenetic trees. In Fig. 3B, all Marasmioid (*L. edodes* and *F. velutipes)* genes were outside the clade formed by *P. ostreatus* and Agaricoid laccase subfamily A. This is congruent with Binder's phylogeny of species [28] unlike the corresponding part of the tree in Fig. 3A and laccase trees published earlier.

Moreover *L. edodes* laccase genes were in two clades. In one of them there are *F. velutipes* and *P. rudis* (Polyporales) laccase genes. Four intron positions are shared by *P. rudis*

lac A and *F. velutipes* laccase, three of them are shared with *L. edodes*. As they are present in Agaricales and Polyporales, these positions constitute a plesiomorphic (ancestral) character for these two orders. The position of *P. rudis* lacA inside a clade of Agaricales laccase genes is a problem. This clad emight be constituted by genes that evolved very little. Te presence of few and mostly ancestral introns in Pru_lacA (12 introns) and Fve_laccase (9 introns) might have resulted in a wrong place of Pru_lacA in the phylogenetic tree, unlike in Fig. 3A. This problem is not resolved here.

Identification of a conserved, basidiomycete specifique laccase mutation. Fig. 4 shows a difference of 5 amino acid positions between aligned sequences of basidiomycete laccases and other multicopper oxidases. Very early divergent multi copper oxidases (mco) such as plants LMCO, insect laccases, archae or bacterial mco and two sub-unit mco don't possess most of these 5 amino acids positions. Two exceptions have been found among 240 mco sequences examined, a mco from the Ustilaginomycotina *Malassezia globosa* (XP_001729535) with a much bigger insertion at the same place and a mco from *Rhizopus oryzae* (RO3G_07290.3 at the Broad institute www.broadinstitute.org/annotation/genome/rhizopus_oryzae/MultiHome.html) that has the same 5 amino acid position as basidiomycete laccases, but with no apparent homology. that results probably from an independent mutation. All basidiomycetes *bona fide* laccases actually published possess these 5 amino acids positions with some substitutions and none of ascomycete laccase actually known possess them. Consequently the absence of these five amino acids positions is the plesiomorphic state of this character and the presence of these 5 amino acids positions is a synapomorphy for all *bona fide* basidiomycete laccases. Neither the exact position of the insertion nor the exact number of amino acids inserted (4 or 5) were already known.

The presence of these 5 amino acids results from an insertion of 15 bp in the common ancestor of all Basidiomycetes possessing a laccase gene *sensu stricto, i.e.* all homobasidiomycetes clades. This putative insertion, as it is present in *Thanatephorus cucumeris,* took place before the divergence between chantarelloid fungi, Auriculariales and all other homobasidiomycetes [14]. It is the first time that a structural difference is demonstrated between basidiomycete laccases and all the other laccases, and especially ascomycetes laccases. Some parts of the laccase sequences contain variable indels depending of the gene in the same species or depending of the species: e.g. the substrate binding loops. Nevertheless it's the first time that a difference is detected at the level of a whole fungal clade.

To our knowledge, these amino acid positions have never been described as important for laccase catalytic process, neither for substrate affinity. If this mutation has been conserved in all homobasidiomycetes it can result from the neutral evolution and the genetic drift or it gives a selective advantage. Which type of advantage it may give (consequence on the catalytic site, on the substrate affinity) will have to be looked for. Consequence of this mutation on the structure and function of the protein has to be elucidated. Consequence on the evolution of the protein is also interesting: has the mutation given rise to accelerate evolution in other parts of the protein? The origin and date of the mutation should be elucidated.

The consequence of this mutation, if there is one, on the lignin degradation or on the detoxification of its by-products by basidiomycetes, may give us a new explanation for the different fitness of ascomycetes and basidiomycetes. This discovery could also have an interest for biotechnological applications: e.g. to improve ascomycete laccases. Many hypotheses have now to be tested.

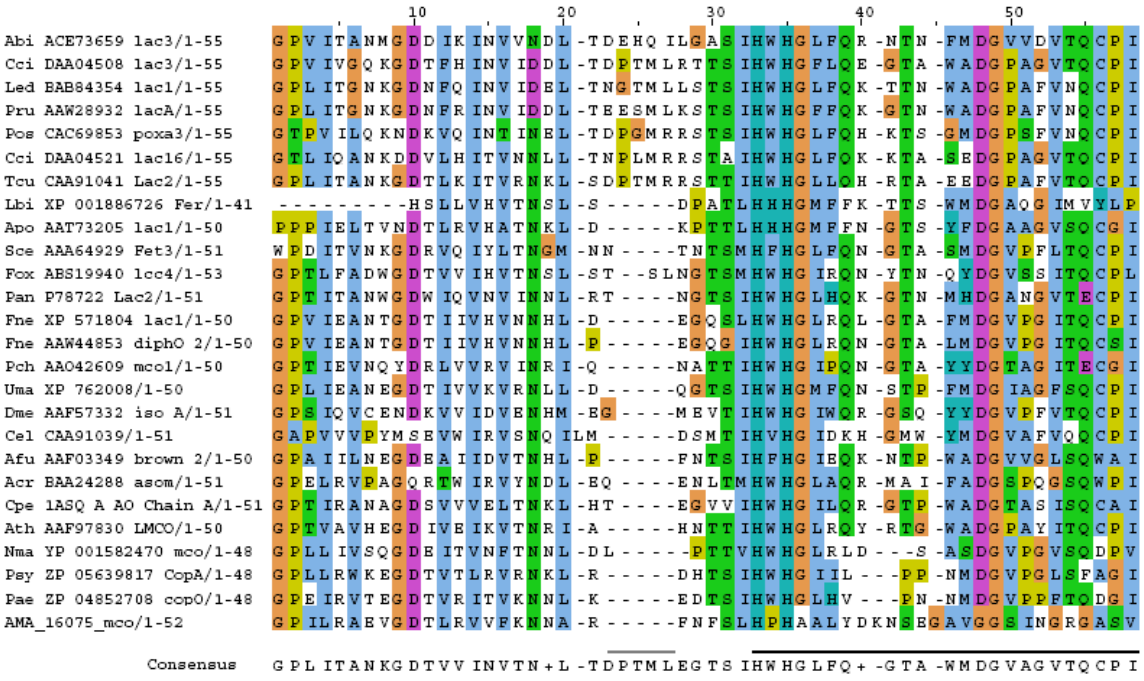
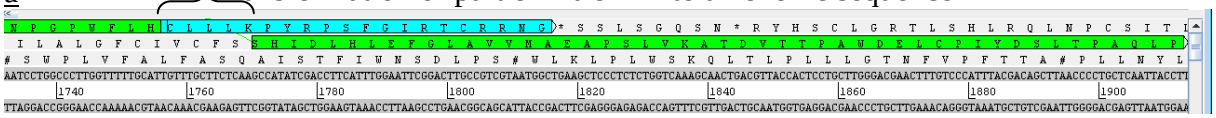


Figure 4 : Alignement of partial sequences of basidiomycete laccases and other multi copper oxidases showing the position of a putative insertion of 5 amino acids in the first ones (grey line) just before the L1 signature of laccases (black line).

Sequence names consist of species code (first letter of genus and first two letters of species name), GenBank protein accession number and protein name (when necessary, laccase is abbreviated to lac and the number used for the gene is added, ferroxidase to Fer, diphenol oxidase to diphO, ascorbate oxidase to AO, copper oxidase to copO, multi copper oxidase to mco) when it exist or gene name. For species name see Table 1 or see below. **Basidiomycetes laccases** sub-family **A** - Abi_lac3, Cci_lac3, Led_lac1, Pru_lacA, sub-family **C** - Pos_poxa3, sub-family **B** - Cci_lac16, **Cantharellales** sub-family -Tcu_lac2 (*Thanatephorus cucumeris*), **ferroxidase** - Lbic_Fer, Apo_lac1, Sce_Fet3, **ascomycete laccases** - Fox_lcc4 (*Fusarium oxysporum*), Pan_Lac2 (*Podospora anserina*) **ferroxidase** - **laccase** - Fne_lac1(*Filobasidiella neoformans*), Fne_diphO, Pch_mco1 (*Phanerochaete chrysosporium*), Uma (*Ustilago maydis*), **insect laccase** - Dme_isoA (*Drosophila melanogaster*), **nematode multi copper oxidase** Cel (*Caenorhabditis elegans*), **fungal pigment** - Afu_brown (*Aspergillus fumigatus*), **ascorbate oxidase** – Acr_asom (*Acremonium sp.*), Cpe_AO (*Cucurbita pepo*), **Plant LMCO** - Ath_LMCO (*Arabidopsis thaliana*), **Archae mco** - Nma_mco (*Nitrosopumilus maritimus*), **Bacteria copper resistance protein** – Psy_CopA (*Pseudomonas syringae*), **Bacteria copper oxidase** – Pae_copO (*Paenibacillus sp.*), **two subunit fungal mco** – AMA_mco (*Allomyces macrogynus* http://www.broadinstitute.org/annotation/genome/multicellularity_project/MultiHome.html)

First time discovery of the presence and absence of three introns in a gene in the same species. We compared some *L. edodes* laccase protein sequences from various origins and available in Genbank database (AAF13037[29], AAF13038[29], BAB83132, BAB84355, BAB83133, AAT99286, ACR24356). These sequences were initially described as different laccases. We observed that all these sequences are probably encoded by different alleles of the same gene *lac1* published first by Zhao *et al.* [29]. However, we observed some differences between the first published sequences [29] and the GenBank sequences. Moreover these GenBank releases were without intron sequences and, probably introns have not been correctly spliced during the annotation. ACR24356 has 99% identity with BAB84355 at the protein level (2 / 533 amino acid differences), but the encoding gene has 3 introns less (10 instead of 13), the three last introns (positions 14, 15 and 16 in Fig. 2). Moreover, BAB83133 don't have the three last introns but had a mutation in intron 14 that leads to disappearance of part of it and to transformation of another part to an exonic sequence, this resulted to a frame shift leading to an early stop codon (Fig 5).

a Transformation of part of intron 14 to an exonic sequence



b

NPGPWFLHCHIDLHLEFGLAVVMAEAPSLVKATDVTTPAWDELCPIYDSLTPAQLP

c

NPGPWFLHCHIDLHLEFGLAVVMAEAPSLVKATDVTTPAWDELCPIYDSLTPAQLP

Figure 5 : Alignment of 3 *Lentinula edodes* lac1 alleles showing two types of mutations affecting introns. In a is presented the BAB83133 protein sequence on the top line (NPGP … RRNG). A mutation in intron 14 leads to disappearance of part of it and to transformation of another part to an exonic sequence. The resulting frame shift stops the protein very early (blue segment). The three first lines correspond to the three reading frames obtained with the gDNA sequence present in the fourth line (Artemis software). In b ACR24356 sequence. In c, BAB84355 sequence with the position of the three introns sharing the gene coding for this sequence**.**

The conservation of the synteny of the flanking genes or the all genome sequencing might confirm that these are alleles of the same gene *lac1*. It is the first time, to our knowledge, that the presence and absence of three introns are found in one gene in the same species. Presence and absence of one intron in the same species have been found very rarely within a living organism [30, 31].

In addition to the interest of this *L. edodes* gene for his splicing variants, it might be very instructive to study it in different strains, as it is an opportunity to study the mechanism of the putative linked insertion or deletion of three introns in a gene. This could help to better understand the mechanism of appearance or disappearance of splicosomal introns which are still not well understood.

CONCLUSION

The identification of a new laccase sub-family (A) in addition to laccase sub-family C in *A. bisporus*, as in *P. ostreatus*, has led us to study intron positions and protein structure of mushroom laccases. Phylogeny based on intron positions compared to phylogeny of species could give complementary information to that based on amino acid sequences. We propose the existence of at least three very old laccase sub-families (A, B and C) in Agaricales and Polyporales in addition of Cantharellales laccase sub-families. The specific laccase roles in each sub family are no yet known but clade specific laccase functions will probably be find in the future. The expression of *lcc1*, *lcc2* and *lcc3* have been studied by Largeteau *et al.* [32] to find if one of them was more expressed on sporophores contaminated by *Leucanicillium fungicola.* None of these genes where over expressed under this condition compared to healthy sporophores. Comparative position of each laccase sub-family on chromosomes and all genome sequences may give confirmations of the phylogenetic hypotheses proposed here, as well as new data. A five amino acid insertion was probably at the origin of all basidiomycetes *bona fide* laccases. We identified a *L. edodes* gene with presence or absence of 3 introns depending of the allele. Studies of intron positions and comparative analysis of protein structure in laccase sub-family in relation to species phylogeny may generate many original data on the evolution of this protein and gene family and also on evolution of introns. New hypotheses have been proposed and are now to be tested.

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BIOBASED ANTIBIOTICS FROM BASIDIOS: A CASE STUDY ON THE IDENTIFICATION AND MANIPULATION OF A GENE CLUSTER INVOLVED IN PLEUROMUTILIN BIOSYNTHESIS FROM *CLITOPILUS PASSECKERIANUS*

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ABSTRACT

With bacteria becoming resistant to antibiotics, there is a growing need to find new sources of antibiotics. Our work has focussed on the organism *Clitopilus passeckerianus* which produces a natural antibiotic, pleuromutilin. Recently, a derivative of pleuromutilin, retapamulin (developed by GSK) was approved for use in humans. Clinical trials have demonstrated its efficacy against certain Gram-positive bacteria including MRSA. We have developed all the tools to manipulate this important organism, and will present results on transformation, gene manipulation and enhancement, as well as gene isolation and mapping. These tools have allowed us to isolate the pleuromutilin gene cluster. Using the molecular tools we have been able to identify all genes involved, their roles, and perhaps most importantly, the ability to manipulate to elevate levels of antibiotic production and deliberately alter products produced. These results demonstrate that we are able to manipulate and control the *Clitopilus* genome. This provides a molecular toolbox which makes it possible to identify and manipulate individual genes of this fungus, and leading to some major new drugs which are not compromised by antibiotic-resistant strains of bacteria. The results will open up major opportunities for other previously intractable systems and antibiotics in fungi.

Keywords: *Clitopilus passeckerianus*; antibiotic; pleuromutilin; diterpene; GGS

INTRODUCTION

The high incidence of antibiotic resistance in clinical isolates of bacteria is making the control of infection more and more difficult. This is illustrated by the frequency with which infections such as those caused by *Staphylococcus aureus* or *Clostridium difficile* are encountered in hospital situations where they can become life-threatening due to the limited range of control agents to which they are susceptible. This has resulted in increased interest in developing antibiotics that demonstrate new modes of action or overcome existing resistance mechanisms.

One such class of antibiotics is the mutilins, which although used in veterinary medicine for many years, had not been developed for human therapeutics. Retapamulin was approved for human use in 2007, and is a semisynthetic derivative of pleuromutilin (Fig. 1), an antibiotic first reported in 1951 from the homobasidiomycete *Pleurotus passeckerianus*, later reclassified as *Clitopilus passeckerianus* [1, 2]. It is a potent and highly selective antibiotic active against a

range of Gram positive bacteria. Pleuromutilin inhibits protein peptidyl transferase by binding to domain V of 23S rRNA on the 50S ribosomal subunit. Whilst other antibiotics also target this region (e.g. carbomycin), they interact with different target nucleotides so there is no cross resistance to currently used antibiotic classes. This has led to the development of many semi-synthetic analogues, such as valnemulin, tiamulin and retapamulin.

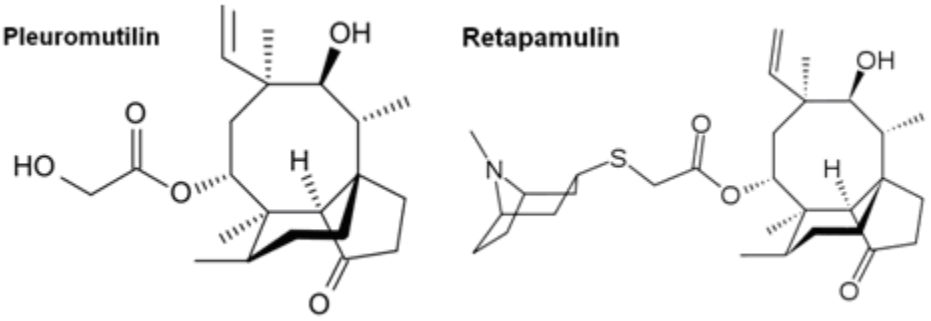

Figure 1: The structure of pleuromutilin and retapamulin.

Whilst retapamulin is only approved for use in creams and other methods of external application, there is ongoing research in a number of pharmaceutical companies seeking to develop derivatives which are suitable for oral administration. Pleuromutilin is a tricyclic diterpene which is commercially produced by fermentation of *C. passeckerianus*, however production is limited due to poor fermenter growth of the fungus, coupled with low titres of the antibiotic. If the present research into orally available derivatives is successful, there may be a substantial demand for the antibiotic, to the extent that production levels could become limiting, or add significantly to the production costs. To date, attempts to increase titre by conventional strain improvement have only met with limited success [3-5], likely due to the dikaryotic nature of the producing organism. It was therefore desirable to develop new methods to increase the titre of antibiotic production within the native host, or to isolate the genes responsible for its production to facilitate pleuromutilin production in a species more amenable to bulk fermentation.

We have recently explored the taxonomy of this group, showing that the various species reported to produce pleuromutilin may in fact be the same on the basis of ITS sequence, with pleuromutilin being made by only a small clade within the Entolomataceae [6, 7]. On the basis of transformation systems established for *Coprinopsis cinerea* and *Agaricus bisporus* [8-10], we then set up methods for transformation of *C. passeckerianus*, using both Agrobacterium and protoplast-based techniques [11]. As is typical in many such basidiomycetes [12], efficient expression of heterologous genes has been shown to need the presence of a 5' intron. We have previously used GFP as a target for establishing silencing in other fungi [13-15] and applied this to *C. passeckerianus* showing that gene silencing could be used to suppress activity of a GFP transgene [11]. We therefore have a set of tools which would allow investigation into the molecular basis of pleuromutilin in this fungus.

In other fungi, it is common for all the genes needed for production of a secondary metabolite to be clustered together within the genome, meaning that if you can identify one key gene, the remaining genes ought to be adjacent. Diterpene pathways have been characterized in several ascomycete fungi, and in most cases they contain a gene encoding a pathway-specific

geranylgeranyldiphosphate synthase. We therefore set out to use this as the basis for isolation of the pleuromutilin biosynthetic pathway. We also sought to further characterize *C. passeckerianus* since improved understanding of its biology and genetics may serve to underpin future development of antibiotic production in this species.

MATERIALS AND METHODS

Strains and growth conditions. *C. passeckerianus* DSMZ1602 was obtained from the Deutsche Sammlung von Microorganism and Zellkulturen and was cultivated on Malt Extract Agar (MEA) at 25^0C.

Pleuromutilin assays. For plate-based bioassays, the fungus was cultured on plates containing 20ml of Tryptic Soy Agar for four days and then overlaid with 5ml agar containing spores of *Bacillus subtilis* ATCC 6633, as detailed in [6]. Colony diameters and clearing zones were measured after an additional 48h incubation at 30^0C. Cultures for HPLC analysis were prepared as in [6]. Briefly, a starter culture of CS01 medium was inoculated with five 4mm plugs from an agar plate and incubated until uniform mycelial slurry was obtained. This was used to inoculate 100 ml of proprietary high production medium and cultured for six days at 25^0C with shaking at 220rpm. Aliquots were then homogenised and mixed with three volumes of acetonitrile before centrifugation to remove matriculate matter. This was then analysed by HPLC as described previously [6].

Classical genetics methods. Fruiting bodies of *C. passeckerianus* were obtained after 28 days incubation of MEA plates at 20^0C under a white light illumination regime of 16h day, 8h night. Fruiting bodies were excised and placed in a Petri-dish to allow spores to be shed. Spores were then harvested in water and diluted appropriately before plating onto MEA to obtain discrete colonies. These were observed by microscopy after 24h and those colonies thought to originate from single spores were identified and sub-cultured. For attempted mating, agar plugs of different lines were placed 5mm apart on MEA plates and incubated for up to 21 days, with frequent observation to note any morphological changes upon mycelial contact. Small-scale DNA extractions and PCR analysis of monokaryons was based on [6] using 30b allele-specific PCR primers designed on derived sequences [16] and with annealing conditions optimised for each primer combination.

Gene isolation and analysis. Genomic DNA was extracted from *C. passeckerianus* as previously described [11] and a lambda genomic library prepared in λGem11 (Promega) by partial digested of gDNA with *Sau*3A, partial fill-in of the ends and ligation into pre-prepared vector following manufacturers recommended methods. The ligation mixture was packaged into phage particles and aliquots were transfected into *E.coli*. Approximately 25000 phages were screened in each round of analysis as outlined in [17]. Positive plaques were subcultured and then insert-containing plasmids excised for subsequent analysis.

Degenerate primers were used to amplify fragments of target genes from *C. passeckerianus* by PCR. The products were gel purified (Promega Wizard) and cloned into pCR2.1topo (Invitrogen). Plasmids were extracted (Qiagen miniprepII) and 10µg used for sequencing with universal primers (Agowa).

For Northern blotting, RNA was isolated using the method of [17]. Gels were electrophoresed, blotted and probed according to [17] using appropriate α^{32}PdCTP-labelled PCR fragments as probes.

Fungal Transformation. Plasmids for fungal transformation were prepared by yeast-based recombination, where individual fragments (either restriction fragments or PCR products) were designed to include 30bp of homology between each piece. These were co-transformed into *S. cerevisiae* Y10000 along with appropriate linearised pYES-based vectors [9, 10] and uracil-independent transformants were selected. Plasmids were extracted from yeast colonies using Zymoprep II (Zymogen) and rescued into *E. coli* prior to confirmation by PCR, restriction digestion or sequencing.

Protoplasts of *C. passeckerianus* were prepared from CS01A starter cultures and transformed as described in [11], with hygromycin-resistant transformants being selected on PDA supplemented with 0.6M sucrose, 100µg/ml hygromycin. These were subjected to three rounds of subculture on PDA with 50µg/ml hygromycin before analysis of pleuromutilin titre.

RESULTS AND DISCUSSION

Isolation of the pleuromutilin pathway. Alignments of the available GGS enzymes identified conserved regions which were suitable for the design of degenerate oligonucleotide primers for a PCR-based gene isolation approach. PCR using genomic DNA from *C. passeckerianus* as the template, yielded a mixed PCR product. This was cloned and sequencing of numerous clones revealed the presence of four different families of products. One of these showed an expression profile on Northern analysis that was closely correlated with the production of pleuromutilin. The other two did not show increased levels during pleuromutilin production and so were assumed to have housekeeping roles or to be involved in other secondary metabolism pathways.

The pleuromutilin-associated GGS region was used to probe a genomic lambda library resulting in the isolation of several clones. Probes derived from these were then used to walk into the library in each direction and eventually a region spanning 32kb was isolated and fully sequenced. Bioinformatic analysis of this region highlighted the presence of not just the GGS gene, but twelve additional genes (Fig. 2).

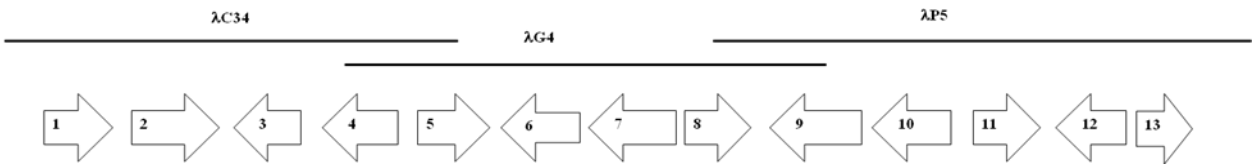

Figure 2: The pleuromutilin locus. ORF 5 is the pleuromutilin associated GGS

In addition to isolation of the suspected pleuromutilin gene cluster other target genes were amplified by degenerate PCR and genomic clones identified and sequence, including α-actin (AAGTACCCCATCGAGCACGG and AAGATGACTCAAATCATGTTCGAGAC), β-tubulin (CGATTCCCTGGTCAACTCAACTC and GTGAACTCCATCTCGTCCAT) to use as controls in expression studies and sources of constitutive strong promoters, and the farnesyl diphosphate

synthase (FDS) which may be involved in substrate supply for the pathway. In all cases, two alleles were isolated for each gene.

Confirmation of involvement in pleuromutilin biosynthesis. Genes 1 and 11-13 were unlikely to be involved in pleuromutilin biosynthesis on the basis of similarity to other known proteins, but genes 2-10 were all similar to those involved in secondary metabolite biosynthesis in other species, many of them with functions predicted to be needed for pleuromutilin biosynthesis. Northern analysis was performed using each of these genes to probe expression levels during induction or repression of pleuromutilin biosynthesis. The northern for the pleuromutilin-associated GGS is shown in Figure 3 and was typical of the induced expression during production. This showed that genes 2-8 were coordinately expressed, with increased titres during pleuromutilin biosynthesis, and so are all likely to be part of the pleuromutilin pathway.

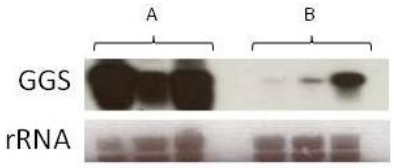

Figure 3: Northern analysis using the candidate pleuromutilin-specific GGS product to probe mRNA from growth conditions with induction (A) and repression (B) of pleuromutilin production at days 3, 4&5, showing induction of expression during pleuromutilin production.

Whilst the gene cluster contained all the genes believed necessary for pleuromutilin biosynthesis, coordinated expression alone was not sufficient proof that this was the correct gene cluster. Therefore gene silencing was used to knock down transcript levels to determine whether this resulted in reduced pleuromutilin titre. Central regions of each gene were amplified using primers designed to allow yeast-based recombination into a suitable vector to express each in an antisense orientation. The vector pYes hph 004cbx [9] was digested with *Xho*I and *Bam*HI to remove the cbx open reading frame and the antisense GGS or other target genes integrated in its place. When transformed into *C. passeckerianus*, approximately 20% of the GGS antisense transformants showed reduced pleuromutilin production as indicated by smaller clearing zones on plate-based bioassay (Fig. 4). Similar results were obtained for some of the other genes believed to be involved in biosynthesis. Where plate-based bioassay indicated reduced titres, this was also observed by HPLC analysis. The reduction in pleuromutilin yields upon this specific gene silencing confirmed that this cluster was indeed responsible for pleuromutilin production.

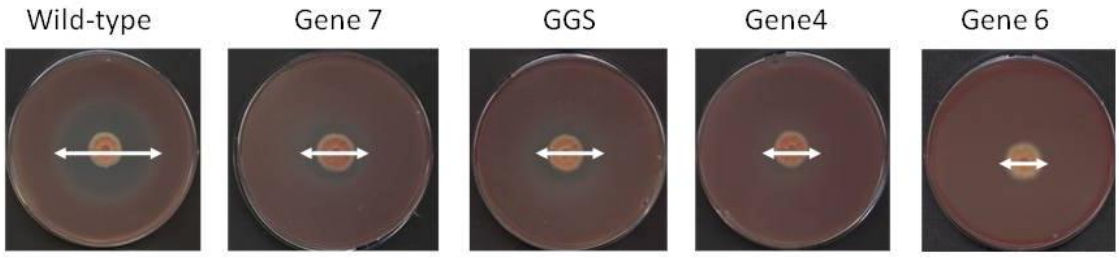

Figure 4: plate-based bioassays showing the reduced titre of pleuromutilin (indicated by arrows) for selected silenced lines.

Reduced titres were not seen for all of the genes analysed, however silencing was not expected to cause a reduction in titre for all the genes, given that some of the later pathway intermediates still have antimicrobial activity. Therefore even if the later stages of the pathway were completely blocked, the accumulating intermediates should still give good clearing zones when assessed by plate-based assay.

Classical genetics on *C. passeckerianus*. The wild-type isolate DSMZ1602 is the strain on which commercial production has been based. During *in vitro* culture we were able to produce fruiting bodies (Fig. 5) which yielded viable basidiospores, indicating that this isolate is a fertile dikaryon as was suspected from the presence of two allelic forms of each gene that had been sequenced. 100 individual lines have been raised from these basidiospores. These strains show variation in growth rates and morphology as would be expected from sexual progeny.

Figure 5: A fruiting body of *Clitopilus passeckerianus*. This is a mature basidiocarp (spore producing), approximately four weeks after plate inoculation. The colouration below the basidiocarp is the brown-pigmented spores.

Allele-specific PCR was performed on 24 progeny to investigate their karyotype. In 20/24 cases these have been confirmed to be monokaryons as determined by the presence of single allelic types for loci including tubulin, actin, FDS as well as various locations within the pleuromutilin cluster, with just four lines giving both patterns suggesting possible dikaryons. Amongst the 20 presumed monokaryon progeny, no recombination events were detected between markers separated by ~11kb within the pleuromutilin gene cluster, indicating that recombination frequencies are low in this region. This low level of recombination means that natural mating and selection may not be a realistic means of strain improvement using alleles within the gene cluster, however it may be a means to alter the genetic background to modify factors such as substrate supply or regulatory processes.

We found that monokaryon lines were able to produce pleuromutilin, often with titres similar to, or only slightly less than the dikaryotic parental strain. These monokaryotic strains were amenable to transformation and to gene silencing. Given that these contain just one nuclear type, such isolates might prove to be more appropriate hosts for genetic modification aimed at strain improvement.

Over-expression of GGS. Whilst gene-silencing showed it was possible to reduce antibiotic titre, thus confirming the involvement of particular genes in pleuromutilin biosynthesis, the overall aim of this research was to produce a strain with an increase in antibiotic yield. The first committed step in pleuromutilin biosynthesis is the pathway-specific GGS, so we set out to over-express this gene in an attempt to increase the rate of entry of precursors into the pathway and hence increase final titre. The GGS cDNA was fused with a 5'intron and placed under the control of the *gpd*II promoter of *A. bisporus*. This promoter plus 5' intron system has previously been show to give high-level expression in various basidiomycetes [11, 12]. The plasmid was transformed into protoplasts of *C. passeckerianus*, and the resulting transformants screened for antibiotic production. Some of the transformants were found to have increased clearing zones on plate-based bioassay. These were then investigated in more detail by fermentation and HPLC. One such transformant, GGS 16 was show to have a 116% increase in titre. This is a dramatic increase in yield, given that GGS is already expressed at comparatively high levels during production as assessed by Northern analysis. This highlights the potential for further increases in titre by manipulation of the other genes in this pathway. It may be that increasing the transcripts of some or all of the pathway could lead to even greater increases in titre, particularly if the growth conditions and media were then optimized for such improved strains.

CONCLUSIONS

By means of PCR with degenerate primers we were able to isolate a GGS-like gene that had an expression pattern corresponding to pleuromutilin production and so was likely to be involved in its biosynthesis. This enabled the cloning, sequencing and characterization of a cluster of genes responsible for the production of the diterpene antibiotic pleuromutilin from *Clitopilus passeckerianus*. Co-localisation of these genes shows that the phenomenon of gene clusters for secondary metabolite pathways does occur in basidiomycetes and could be valuable in isolation such pathways from other fungi.

The wild type fungus is a fertile dikaryon and enabled the isolation of monokaryotic sexual progeny, which displayed a range of phenotypes including variable pleuromutilin production, highlighting that conventional mating might feasibly be a method to increase production, but recombination was rather limited and there may not be sufficient natural variants available to make such a route productive. Gene silencing has proven to be very effective in confirming involvement of several of the genes in pleuromutilin biosynthesis, whilst over-expression has been used to create strains with greatly increased titre. Should pleuromutilin derivatives be developed as oral antibiotics, techniques such as these will be invaluable for making production commercially viable. This highlights that basidiomycete fungi are amenable to strain improvement by means of genetic modification and opens the way for future development of this and other pathways using such methods. The development of the necessary tools for manipulating this species should be appropriate to other basidiomycetes.

ACKNOWLEDGEMENTS

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ANTIOXIDANT ACTIVITIES OF EXTRACTS FROM THE GENUS *PHELLINUS* SPECIES

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ABSTRACT

Ethanolic extracts of fruit bodies of five species (eight strains) of the genus *Phellinus*, and five solvent extracted fractions derived from one of these extracts (*Ph. baumii* PB-10), were evaluated for antioxidant activity and the capacity to protect PC12 cells against H_2O_2-induced oxidative damage. Extracts of all eight strains of *Phellinus* spp. exhibited antioxidant activity and protected PC12 cells against oxidative damage at different magnitudes of potency. Strongest antioxidant activity was exhibited by extracts of *Ph. baumii* PB-10, with recorded IC_{50} values for superoxide radical and hydrogen peroxide scavenging activity of 3.76 μg/mL and 4.24 μg/mL, respectively. Radical scavenging activities, reducing potential and anti-lipid peroxidation capability of five different extracts (petroleum ether, chloroform, ethyl acetate, n-BuOH and ethanol-petroleum phase) derived from *Phellinus baumii* were studied. The results showed the ethyl acetate and n-BuOH extracts exhibited stronger antioxidant capacity. Radical-scavenging activity and protection levels against H_2O_2-induced damage to PC12 cells were highly correlated with the flavonoid content of the extracts and isolated fractions.

KEY WORDS: Species of the genus *Phellinus*, Extracts, Antioxidant activities.

INTRODUCTION

Reactive oxygen species (ROS) are among the major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro* [1]. The triplet state oxygen can react with other molecules to yield ROS such as hydrogen peroxide (H_2O_2), superoxide (O^{2-}), and hydroxyl radicals (OH) [2]. ROS have been associated with the beginning of many diseases and degenerative processes in ageing [3]. Other free radicals, such as DPPH and ABTS, can also lead to the oxidative damage. Natural products with antioxidant activity may be used to help the human to reduce oxidative damage. Many natural antioxidants have already been isolated from different kinds of plant materials [4].

Species of the genus *Phellinus* Quél. (Hymenochaetaceae, Aphyllophoromycetideae, higher Basidiomycetes) have been shown to contain various bioactive substances including triterpenoids, polysaccharides and flavones [5]. *Phellinus* species are believed by traditional Chinese herbalists to be effective in treating many gynecopathic ailments [6]. Much of the research carried out on *Phellinus* in recent years has focused on the chemical composition, structure and pharmacology of polysaccharides and proteoglycans [7-9] derived from mushroom fruit bodies, while the flavone components have received relatively little attention. We have now prepared ethanolic extracts and other solvent extracted fractions rich in flavonoids from the fruit bodies of different *Phellinus* species and measured their antioxidant activities. Our aim is to provide a scientific basis for earlier empirical observations, and to promote the future development of *Phellinus* species as important medicinal mushrooms.

MATERIALS AND METHODS

Materials. Fruit bodies of eight different strains of five species of the genus *Phellinus* were cultivated at the Institute of Edible Fungi (IEF), Shanghai Academy of Agricultural Sciences (SAAS). Strains were obtained from the Herbarium of the Edible Fungi Culture Collection Center, Branch of China Culture Collection Center of Agricultural Microorganisms, and maintained at the Research Center of Medicinal Resources, IEF. Designation and origin of the strains were as follows: *Phellinus baumii* PB-10, (Korea); *P. gilvus* KSH, (Korea); *P. linteus* PLSH, (Korea); *Ph. igniarius* JSH, (Japan); *P. badius* NFSH, (South Africa); *P. igniarius* PI-12, (China); *P. linteus* MYSH, (China); and *Phellinus* sp. CSH, SA06 (China). The designation of CSH was based on our ITS1-ITS4 sequencing data and matching with an identical sequence in GenBank.

Rutin, luminal and nerve growth factor (NGF) were purchased from Sigma-Aldrich. Superoxide dismutase was purchased from LanJi Science and Technology Development Co. Ltd (Shanghai, China), while all other chemicals were from local suppliers and of AR grade.

Preparation of Ethanol extracts and other Solvent Fractions. Fruit bodies of all the studied *Phellinus* strains were cut into small pieces and dried at 50-55 °C for 48 h. Dried material (2000 g) was immersed twice in 10 vols 60% (v/v) ethanol and refluxed at room temperature (~24 ºC) for 24 h. Combined aqueous ethanolic extracts were centrifuged (3,500 g, 10 min, 20 °C) to removed suspended impurities, concentrated by vacuum evaporation and then freeze-dried.

The dried ethanol extracts 60 g was resuspended with 500 ml 20 % ethanol. 1 L of petroleum ether was used to extract the sample for three times. The collected extracts were combined. And then the samples were fractionated successively with 3 L of chloroform, 3 L of ethyl acetate (EtOAc), and 3 L of n-BuOH in the same ways. All the solvent fractions were removed under reduced pressure to be dried. Finally, five different extracts (petroleum ether, chloroform, ethyl acetate, n-BuOH and ethanol-petroleum phase) were collected.

Determination of Flavone Content. Total flavone content of the crude ethanolic extracts was determined by the $NaNO_2$-$Al(NO_3)_3$ colorimetric assay using rutin as the standard [10].

Superoxide Radical Scavenging Activity. Superoxide radical scavenging activity of the crude ethanolic extracts was measured by the chemiluminescence-based method according to Guo and Wang [11]. Test samples (2 μL) dissolved in 70% ethanol to various concentrations (5, 10, 20, 40 and 60 μg/mL) were dispensed into each well of a 96-well plate. K_2HPO_4-KH_2PO_4 buffer (0.05 mol/L) served as the control. Pyrogallol (8 μL, 6.25×10^{-4} mol/L), luminal (50 μL, 1 mM) and sodium carbonate buffer (100 μL 0.05 mol/L) were then added to each well and the intensity of luminescence determined at 0.6 s intervals for a total of 30 s using a Clarity Microplate Luminometer (Bio-Tek, USA). Superoxide radical scavenging activity was calculated using the following equation:

Scavenging ratio (%) = (luminescence value of control - luminescence value of test sample) / luminescence value of control × 100%

IC_{50} refers to the sample concentration when the scavenging ratio was 50%.

Hydroxyl Radical Scavenging Activity. The radical scavenging activity was determined through the CuCl-phenanthroline-H_2O_2 system according to the method established by HongFei

Fu *et al* [12] with some modifications. The Clarity Microplate Luminometer was used in the assay. The mixt composed of 10 μL of sample solution and positive control, 10 μL of a 1.0 mmol/L CuCl solution, 10 μL 1 mmol/L 1,10-phenanthroline solution were added in the 96 microwell plate, and then 10 μL 0.6 % H_2O_2 was added in every cell from pump one and 150 μL Na_2CO_3-$NaHCO_3$ (pH=8.5, 0.05 mol/L) were added from pump two to process the reaction. The chemiluminescence intensity was recorded with a program in the processor once every 6 s and was kept 30 s. 70 % ethanol was used as a control. Hydroxyl radical scavenging activity was calculated using the following equation:

Scavenging ratio (%) = (luminescence value of control - luminescence value of test sample) / luminescence value of control × 100%

IC_{50} refers to the sample concentration when the scavenging ratio was 50%.

Hydrogen Peroxide Scavenging Activity. Hydrogen peroxide scavenging activity of the crude ethanolic extracts was measured by the chemiluminescence-based method according to Qin *et al* [13]. Aliquots of H_2O_2 (10 μL) were first dispensed into each well of a 96-well plate. Test samples (10 μL) dissolved in 70% ethanol to various concentrations (2, 5, 10, 20, 40, 60 and 80 μg/mL), luminol and sodium carbonate buffer solution (150 μL) were then added to each well. Double distilled water served as the control. The intensity of luminescence was read every 0.6 s for 30 s and the scavenging ratio value for each sample was calculated as follows:

Scavenging ratio (%) = (luminescence value of control - luminescence value of test sample) / luminescence value of control × 100%

IC_{50} refers to the sample concentration when the scavenging ratio was 50%.

DPPH Radical Scavenging Activity. The scavenging activity of the DPPH free radical was assayed according to the method of Brand-Williams *et al* [14] with slight modification. 100 μL of various concentrations of five different phases samples were added to 100 μL of 6×10^{-4} mol/L DPPH· radical solution mixed with 900 μL of 70 % ethanol (final concentration of DPPH· was 6×10^{-5} mol/L). The mixture was shaken and stood at 25 °C for 20 min; the absorbance of the solution was measured at 517 nm. 100 μL of 70 % ethanol was used as a control. The radical scavenging capacity of the tested samples was measured as a decrease in the absorbance of DPPH· radical and was calculated by using the following equation [15]:

Inhibition (%) = $[(A_{control}-A_{sample})/ A_{control}]\times100$

Reducing Potential. The reducing power was described by a modified method of Neeraj Mishra *et al* [16] and use of ascorbic acid as a standard. 50 μL different concentration of samples were mixed with 500 μL of 0.2 mol/L sodium phosphate buffer (pH=6.6) and 500 μL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After 500 μL of 10% trichloroacetic acid were added, the mixture was centrifuged at 4000 g for 10 min. The upper layer (500 μL) was mixed with 400 μL of distilled water and 100 μL of 0.1% of ferric chloride and the absorbance of the solutions at 700 nm was then measured. The absorbance is proportional to reducing power.

Anti-Lipid Peroxidation Capability. Anti-lipid peroxidation capability was assayed as described previously (Yu Cao and Isao Ikeda [17]) with minor modifications. The fresh rats' hepatic tissue were dissected and homogenized in 4 °C PBS (20mM, pH 7.4) to produce a 10% homogenate (v/v). The homogenate was mixed with 20 μL $FeSO_4$ (6 mmol) and 20 μL H_2O_2 (60 mmol). The mixture was incubated with the test samples in the presence of sample at 37 ◦C for 1.5 h. The reaction was stopped by addition of 200 μL trichloroacetic acid (TCA, 20%, w/v). The

mixture was centrifuged at 4000 g for 10 min to remove precipitation. 500 µL thiobarbituric acid (TBA, 1%, w/v) added to 300 µL supernatant were heated at 100 °C for 15 min. After centrifugation at 4000 g for 10 min, the absorbance (A_1) was measured at the wavelength of 532 nm. A_0 was measured by using 70 % ethanol as the control. The inhibition of lipid peroxidation was determined by quantification of MDA. The inhibitory rate was obtained according to the formula [18]:

Rate of inhibition of lipid peroxidation (%) = $(1-A_1/A_0)\times100$

Protection of PC12 Cells against Oxidative Damage. PC12 cells were grown in DMEM culture medium and harvested during the logarithmic phase by centrifugation after culturing at 37 °C under 5% CO_2. Cell pellets were resuspended in sterile culture medium to a concentration of 2×10^4 cells/mL and 190 µL of this suspension were added to each well of a 96-well plate. Ten µL H_2O_2 (0.75%) were then added to each well and, after standing under the test conditions for 4 h, 199 µL DMEM culture medium were added to each well followed by 1 µL sample of various concentrations (10 µg/L, 50 µg/L, and 150 µg/L). Controls consisted of a normal control group (without H_2O_2), a H_2O_2 group (without sample) and a positive control group (1 µL 100 µg/L NGF). Well plates were incubated at 37 ºC in a 5% CO_2 atmosphere for 48 h after which aliquots (20 µL) of Alamar Blue reagent (Biosource Nivelles, Belgium) were added to each well and the incubation continued for a further 12 h. Changes in extinction values at 570nm and 600nm were measured using a micro ELISA autoreader **(**Bio-Tek Instruments, Winooski, VT, USA).

Cell viabilities were calculated according to the Biosource protocol:

Cell viability (%) = $[117216 \times A_{\lambda570}\,(\text{sample}) - 80856 \times A_{\lambda600}\,(\text{sample})]/$
$[117216 \times A_{\lambda570}\,(\text{control}) - 80856 \times A_{\lambda600}\,(\text{control})] \times100$

Statistical Analysis. All data were given as means±standard deviation (SD). Comparisons between the means of various treatment groups were analyzed using SPSS 13.0 followed by analysis of variance (ANOVA). $P < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Flavone Content of Ethanolic Extracts. Large variations were observed in the flavone content of the eight *Phellinus* ethanolic extracts (Table 1).

Table 1: Flavonoid Content of Different *Phellinus* Strains

Strain	Yield of ethanol extract	Total flavone content of ethanolic extract	Total flavone content of fruit body
PB-10	26.5 ± 4.9	59.6 ± 2.1	15.8 ± 1.9
PI-12	20.2 ± 7.1	30.4 ± 1.2	6.1 ± 2.7
NFSH	15.1 ± 5.4	30.4 ± 2.7	4.6 ± 2.3
MYSH	17.1 ± 4.7	28.0 ± 1.2	4.8 ± 0.6
CSH	20.0 ± 1.7	32.1 ± 1.5	6.4 ± 3.5
JSH	18.1 ± 7.2	22.4 ± 0.8	4.1 ± 4.3
KSH	17.7 ± 1.8	28.0 ± 2.3	4.9 ± 2.6
PLSH	6.9 ± 3.9	42.5 ± 0.9	2.9 ± 1.7

Values are expressed as % dry weight and represent the mean ±S.D (n = 3)

Highest extract yields (26.5%) and flavone levels (59.6%) were recorded in *P. baumii* PB-10. The flavone content of *P. linteus* PLSH was higher than the remaining samples (up to 42.5%), but the yield of ethanolic extract was the lowest (6.9%). Fruit bodies of *P. baumii* PB-10 had the highest total flavone content (15.8%), and ethanolic extracts of this strain were subjected to further fractionation.

Radical Scavenging Activities of Ethanolic Extracts From different *Phellinus* Fruiting bodies. Ethanolic extracts of *P. baumii* PB-10 exhibited the strongest antioxidant activity with IC_{50} values for superoxide radical and hydrogen peroxide scavenging activity of 3.76 μg/mL and 4.24 μg/mL, respectively (Table 2). Antioxidant activity of *P. linteus* PLSH extracts was also strong with corresponding IC_{50}-values of 5.94 μg/mL and 7.88 μg/mL, whereas IC_{50} values for the other samples were significantly higher. The stronger radical scavenging activity of *P. baumii* PB-10 and *P. linteus* PLSH extracts correlated well with the higher flavone contents and suggested that flavones were the main antioxidants in *Phellinus* fruiting bodies.

Table 2: *In vitro* Antioxidant Activity of Ethanolic Extracts of Different *Phellinus* Strains

Strain/Sample	Scavenging activity	
	Superoxide	H_2O_2
SOD	0.02 ± 0.01^{a}	
Vitamin C		14.2 ± 1.1^{d}
PB-10	6.9 ± 0.7^{b}	5.0 ± 0.4^{a}
PI-12	7.3 ± 0.6^{b}	14.7 ± 0.3^{d}
NFSH	9.4 ± 1.3^{c}	14.6 ± 0.5^{d}
MYSH	8.9 ± 0.9^{b}	16.4 ± 0.7^{e}
CSH	7.2 ± 1.1^{b}	10.9 ± 0.8^{c}
JSH	9.7 ± 2.1^{c}	14.9 ± 1.2^{d}
KSH	8.7 ± 1.6^{c}	12.9 ± 1.4^{c}
PLSH	5.9 ± 0.8^{b}	7.9 ± 1.7^{b}

All values are the mean (n = 3) IC_{50} values (μg/mL) ±S.D. Superoxide dismutase (SOD) and vitamin C served as positive controls. Significant differences determined by the Tukey HSD test ($P<0.05$) are indicated by different letters (a-e).

Table 3: Radical-scavenging activities of extracts of *Phellinus baumii*

Extract standard antioxidants	Total flavone content (% dry wt)	IC50 (μg/ml)			
		Superoxide anion radical scavenging	Hydroxyl radical scavenging	Hydrogen peroxide scavenging	DPPH radical scavenging
Petroleum ether	1.78±1.12	max	1.16±1.37	max	2870.13±5.59
Chloroform	27.04±3.01	25.91±0.75	0.12±0.01	2.49±0.21	14.77±0.21
Ethyl acetate	89.61±1.32	2.98±0.01	0.041±0.001	0.24±0.07	3.89±0.02
n-BuOH	62.01±0.98	4.39±0.11	0.13±0.01	0.38±0.02	5.12±0.04
Ethanol petroleum	9.55±2.34	26.11±2.21	0.41±0.03	225.30±12.4	27.14±0.42
Vc		7.54±0.07	/	/	/
BHA		/	0.021±0.03	/	/
catechin		/	/	0.11±0.04	4.32±0.09

Max stands for too huge to count. Values are means ± SD of three determinations. Vc, BHA, catechin were used as positive compounds to compare with five different extracts.

Radical Scavenging Activities of Different Solvent Fractions from *Ph. baumii* PB-10. The ethyl acetate fraction showed the most potent scavenging ROS radical activities (Table 3) and indicated that compounds with strongest radical-scavenging activity in this species are of medium polarity. The Petroleum ether fraction had the lowest antioxidant capacity, even the IC50 values on superoxide anion radical scavenging and hydrogen peroxide scavenging were too huge to count. It illustrates the petroleum ether fraction has little antioxidant capacity. Except for Hydroxyl radical scavenging, the n-BuOH fraction showed higher antioxidant capacity than the chloroform fraction. The ethyl acetate fraction and the n-BuOH fraction had a higher flavone content and a lower IC_{50} value than the other extracts (Table 3), further supporting a link between flavone constituents and antioxidant activity.

Reducing potential. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [19]. The reducing power of five different extracts increased with high concentration (Fig 1). The ethyl acetate and n-BuOH extracts exhibited strongest ability among five different extracts. The petroleum ether phase fraction had the lowest reducing potential.

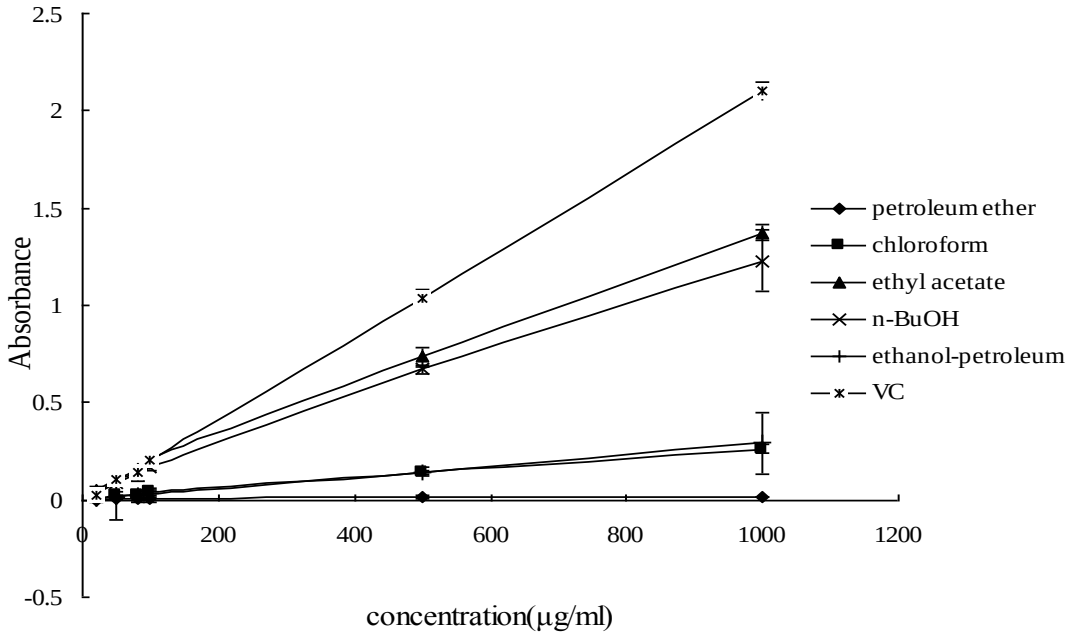

Figure 1: Reducing potential of different extracts from *Phellinus baumii*
Values are means±SD of three determinations, Vc were used as positive compounds

Anti-lipid peroxidation capability. The initiation of lipid peroxidation is carried out mostly by free radicals, such as superoxide, hydroxyl radicals and other reactive oxygen species. Lipid peroxidation causes cellular injury by inactivation of the enzymes and receptors in membrane, and depolymerization of DNA/RNA as well as protein cross-linking and fragmentation [17]. The ethyl acetate extracts exhibited strongest anti-lipid peroxidation capability（Fig 2）and followed by the chloroform extracts. Anti-lipid peroxidation capability of five different extracts increased with high concentration.

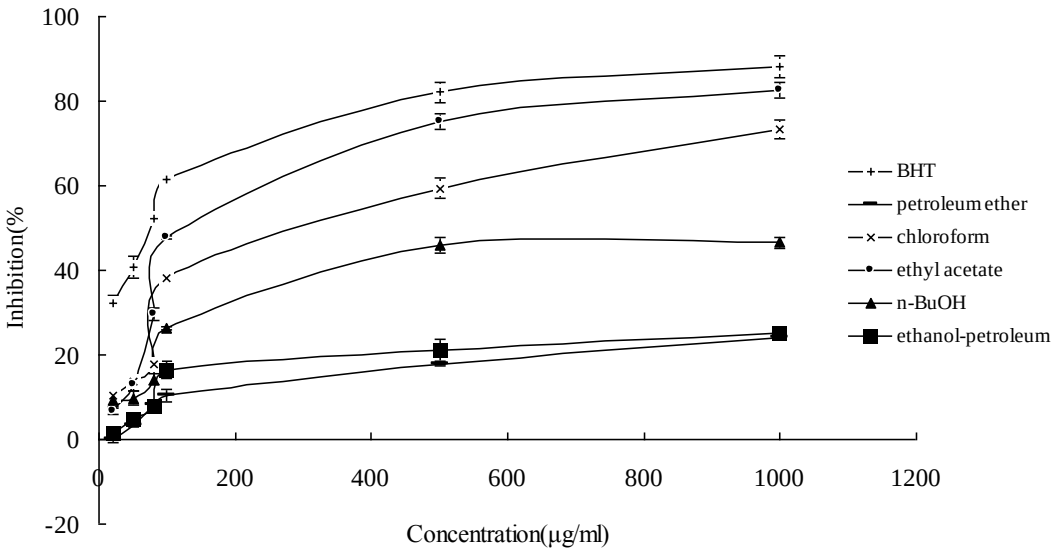

Figure 2: Anti-lipid peroxidation capability of *Phellinus baumii* Values are means±SD of three determinations

Protection of PC12 Cells against Oxidative Damage. Ethanolic extracts of all eight *Phellinus* strains protected PC12 cells against H_2O_2-induced oxidative damage in a dose-dependent manner (Table 4). Highest cell survival rates, 72.47 ±0.3% at low concentrations (10 μg/mL) and 90.73 ±3.6% at high concentrations (150 μg/mL), were recorded with ethanolic extracts of *P. baumii* PB-10. Ethyl acetate and n-BuOH extracts derived from ethanolic extracts of *P. baumii* PB-10, also protected PC12 cells against H_2O_2-induced oxidative damage in a dose-dependent manner (Fig. 3). In addition to involvement of direct free radical scavenging activity, a reduction in free radical generation may also contribute to the protective action of ethanolic extracts [20].

Table 4: Protective Effect of Ethanolic Extracts of Different *Phellinus* Strains Against H_2O_2-induced Oxidative Damage to PC12 Cells

Strain/Sample	**Cell viability (%) at different sample concentrations**		
	10 μg/mL	**50 μg/mL**	**150 μg/mL**
PB-10	72.47 ± 0.3^{b}	80.10 ± 1.7^{a}	90.73 ± 3.6^{a}
PI-12	57.10 ± 1.8^{c}	72.15 ± 1.8^{b}	81.14 ± 1.3^{c}
NFSH	55.77 ± 3.5^{c}	72.89 ± 2.1^{b}	83.06 ± 2.9^{c}
MYSH	56.43 ± 0.5^{c}	69.88 ± 1.2^{b}	81.11 ± 2.3^{c}
CSH	64.01 ± 1.6^{b}	71.10 ± 0.7^{b}	80.67 ± 2.5^{d}
JSH	51.80 ± 1.3^{d}	63.64 ± 3.1^{c}	70.33 ± 3.0^{e}
KSH	59.40 ± 6.7^{c}	67.83 ± 2.2^{b}	86.03 ± 2.8^{b}
PLSH	68.87 ± 2.9^{b}	75.30 ± 6.4^{a}	85.56 ± 2.8^{b}
Control		100.00 ± 6.7	
H_2O_2		$44.17 \pm 2.8^{*}$	
NGF （100 μg/mL）		93.52 ± 2.8^{a}	

All values are the mean (n = 3) ±S.D. NGF served as the positive control. Significant differences determined by the Tukey HSD test ($P<0.05$) are indicated by different letters (a-e) in each concentration column; *compared with control group: $p<0.01$.

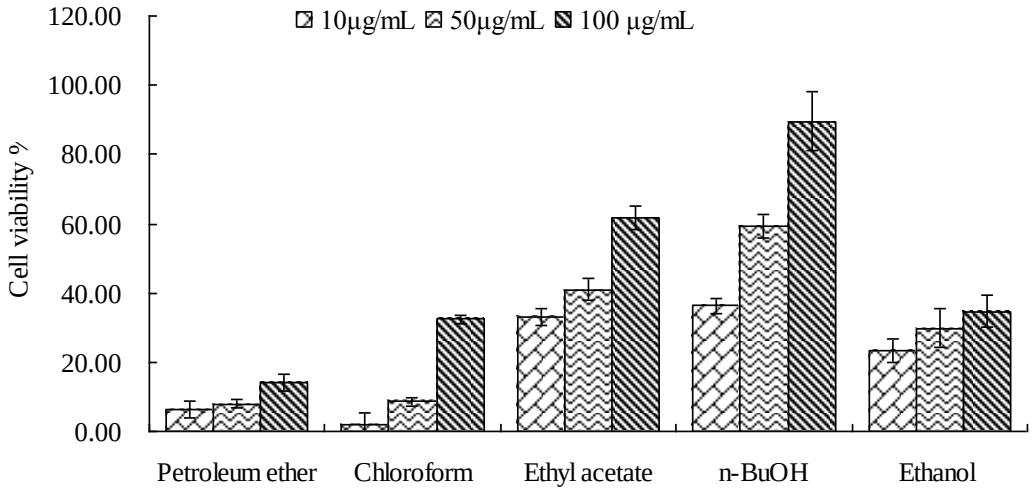

Figure 3: Protection of PC12 cells against H_2O_2-induced oxidative damage by extracted fractions from *Phellinus baumii* PB-10 fruit bodies.

Conclusion

Oxidative damage involving reactive oxygen species (ROS) and other free radicals are involved in a variety of pathological events. Excessive generation of ROS can damage proteins, carbohydrates, polyunsaturated fatty acids, and DNA, thereby leading to oxidative stress and to a variety of degenerative processes and diseases such as ageing, neurological disorders, inflammation, atherosclerosis, coronary heart disease and certain cancers [21-23].

Of the strains examined in our study, PB-10 (*P. baumii*) appeared to have the highest potential value for antioxidant and anti-aging. Our results showed the ethyl acetate and n-BuOH extracts derived from *Ph. Baumii* ethanol extract exhibited stronger antioxidant capacity. This order is similar to the total flavonoids contents of the extracts. Several published reports [24-26] showed the radical-scavenging activity of extracts could be related to the nature of phenolics, thus contributing to their electron transfer/hydrogen donating ability. Further works should be performed on the isolation and identification of the components in the extracts, especially in the ethyl acetate and n-BuOH extracts. In addition, further investigation of individual flavonoid compounds, their *in vivo* antioxidant activity and the different antioxidant mechanisms should be studied.

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SELENIUM-ENRICHED POLYSACCHARIDE FRACTION ISOLATED FROM MYCELIAL CULTURE OF *LENTINULA EDODES* (BERK.) – PRELIMINARY ANALYSIS OF THE STRUCTURE AND BIOLOGICAL ACTIVITY

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ABSTRACT

We hypothesized that enriched in selenium polysaccharide fraction extracted from *Lentinula edodes* would possess higher biological activity than non-enriched, currently used to treat cancer. Se-enriched exopolysaccharide fraction was isolated from *L. edodes* mycelium, cultivated under submerged conditions in selenium-enriched medium. The monosaccharide composition of selenated polysaccharide, the amino acid content and the total concentration of Se was determined by reversed-phase high-performance liquid chromatography (RP HPLC) methods. X-ray absorption spectroscopy (XAFS) was used to probe the oxidation state and chemical speciation of selenium. The molar weight of Se-enriched polysaccharide fraction was determined by gel permeation chromatography (GPC). IR and NMR spectra were used to determine the type of glycosidic bonds. Se-enriched polysaccharide was tested for its antioxidant, cytotoxic and immunomodulating properties. All results regarding Se-enriched fraction were compared with that of reference polysaccharide fraction, extracted from mycelium not enriched in Se.

Concentration of selenium in tested polysaccharide was 67μg/g. Se-enriched fraction contained mainly glucose (89%) and mannose (8%). The molecular weight of the polysaccharide was 200 KDa; it contained 3% of protein. The type of glycosidic bounds was mainly β. XAFS analysis showed that the degree of Se oxidation in the polysaccharide was equal to –II and 0.

The comparison of cytotoxic profiles of tested fractions revealed that polysaccharide was not toxic toward HeLa (cervix carcinoma) and HUVEC (normal) cells. However, the Se-enriched mycelial polysaccharide fraction of high molecular weight significantly enhanced viability of cells; that may be an effect of the enhanced antioxidant activity. When assayed at concentrations 1-100 ug/ml, Se-enriched fraction caused no activation of lymphocytes T and B

Keywords: *Lentinula edodes*, selenium, exopolysaccharides, biological activity

INTRODUCTION

Lentinula edodes (Berk.) Pegler, known commonly as shiitake mushroom, is one of the medicinal mushrooms postulated to exert highly potent enhancement of the immune system. The significant antitumor activity of *lentinan* and other pharmacologically-active polysaccharides and polysaccharide-protein complexes contained in shiitake extracts results from activation of the host immune system. The mechanism by which selenium – one of the trace elements of fundamental importance to human health - exerts anticancer and immunomodulating activity differs from that of *L. edodes* polysaccharide fractions, but a similar pharmacological effect suggests a possible synergism of these two agents. We hypothesized, therefore, that high concentrations of selenium in mycelial biomass would enhance the antioxidant and immunomodulating activity of mushroom mycelial extracts.

In a previous study, we demonstrated that *L. edodes* mycelium effectively accumulated Se from the cultivation medium [1, 2]. We found, that selenium is also incorporated into the mycelial polysaccharides [3, 6].

Thus, the present study deals with the isolation, structural analysis and examination of cytotoxic, antioxydant and immunomudulating activity polysaccharide-protein complexes isolated from the *L. edodes* Se-enriched and not enriched mycelial biomass.

MATERIALS AND METHODS

Microorganism and cultivation media. The *Lentinula edodes* (Berk.) Pegler strain used in this study was ATCC 48085. The mycelial cultures were grown under the same conditions described in our previous reports [2, 4]. The fermentation medium was Se enriched to a concentration of 20 μg/ml by the addition of sodium selenite (Na_2SeO_3, Sigma, Cell Culture Tested).

Extraction and isolation of Se-enriched exopolysaccharide fraction. Se-enriched polysaccharide fraction was isolated from the mycelial biomass by use of Chihara method [5]. Reference fraction was extracted from not enriched in Se mycelium *of L. edodes*.

Structural analysis of polysaccharide fractions.

RP-HPLC determination of monosaccharide composition. A freeze-dried sample of polysaccharide fraction was hydrolyzed with 3M TFA at 120^{o}C for 5 hrs. Samples were evaporated and neutalized. Monosaccharide composition was determined by use of RP HPLC method described in our previous paper [6].

RP-HPLC determination of Se. RP HPLC method of selenium determination after precolumn derivatization, described in our previous reports was used [2, 4].

RP-HPLC determination of amino acids. Amino acids were determined in hydrolysates of the polysaccharide dry weight, by high performance liquid chromatography of o-phthaldialdehyde derivatives (OPA method).

IR and NMR spectral analysis. The IR spectrum was recorded with a Nicolet Fourier transform infrared (FTIR) spectrometer (Shimadzu). Test specimens were prepared by the KBr-disk method.

NMR Analysis. ^{1}H-NMR spectra were determined at room temperature. Fractions were dissolved in 6% NaOD solution in D_2O.^{13}C-NMR spectra were determined in solid state by use of CP MAS technic. Bruker DMX400 WB instruments was used.

X-ray absorption spectroscopy (XAFS). XAFS analysis was performed according to the method described by Lee *et al* [7].

Tests of biological activity of polysaccharide fractions. Cells viability assay was performed by MTT test The cytotoxicity of all compounds was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Briefly, after 24 h or 48 h of incubation with polysaccharides, the cells were treated with the MTT reagent and incubation was continued for 2 h. MTT-formazan crystals were dissolved in 20 % SDS and 50 % DMF at pH 4.7 and absorbance was read at 562 and 630 nm on an ELISA-PLATE READER (ELX800, Bio-Tek, USA). As a control (100 % viability), cultured cells treated with vehicle (saline or DMSO) were used. The DMSO concentration in cell cultures didn't exceed 2%.
Antioxidant activity of polysaccharides in cell cultures. The HeLa cells grown as above were pre-incubated with polysaccharides or crude extracts from *L. edodes* (at the final concentration of 50 or 200µg/ml, respectively) for 30 minutes. Following this incubation, H_2O_2 was added to the cells (final concentration 100µM or 300µM) for 24 hours. Cell viability was determined as above using MTT assay.
Tests of immunomodulating activity of polysaccharides.
Cells isolation and L. edodes fractions preparation. Mononuclear cells (MNC) and granulocytes were isolated from peripheral blood of healthy blood donors by centrifugation in ficoll gradient (1077 g/l for MNC and 1119 g/l for granulocytes) and then were counted in Turk medium in Bűrker counting chamber. MNC were suspended in Parker medium with addition of L-glutamine (2mM), 3-β-mercaptoethanol, Hepes (0,23%), fetal bovine serum (FBS, 10%) and gentamycin (0,1 mg/ml), and granulocytes in PBS containing glucose (0,036%) and bovine serum albumin (BSA, 0,1%). Fractions of *L. edodes* monosaccharide were diluted in 0,9%NaCl to 0,1-0,001 mg/ml concentration.
Proliferation assay. Lymphocytes cultures were established in 96- well flat-bottom microplate (concentration 1×106 cells/well) and induced with specific mitogen: anti-CD3 mAb (OKT3, 1µg/ml), phytohemoagglutinin (PHA,20 µg/ml) and suspension of *Staphylococcus aureus Cowan* strain (0,004% w/v). *L. edodes* monosaccharide fractions were added in a proportion of 100µl of prepared dilution per well. Control cultures contained an equivalent amount of NaCl. Lymphocytes were cultured for 3 days at 37°C in a humidified atmosphere with 5% CO2, then 17 hours more with tritiated thymidine and then harvested. Lymphocytes DNA, with incorporated thymidine, was transferred onto a paper filters and read off in scintillation counter.

RESULTS AND DISCUSSION

Structure of the polysaccharides. Total selenium amount in the *L. edodes* mycelium cultivated in medium enriched with 20 ppm Se medium was 748 µg/g. Approximately 13% of total mycelial selenium was combined to the polysaccharide fraction. Concentration of selenium in the isolated polysaccharide fraction corresponding to the Japanese drug *Lentinan* was 67µg/g.
Examined Se-enriched polysaccharide fraction contained mainly glucose (89%) and mannose (8%). Monosaccharide composition of reference fraction not enriched in selenium was similar. Isolated fraction contained 3% of protein. Main amino acids present in the protein component of the isolated fraction were lysine, arginine, serine, glutamic acid and glycine. The IR and NMR spectra confirm the type of glycosidic bounds was mainly β. The samples exhibited

characteristic absorption at 890 cm^{-1} for the β-configuration. Assignment of the carbon-13 NMR spectra of polysaccharide fractions was made by comparison with previously published spectra [8]. The anomeric carbon signal apparent around 103 ppm was assignable as a β –configuration of glucan. XAFS spectra suggest the selenium in the isolated Se-exopolysaccharide is present at –II and 0 oxidative stage. Important problem to solve is the way the selenium is combined to the polysaccharide structure. The continuation of examination of the structure of isolated Se-polysaccharides is in progress.

Biological activity of the polysaccharides. In general isolated EPS fractions (selenated and not selenated) displaied no cytotoxic activity. Based on the dose-response curves, the IC_{50} values were determined. The IC value for Se-exopolysaccharide fraction was >25 whereas it was >50 for not enriched in selenium exopolysaccharide. The comparison of cytotoxic profiles shows that the polysaccharides are not toxic toward HUVEC or HeLa cells. Cell viability in the presence of both, selenated and not selenated, fractions was higher than the control. It could be the result of the antioxidant activity of tested fractions, so the test of the protection of cells against oxidative stress by polysaccharide fractions was carried out.

Antioxidant activity of polysaccharides in cell cultures was expressed by the higher cells viability after H_2O_2 exposure. Both tested exopolysaccharide fractions displayed antioxidant activity. Cell viability in the presence of the selenated polysaccharide was approximately two times higher than with not selenated polysaccharides. This is particularly evident for cells exposed to 300μM H_2O_2 (Fig1).

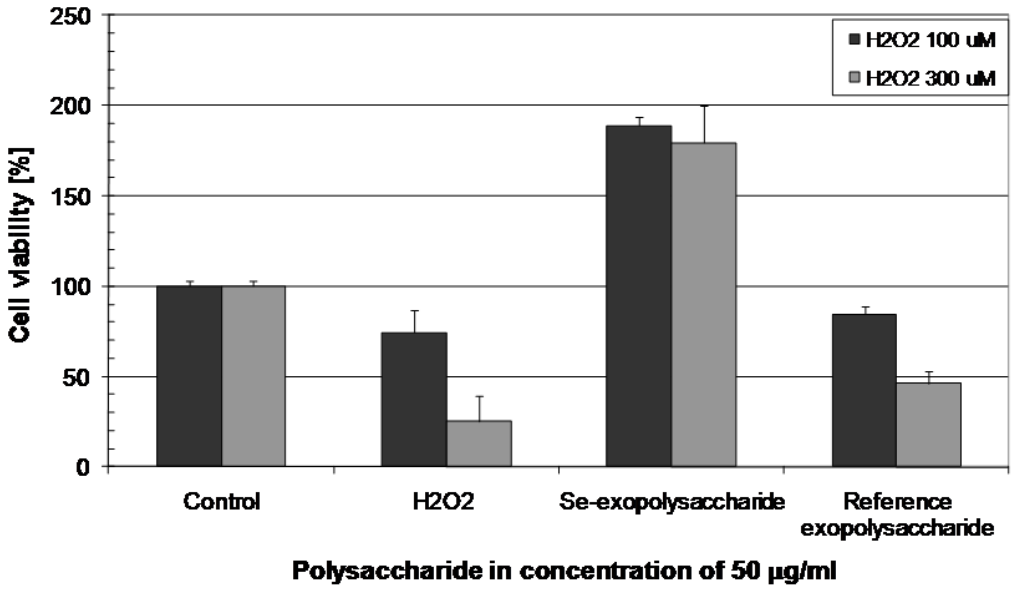

Fig.1 :Anti-oxidative properties of selenated and not selenated exopolysaccharides in HeLa cell cultures exposed to H_2O_2 to induce the oxidative stress (24h)

These results strongly suggest the presence of selenium in polysaccharide enhance it's antioxidative properties.

When assayed *in vitro*, *L. edodes* fractions in concentrations 1-100 ug/ml caused no stimulation of human T and B lymphocyte activation, even immunosuppressive activity was observed.

Selenium-enriched polysaccharide fraction display higher immunomodulating activity than not enriched fraction.

CONCLUSIONS

Selenium- enriched exopolysaccharide fractions isolated from *L. edodes* mycelium cultivated in selenium enriched medium show interesting biological activities, not characteristic for mushroom polysaccharides. The continuation of the structural and biological activity tests is necessary.

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CHANGES IN ANTIOXIDANT ACTIVITIES AND COMPOUNDS DURING CULTIVATION OF SHIITAKE (*LENTINULA EDODES*)

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ABSTRACT

Mushrooms contain a variety of secondary metabolites, including various phenolic compounds, which have been shown to act as excellent antioxidants. Recently, a specific antioxidant, ergothioneine (ERG) has been identified in various genera of mushrooms, including shiitake (*Lentinula edodes*). Shiitake is the second most highly consumed mushroom in the world, and present several functional properties, such as antitumor, hypocholesterolemic, antioxidant and antimicrobial potentials that have been intensively investigated. Although research was focused on its therapeutic effects, little information is available about the compounds responsible of the antioxidant properties. Our objectives were to identify changes in quality of shiitake as antioxidant source during cultivation cycles on oak woodships. The antioxidant activities and contents of potential antioxidant components, including total phenolic (TP) compounds and ERG, were analyzed in two parts of mushrooms (stipe and cap) from the successive flushes. In addition, the seasonal variations were studied. The Folin-Ciocalteu reagent was used to quantify the TP, and ERG was quantified by HPLC-DAD. The antioxidant activity was measured using the ORAC assay. Our analysis revealed only minor seasonal variations in TP and ERG contents as well as the ORAC value, excepted during the winter where these contents were 50% higher in the caps. Regarding the age of the culture, we observed a progressive decrease in the contents of TP and ERG all along the flushes, whatever the part of the mushroom. Surprisingly, the ORAC value increased in the whole mushrooms, whereas it decreased in the caps and strongly increased in the stipes. We concluded that the antioxidant activities could be largely dependent on other molecules than ERG, perhaps specific phenolic compounds. Having established the main variations in the antioxidant activity of shiitake, the chemical characteristics of the antioxidative components will be now further investigated. This study could provide valuable new opportunities for mushroom growers, since shiitake can serve as a good source of antioxidants in the human diet.

Keywords: *Lentinula edodes*; Shiitake; Antioxidants; Ergothioneine; Phenolic compounds

INTRODUCTION

Shiitake, *Lentinula edodes* (Berk) Pegler, is the second largest cultivated and most popular edible mushroom in the world, comprising about 25% worldwide production. Shiitake mushroom contains several therapeutic actions such as antioxidant, antitumoral and antimicrobial properties, carried by the diversity of its components. Shiitake mushrooms are a very good source of three key antioxidant minerals: manganese, selenium and zinc. They also contain some

unusual phytonutrient antioxidants. One of the best studied is ergothioneine (ERG). This antioxidant is derived from the amino acid histidine, and acts as an antioxidant by scavenging most reactive oxygen species, chelating various divalent metallic cations and suppressing the oxidation of homoproteins. Polyphenolic compounds have also been detected in shiitake, and may contribute to the antioxidant potential of this mushroom.

There is a growing interest to measure the antioxidant capacity in foods, since the compounds exhibiting such properties could be isolated and used for the prevention of free radicals mediated pathologies, such as cardiovascular and neurodegenerative diseases. In this study, the antioxidant capacity (radical scavenging) of shiitake cultivated on oak sawdust from the successive flushes was investigated in two parts of mushrooms (stipe and cap) using the oxygen radical absorbance capacity assay (ORAC). In addition, total phenolic compounds (TP) and ERG were quantified in order to evaluate their contribution to the antioxidant capacity of mushrooms.

MATERIALS AND METHODS

Chemicals. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein, 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), ergothioneine, phosphate buffer and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (France). All the solvents were of HPLC grade (Scharleau).

Sample collection and preparation.
The *Lentinula edodes* strain 3706 (Mycelia) was cultivated by EuroShiitake Company in insulated green houses in a temperate region, Dordogne, France. The composition of the cultivation substrate was: 85 Kg of oak woodchips and sawdust, 10 kg of wheat bran, 1 kg of gypsum, water to reach 62-65% water content. Substrate sterilization was performed with live steam for 4 hours in a tanker. After cooling 4 % (w/w) of spawn were added aseptically and mixed in the tanker. Polyethylene bags were filled with 6 Kg of spawned substrate. Incubation was at 22 ± 2 °C for 3 to 4 weeks. After removing of the bags, browning was induced by and placing them on shelves in a greenhouse with temperature from 16 to 19 °C and 92-98 % relative humidity, for 3 to 4 weeks. Fruiting was induced by soaking the blocks into cooled tap water (12-15 °C) until they reached their initial weight (6 Kg). This was done after each flushed of harvest.

Mushrooms were harvested at the French commercial stage of development (before veil had broken). For the studies of seasonal variations, mushrooms were randomly harvested one day of collect in October, January, April and July (3 Kg). For the studies of culture cycles variations, mushrooms were collected in winter during 4 successive flushes.

Harvested mushrooms immediately placed at 4°C and then freeze within 4 hours. In the laboratory they were freeze-dried, ground to a fine powder and stored in the dark at room temperature prior to analysis. Mushroom extracts were performed as followed: 100 mg of dried powder were extracted with 5 ml of MeOH overnight. The extract was centrifuged at 3000 rpm for 10 min. and the supernatant was used for analysis. All the extractions were performed in triplicates.

Determination of antioxidant capacity. The ORAC assay was applied as described previously [1]. The reaction was carried out in 75 mM phosphate buffer (pH 7.4) in a 96-well plate. 30 µl of mushroom extract or pure phenolic compounds solutions and 180 µl of fluorescein solution (70 nM final concentration) were mixed and preincubated for 5 minutes at 37°C. 90 µl of APPH solution (12 mM final concentration) were then added and the fluorescence was recorded for 60

minutes at excitation and emission wavelengths of 485 nm and 520 nm respectively using a Fluostar Optima plate reader (BMG Labtech, Germany). A blank sample and six calibration solutions of Trolox (0.1 to 4 µM, final concentration) were also tested in each assay. All samples were analyzed in triplicate. Area under curve (AUC) was calculated for each sample by integrating the fluorescence curve. Net AUC was calculated by subtracting the AUC of the blank. Regression equation between net AUC and Trolox concentration was determined and ORAC values were expressed as equivalent concentration of Trolox per dry weight.

Determination of total phenolic content. The total phenolic concentration in mushroom extracts was determined according to the Folin-Ciocalteu method using gallic acid as standard. 20 µl solution of mushroom extract and 80 µl of sodium carbonate solution (7.5% in deionized water) were added to 100 µl of Folin-Ciocalteu reagent (diluted 10 fold in deionized water) in a 96-well plate. A blank sample and six calibration solutions of gallic acid (0.625 to 20 µg/ml, final concentration) were analyzed under the same conditions. After incubation for 30 min. at room temperature, the absorbance was measured at 760 nm using a Fluostar Optima plate reader (BMG Labtech, Germany). All determinations were carried out in triplicate and results were expressed as mg gallic acid equivalent/100 g of dry weight.

Determination of ergothionein content. The ergothionein content of mushroom extracts was analyzed by reverse-phase analytical HPLC using a Varian Prostar HPLC. The separation was performed using a Prontosil C18 reverse phase column (250 mm × 4 mm, 5 µm particle size), protected with a C18 guard column. Mushroom extracts were injected (100 µl) and the elution (1 ml/min) was performed using a solvent system comprising solvents A (0.1 % trifluoroacetic acid (TFA) in water) and B (0.1 % TFA in acetonitrile) mixed using a gradient starting with 0% B and linearly increasing to 72% B in 50 min. ERG was quantified by monitoring absorbance at 254 nm and comparing the peak area of the sample to peak areas obtained from different concentrations of the authentic standard.

RESULTS AND DISCUSSION

Seasonal variations. Variations in yields are commonly observed in mushroom farms during the year. They might be due to the outside climatic conditions having consequences on how are maintained the temperature and humidity in the cultivation rooms, and to variations in the quality of raw ingredients used to produce the substrate throughout the year. Several studies have shown that the antioxidant activity of mushrooms was correlated with the content of their phenolic compounds [2-4]. Thus, it was important to consider the total phenolic (TP) in relation to the antioxidant activity (ORAC) of methanolic mushroom extracts at various periods of the year.

Figure 1 shows that the amounts of total phenolic compounds in methanolic extracts are in the range of 0.8-1.5 % dry weight (dw) in the caps and 0.8-1.1 % dw in the stipes. There were minor seasonal variations in TP and ORAC values, but a significant difference between spring and summer was observed. The winter values in the caps were twice as high as those at spring.

Differences between the parts of mushrooms had previously been observed with other mushrooms [5] and should be taken into account for the extraction of antioxidants from mushrooms. On another hand, the level of variations in *L. edodes* composition and quality as source of bioactive molecules observed here in a same farm throughout a year of cultivation are significant and should affect the efficiency of mushrooms as source of antioxidant. That also stresses why it is so difficult to compare the data obtained by various authors on samples harvested at only one time under different cultivation conditions.

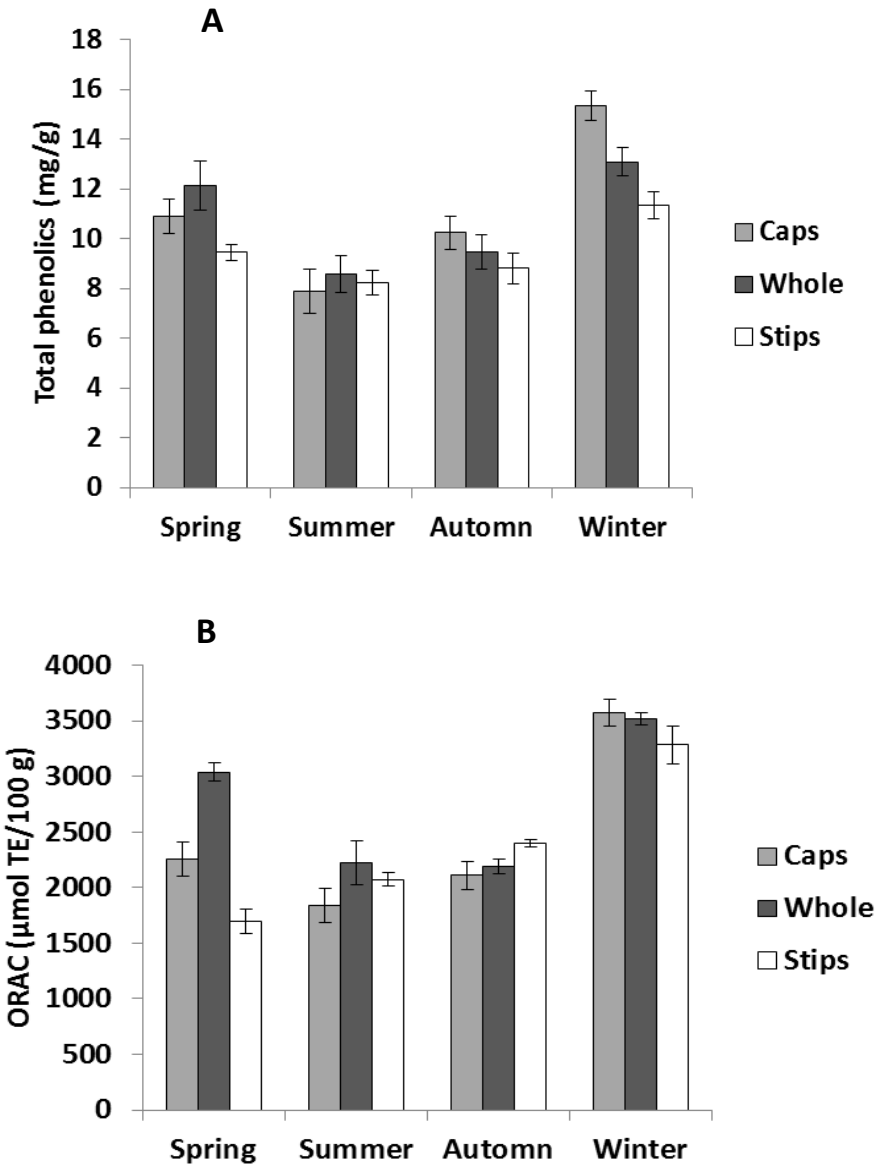

Figure 1: Effects of seasonal variations in the total phenolic contents (A) and antioxidant activities (B) of methanolic extracts from different parts of the mushroom.
Results are expressed as means ± SD (n=3 extractions).

Effect of culture aging on mushroom contents and antioxidant activity. Changes in mushroom quality with fruiting body maturity stage are documented [5, 6]. The changes between flushes of harvest that linked to culture aging are commonly suspected, but less documented.

Regarding the age of the culture, we observed a strong decrease in the contents of TP in the caps during the culture cycles, whereas those in the stipes were constant (Fig. 2). Surprisingly, the ORAC value increased of 30% in whole mushroom, whereas it decreased in the caps and strongly increased in the stipes.

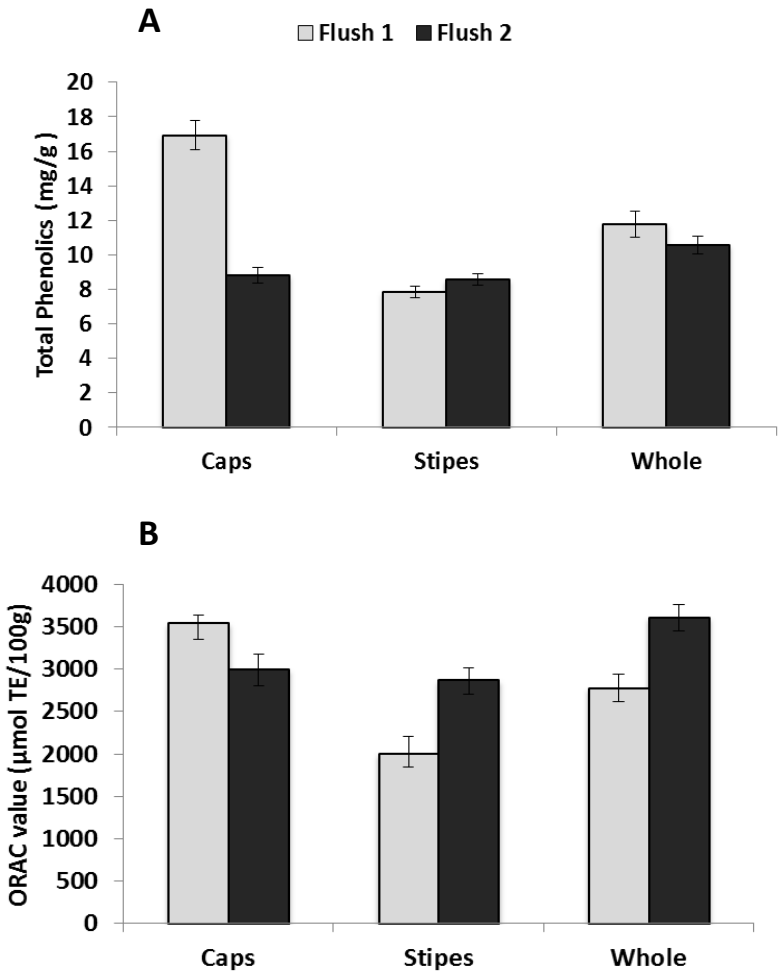

Figure 2: Effect of culture aging on total phenolic compounds (A) and antioxidant activity (B) in different parts of the mushroom.
Results are expressed as means ± SD (n=3 extractions).

A recent study showed that ERG was responsible for the majority of the DPPH scavenging activity of shiitake mushrooms [7]. In this study, we investigated this hypothesis by analyzing the compounds in whole mushroom extracts during successive flushes using HPLC-DAD (280 nm). As shown in Fig.3, the profiles of the different samples are similar but the intensity of several peaks increased or decreased during the culture cycle. Indeed, the ERG content decreased of 45% between flush 1 (36,4 mg/100 g) and 2 (19,7 mg/100 g), whereas the intensities of peaks A and B increased.

These results could explain the increase of the antioxidant activity during aging, and we suggest that the antioxidant activities of mushrooms could be largely dependent on other molecules than ERG, perhaps specific compounds which could be synthetized in the stipes during aging. Work is in progress to identify the compounds responsible for the antioxidant activity of aged cultures.

CONCLUSIONS

In this study, we have established the main variations in the antioxidant activity and selected compounds (ERG and TP) of shiitake during cultivation cycles on oak sawdust. We concluded that the antioxidant activities could be largely dependent on other molecules than ERG, perhaps

specific phenolic compounds. The chemical characteristics of the antioxidative components will be now further investigated. This study could provide valuable new opportunities for mushroom growers, since shiitake can serve as a good source of antioxidants in the human diet.

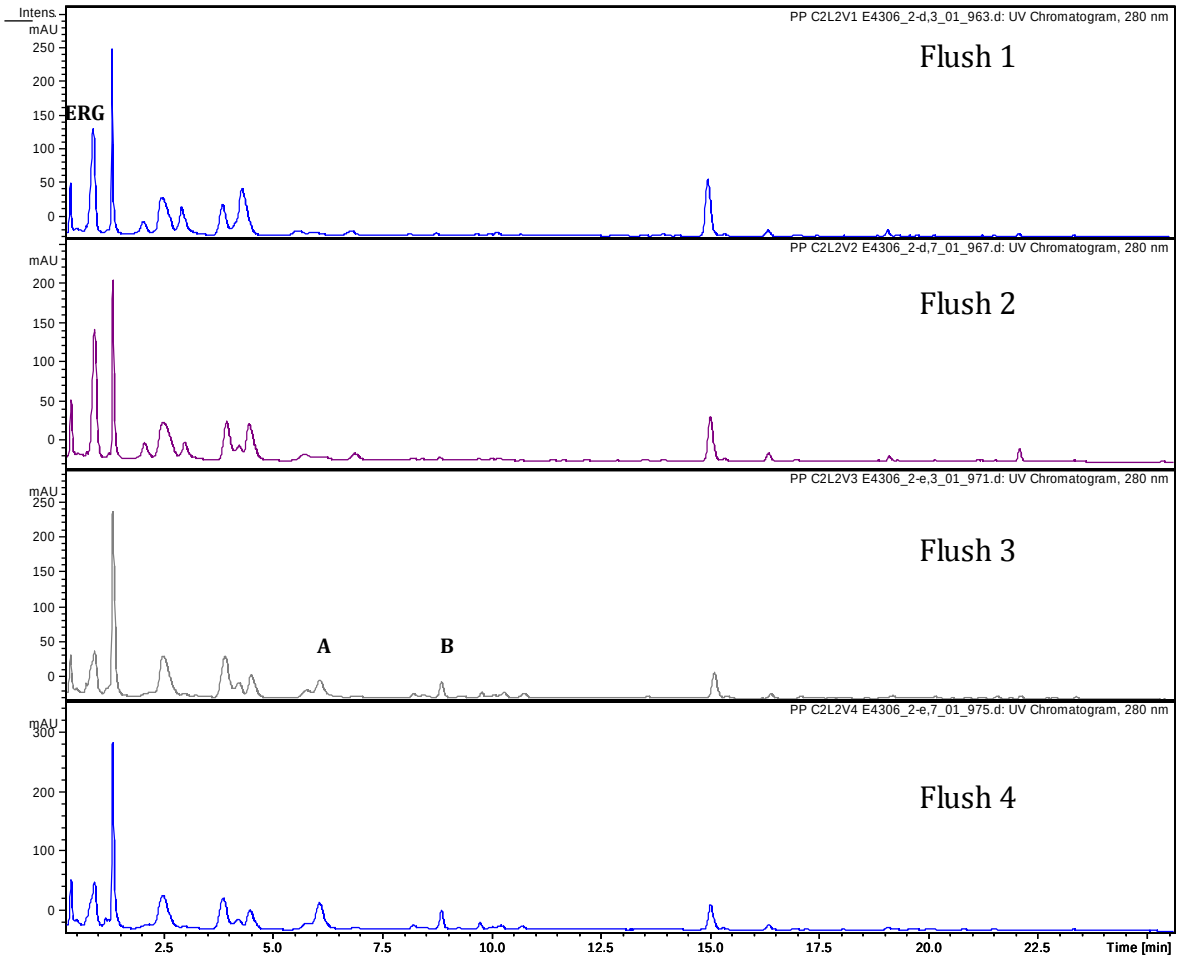

Figure 3: HPLC chromatograms (280 nm) of whole mushroom methanolic extracts from successive culture cycles. ERG: ergothioneine.

ACKNOWLEDGEMENTS

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VARIATION OF BIOACTIVE LENTINAN-CONTAINING PREPARATIONS IN *LENTINULA EDODES* STRAINS AND STORED PRODUCTS

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ABSTRACT

Lentinan, a ß-(1→3)-D-glucan isolated from the common edible mushroom, *Lentinula edodes* (Shiitake), is a biologically active macromolecule with a potential medical application towards immune functions. The immunomodulatory activities of ß-glucans depends on their degree of branching, conformation and the inter- and intra-molecular association of the polysaccharide. Up to now, there is a lack of understanding the relationship between the structure and function of lentinan and no quality controlling methods are available to analyse bioactivity of lentinan containing supplements or for the Shiitake mushrooms. Therefore, we attempted to clarify the relation between structural details and biological responses of lentinan extracts isolated from fresh and stored mushrooms.

From freshly harvested fruiting bodies, the yield of crude lentinan extract varied from 260 to 825 mg/100g fresh weight. After 4 days of cold storage, the variation in crude lentinan extract content was more pronounced among the six strains analysed. As a general tendency, the yield decreased during storage with the exception of one strain for which lentinan content appeared to increase during storage.

The bioactivity of the crude lentinan extracts was studied using the RAW264.7 macrophage cell line and the ability to modulate the NO production of LPS challenged. Results indicated that the crude lentinan extracts could inhibition of LPS-induced NO production but again some strains showed higher immune modulatory effects than others. Size exclusion chromatography (SEC) was applied to examine molecular weight distribution of the crude lentinan extracts. It appeared that in crude lentinan extracts of some of the strains, the polysaccharide consisted of a different conformation or modification of lentinan or contained other polysaccharides. In general, the presence and ratio of some of the different polysaccharide forms that could be distinguished by SEC analysis are correlated to *in vitro* bioactivity.

In conclusion, our study resulted in a broader insight into structure-function relationship and the possible influences of strain, growth and storage condition on bioactivity of Shiitake lentinan. The SEC and bioactivity analyses together presented a possible direction of quality control for lentinan-containing products. The standardisation of lentinan-containing bioactive products should include the background knowledge of strain, and of growth and storage conditions of the mushroom.

INTRODUCTION

Lentinula edodes, the Shiitake mushroom, is well-known for its health-promoting effects, such as antitumor, hypocholesterolemic actions, antimicrobial and antioxidants potentials [1,]2]. Lentinan isolated from Shiitake is recognized as being an effective biological response modifier [3]. The molecular formula of lentinan is $(C_6H_{10}O_5)_n$ with a mean molecular mass of 500 kDa [5]. The backbone structure of lentinan has been reported as a ß-1,3-D-glucan backbone, branched with ß-(1-6)-glucans [4] which show a right-handed triple helix [5].

It is known that the molecular weight, degree of branching, conformation, and intra- and intermolecular association of the polysaccharide chains are important for their biological responses [6].

Previous studies have shown that the biological response of lentinan against infection is host mediated and due to activation of the innate and adaptive immune responses. For the activation of appropriate immune responses, pattern recognition by receptors is important. An important receptor for ß-glucans, including lentinan, is dectin-1 [7]. Dectin-1 is mainly expressed on the surface of macrophages, neutrophils, dendritic cells (DC) and on some T-cells [8].

Several studies reported effects of lentinan on cytokine production of macrophages or monocytes in both mice and human, for instance, production of TNF-α, IL-1β and cytotoxic- or nitric oxide (NO) activation [9]. NO is a noxious, stable and free radical gas which plays an important role in the functions of macrophages. It has been reported that mouse macrophages stimulated with antitumor polysaccharides produced NO [11] and also macrophages stimulated with lentinan released NO [12]. Thus it seems that enhancement of NO production through macrophages may reflect the antitumor activity of lentinan [10]. In addition, Miniato [13] concludes from his investigations that the quality of the Shiitake as a functional food is correlated to the lentinan content. Especially during storage of Shiitake, a decrease in lentinan content was observed, which was caused by enzymatic degradation through exo-glucanase. There are no specified reports yet on the impact of strain or storage conditions on the composition, content and immunomodulating effect of lentinan. In this study, we analyzed the effect of storage on composition changes and immunomodulating effects of lentinan in different Shiitake strains.

MATERIAL AND METHODS

Materials. Purified lentinan (4 mg/ml) was kindly provided by Ajinomoto Co.

Mushroom sample preparation. The shiitake mushrooms strains were selected from the collection of Plant Research International (PRI), Wageningen University and Research Centre. The different Shiitake strains were grown on artificial substrate under Dutch commercial breeding circumstances.

At PRI, Sawdust substrate bags (Hesse 2,8 kg) were inoculated with 30 ml spawn. These spawn was prepared by inoculating pure cultures on sterilized sorghum grain. The bags were incubated using the following climate parameters: 20°C, 1500ppm CO_2 and 90% RH. After 6-11 weeks of vegetative growth, depending on the strain, the plastic was removed from the blocks and the blocks were placed in a production room. The conditions for fruiting body production were 16°C, 1000ppm CO_2, 90% RH and 12 hours light a day. Fruiting body production started, again strain dependent, after 6 to19 weeks of vegetative growth. Immediately after harvesting, the fruiting body's were packed in moisturized boxes and stored for 4 days at 7°C. After 4 days storage, as well as directly after harvest, mushrooms were sectioned into cap and stipe part, frozen with liquid nitrogen and stored at -80°C. Only the cap tissue is discussed in this article because of limitations in available stipe tissue.

Extraction of crude lentinan. The extraction of crude lentinan was modified from the method developed by Yap and NG [14]. To 100 g of frozen cap tissue, boiling water was added with a solid:liquid ratio of 1:3. The samples were homogenized with an Ultrathurax for 1 min and boiled for 3h under continues stirring on a heating plate. After cooling down the extracts to room temperature, the lentinan was precipitated by adding one volume of 95% ethanol followed by an incubation step of 16h at 4°C. The samples were centrifuged at 5000 rpm (Beckman) for 20 min at 4°C. The pellet was snap frozen in liquid nitrogen and lyophilized in a freeze-dryer. One volume of hot water (60°C) was slowly added to the lyophilized pellet and the solute was homogenized using an Ultrathurax for 1 minute at full speed. Subsequently, the homogenate was boiled for 8h under continues stirring, stored overnight at 4°C and centrifuged at 5000 rpm for 20 min at 4°C. The supernatant was collected and precipitated overnight with 1 volume of 95% ethanol. The precipitate was collected by two centrifugation steps at respectively 5000 and 7000 rpm for 20 min at 4°C and subsequently lyophilized. The obtained crude lentinan powder was further dried in an oven at 60°C for 1 day. The samples were weighed (concentration lentinan / g tissue) and stored in a desiccator prior to further analysis.

Molecular characteristics of crude lentinan. To investigate the purity of crude lentinan extract, the protein content using a Bradford method [16] and the total phenolic compounds, with some modifications [17], were determined. Briefly, 5 mg of each extract was dissolved in 1 ml methanol while stirring on a boiling plate at 60°C for 24 hours. To 100µl of crude lentinan extract, 500µl Folin-Dennis reagent (Sigma) and 1ml of saturated sodium carbonate solution were added. A standard curve was prepared using tannic acid (0.1-1.0 mg/100µl), with the addition of 500µl of Folin-Densis reagent (Sigma) and 1 ml of saturated sodium carbonate solution. All the samples were centrifuged and the absorbance values from the clarified supernatants were measured using spectrophotometer at 760nm. The total phenolic content was calculated based on equivalent to tannic acid (ETA). Size-exclusion chromatography (SEC) was used to determine the molecular weight and viscosity of the samples. The SEC measurements were carried out on a triple detection GPC/SEC (Viscotek). Crude lentinan samples (3 mg/ml) were dissolved in dimethylacetamide with 0.5% lithium chloride (DMAc/0.5%LiCl) which was also used as eluent. A homogeneous solution was obtained through continuous stirring at 60°C for 48 hours. All samples were filtered by a 0.45 µM glass microfiber filter (Whatman) before injection (100µl) onto the SEC GMHHR-M + Guard column. The flow rate was 1.0 ml/min. Eight narrow molecular weight pullulan standards in the range 5.8 – 1660 kDa (Shodex standard P82, Showa Denko) were used to calibrate the columns. The TriSec software program version 4 was used for the acquisition and analysis of Viscotek data.

Effect of crude lentinan on NO-production. The effect of crude lentinan on NO production was evaluated. RAW 264.7 cells, a murine macrophage cell line, was cultured in RPMI 1640 medium (Gibco) supplemented with 100 U/ml penicillin, 100µg/ml streptomycin and 10% heat-inactivated fetal bovine serum (Invitrogen). Cells were grown at 37°C under an atmosphere of 5% CO_2. RAW 264.7 cells (10^6 – 10^7 cells/well in 96-well culture plates) were incubated at 37°C (5% CO_2), with or without 10µg/ml LPS (sigma), and complemented with 100 µg/ml crude lentinan extracts for 48 hours. After the incubation period, NO production was determined using a colorimetric test based on the Griess reaction [15]. Briefly, 50 µl of cell supernatant was mixed with 50 µl Griess reagent (Sigma) and the mixture was incubated at room temperature for 10 min. The nitrite concentration was determined by measuring the absorbance at 540 nm in an automated plate reader (Multiskan Spectrum Thermo Labsystems) using the standard curve of $NaNO_2$. The results were expressed as relative percentage of NO production compared to LPS control (100%) with subtraction of the media control.

RESULTS AND DISCUSSION

Variation in lentinan content in different Shiitake strains during storage. Mostly all consumable mushrooms are distinguished as healthy, functional foods, and well-known to contain some types of immunomodulating polysaccharides, specific the polysaccharide called lentinan. In order to investigate the influence of genetic background and the effects of storage on the lentinan content and bioactivity, 13 different Shiitake strains were cultivated and harvested under the same growth conditions. The production was performed only once. As some strains had a very low fruiting body production not all of the analyses could be performed in full matrix (both fresh and stored at 7°C) or in duplicates. Table 1 shows the changes in crude lentinan content from six Shiitake strains with highest fruiting body production, directly after harvest (fresh) and after 4 days of cold storage at 7°C. The content of lentinan of fresh harvested Shiitake varied between 260 and 824 mg/100g fresh weight (fw). Mizuno [22] observed that the content of lentinan did not change drastically during storage at 5°C for 7 days. In this study, we noticed that the decline of crude lentinan content was different per strain. The crude lentinan content of strain Mes02094 decreases minimal (260 to 226 mg/100gr fw). While in strain Mes02007 the crude lentinan content decreased drastically during storage (824 to 229 mg/100g fw). Thus, we can concluded that change in lentinan content does not only depend on storage conditions but also on the Shiitake strain.

Table 1: Lentinan content from different Shiitake strains after harvest and after 4 days at 7°C

Strain	lentinan content mg/100gr fw	
Mes number	fresh	Storage
02007	824	229
02010	727	1478
02054	260	226
02094	244 ± 42	166 ± 4
02121	332 ± 16	229 ± 18
11775	448	385

Molecular characteristics of the crude lentinan containing extracts. Prior to the evaluation of bioactivity of the crude lentinan extracts, the purity of the extracts was investigated. It is known that phenolic compounds (at the range of 16-500µM) cause an inhibitory activity on NO production in LPS activated macrophages of more than 50% NO production [21]. Therefore, the content of phenolic compounds in the crude lentinan extracts was analyzed as show in Table 2. All extracts, contained before and after storage, very low levels of phenolic compounds (< 2µM). As a result, we presumed that the present phenolic compounds in the crude lentinan extracts had no effect on the stimulation/inhibition of NO production. Likewise, the protein levels (Table 2) in the crude lentinan extracts were very low and therefore the present proteins should give no disturbing effect on the bioactivity assay.

Size exclusion chromatography (SEC) was applied to study the composition of the crude lentinan extracts. SEC results revealed that the crude lentinan extracts consisted of several peaks, based on Molecular weight (Mw) (Table 2) and the retention time (data not shown). These peaks correspond to the protein attached triple helix chains and to the fragments of triple helix with a high molecular weight and single chains having low molecular weight [23].

Table 2: Molecular characteristics of crude lentinan extracts and pure lentinan.

Strain Mes number	Phenolic compounds %	Protein %	Mw peak 1 $x10^5$	Mw peak 2 $x10^4$	Mw peak 3	Mw/Mn peak 1	Area peak 1 mV/ml	Area peak 2 mV/ml	Area peak 3 mV/ml
02007-fresh	0.10 ± 0.02	0.75 ± 0.08	2.21	1.27	412	1.9	93.41	15.41	1.75
02010-fresh	0.09 ± 0.04	0.66 ± 0.03	2.87	1.24	431	2.1	85.92	7.71	1.17
02054-fresh	0.12 ± 0.02	0.75 ± 0.02	2.73	1.34	441	2.0	26.54	16.36	3.55
02094-fresh	0.14 ± 0.01	0.54 ± 0.02	2.00	1.26	445	1.6	25.62	10.29	2.08
02121-fresh	0.19 ± 0.02	0.86 ± 0.02	2.12	1.26	462	1.7	62.68	15.36	2.26
11775-fresh	0.17 ± 0.07	1.21 ± 0.04	2.03	1.35	515	1.6	78.30	14.93	2.56
02007-storage	0.26 ± 0.01	1.23 ± 0.00	3.44	1.50	396	2.0	7.13	33.15	5.84
02010-storage	0.06 ± 0.01	0.77 ± 0.00	2.47	1.35	502	1.7	117.7	7.71	0.78
02054-storage	0.32 ± 0.02	0.99 ± 0.03	2.21	1.34	439	2.0	22.35	21.75	4.56
02094-storage	0.19 ± 0.01	0.61 ± 0.01	2.97	1.20	429	2.6	30.20	24.25	5.25
02121-storage	0.18 ± 0.00	1.05 ± 0.04	2.45	1.78	452	1.8	24.79	23.64	4.22
11775-storage	0.24 ± 0.02	1.64 ± 0.03	2.93	1.41	525	2.4	51.18	21.72	3.98
lentinan	0.01 ± 0.01	nd	4.00	1.00		2.6	39.33	7.94	

It was estimated that the lentinan extract derivatives might be modified during the storage of the fruiting bodies, extraction of the lentinan and storage of the extracts, thus causing the degradation of polysaccharides. Cold storage could induce further changes, probably through depolymerisation and oxidation of the polysaccharide. SEC analyses identified that pure lentinan exhibited two peaks with a Mw of 4.0 x 10^5 and 1.0 x 10^4 (Table 2). The Mw of pure lentinan has been determined to be $9.5x10^5$-$10.5x10^5$ [4] and later found to be $2.03x10^5$-$8x10^5$ by SEC analyses [19, 20]. From our results, the Mw of peak1 found in all crude lentinan extracts either from fresh or cold stored fruiting bodies, corresponded with the expected Mw in literature. The Mw from the pure lentinan (received from Ajinomoto and used as reference) is approximately 1.5 times higher than the Mw of the isolated lentinan extracts, probably caused by the conformation of the lentinan due to the isolation and purification procedure used.

The polydispersity (Mw/Mn) of the investigated crude lentinan extracts were found to be between 1.6 and 2.6 (Table 2) and are similar to the finding of Zhang's group [18, 24]; they indicated that the polydispersity of lentinan fractions were between 1.8 and 2.7. In general, the polydispersity of the stored shiitake was higher than from fresh Shiitake. From this we can conclude that partially, the backbones of these modified polysaccharides were more degraded than the polysaccharides in the fresh harvested Shiitake.

Modification of LPS induced NO production. The function of macrophages may be comprised by lentinan via two mechanisms: I) by cell-to-cell contact between macrophages and tumor cells and II) by the release of antitumor factors and mediators such as cytokines and NO [9]. When LPS was administered to RAW264.7 macrophages, the production of NO increased dramatically. To determine the suppressing abilities of crude lentinan on NO production, RAW macrophages were incubated with or without 10µg/ml LPS in combination with or without crude lentinan extracts (100 µg/ml). The inhibitory effect (IE) was expressed as the percentage of decrease in NO production as where $[NO]^a$ represents the NO concentration of cells supplemented with

lentinan and LPS minus $[NO]^b$, the NO concentration of cells supplemented with lentinan alone. $[NO]^c$ represents the NO concentration from LPS activated control macrophages.
$IE(\%) = 100 - ([NO]^a - [NO]^b) / [NO]^c * 100)$.

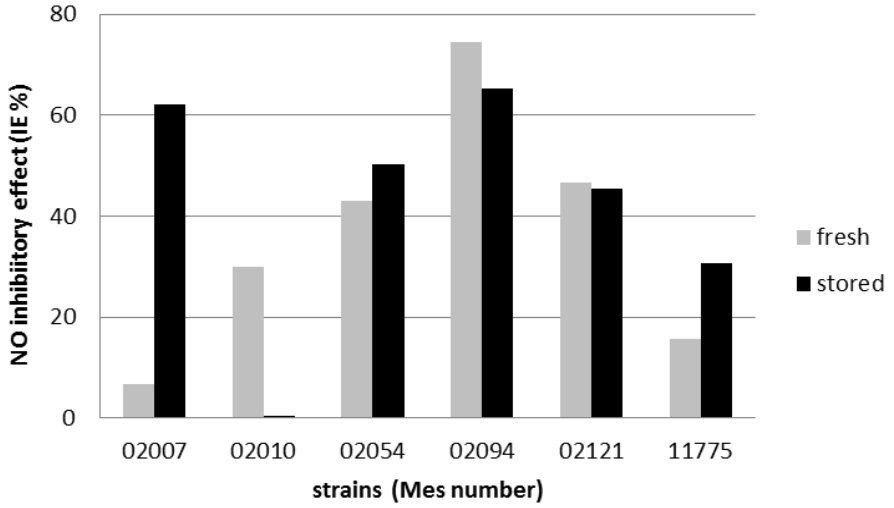

Figure 1: Inhibitory effect on NO production (%) of macrophages by crude lentinan extracts from fruiting body tissue of different strains Shiitake directly after harvest (fresh) and after storage of the fruiting bodies 4 days at 7°C (stored).

Cell viability was assayed to exclude the possibility that the inhibitory effects obtained from crude lentinan extracts might be caused by their cytotoxicity (data not shown). The inhibitory effects of the crude lentinan extract originating from fresh and cold stored fruiting bodies on NO inhibition in LPS activated macrophages is shown in Fig. 1. The crude lentinan extracts of all six strains demonstrate an inhibitory effect of NO production in macrophages, although the effects varied among strains. Crude lentinan extracted from fresh strain Mes02094 showed the highest NO inhibition (75%) while the extract from cold stored Mes2007 showed the least response (7%). Fresh and cold stored Mes02121 showed an equal NO inhibition response while for strains Mes02054, Mes02094 and Mes02121 a difference in response between fresh and stored mushroom extract was seen. The inhibitory effect of strain Mes02010 after cold storage was close to zero, while the inhibitory effect from Mes02007 drastically increased after the cold storage.

In conclusion, the consumption of the diet rich-lentinan –containing Shiitake- may reduce the production of nitric oxide caused by the oxidative stress, thus might increase the protective effects against cardiovascular and chronic inflammatory diseases. When testing the health effects of Shiitake mushrooms in intervention studies, strain and product freshness should be taken into account. Preferably, products should be standardized by using batch wise biochemical and bioactivity analysis.

Relationship between concentration, molecular characteristics and bioactivity of the crude lentinan extracts. The immunomodulating properties of lentinan, observed in NO producing macrophages, are related to the chemical composition, configuration and chain conformation, as well as their physical properties [6]. From our results we have tried to identify a relationship between characteristics of the isolated crude lentinan and NO inhibitory bioactivity. For instant, an exponential negative correlation between NO inhibitory effect and total lentinan content was observed (Fig. 2). This might indicate that higher concentrations of the polysaccharides could reduce the immune modulating competency of the polysaccharide. On the other hand, it could also indicate that, under some conditions, the content of lentinan in crude extracts were

overestimated resulting in the use of lower concentrations in the bioassay than expected based on weight basis.

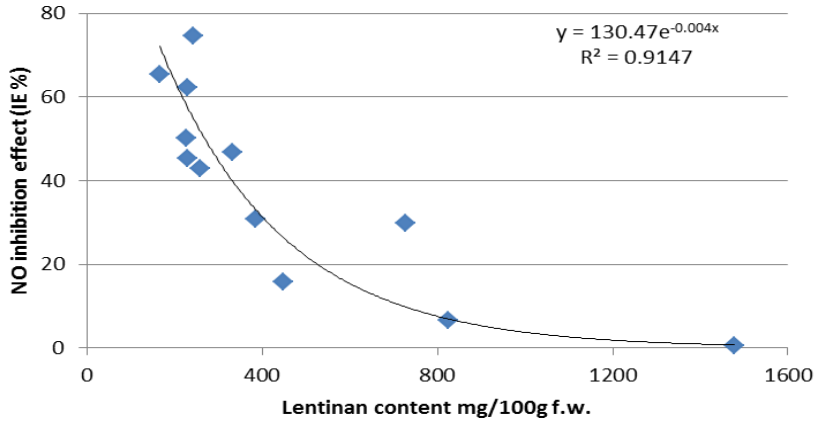

Figure 2: Relationship between the NO inhibition effect (%) and the crude lentinan content (mg/100g fw).

We conclude from that observation that the quantity of the crude lentinan in Shiitake extracts could not be used as a single factor for the immunomodulating capacity of Shiitake mushrooms.

Table 3: Correlation between NO inhibitor activity and peak area after SEC analysis.

R^2	Area peak 1	Area peak 2	Area peak 3	% NO inhibition
Area peak 1	1	-0.783**	-0.896**	-0.833**
Area peak 2	-0.783**	1	0.951**	0.506
Area peak 3	-0.896**	0.951**	1	0.629*
Area peak 2+3	-0.905**	0.935**	0.999**	nd
% NO inhibition	-0.833**	0.506	0.629*	1

p<0.01** , p<0.05*, n=12, nd = not detected

Apart from the lentinan-NO inhibition-relationship, the correlation between NO inhibitor activity and the peaks found after SEC analysis were studied (Table 3). A high interaction between peak areas (equivalent to concentration) was observed, e.g. between peak 1 and 3 in which the concentration of peak 3 increases while peak 1 decreases (R^2 =-0.896; p<0.01). Very likely, this is caused by conformational changes due to degradation. Peak 1, which also has been identified in the pure lentinan from Aijinomoto, as well as peak 3 correlates to the NO inhibition. High concentration of peak 1 in crude extracts relates to lower NO inhibition while increase in peak 3 concentration results in higher NO inhibition effects. Peak 2 showed less correlation with the NO inhibitory effect. However, the sum of peak 2 and peak 3 showed the highest positive correlation with NO inhibition (R^2= 0.905; p<0.01). Some of the strains (Mes 02007, 02054 and 02094) exhibited a fourth peak but the area of this peak was very small and revealed no correlation with the NO inhibitor activity.

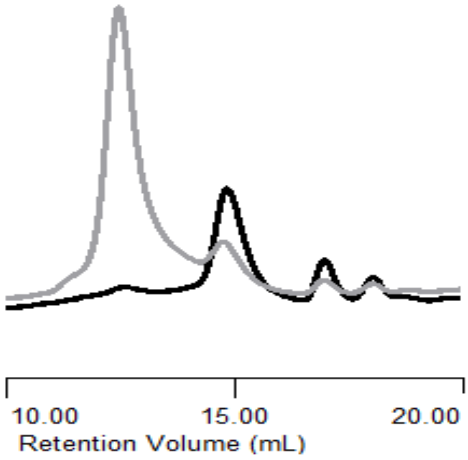

Figure 3: SEC spectra of strain Mes02007 directly after harvest (grey) and after storage of 4 days at 7°C (black).

In general, most strains did not show much change in peak composition when stored as fresh fruiting bodies. However, strain Mes02007 showed a remarkable change in peak area shift from peak 1 towards peak 2 and 3 accompanied by a high NO inhibition bioactivity (Fig. 3). This strain might therefore be an interesting genetic source for fresh Shiitake functional foods as this requires a high storability with remained bioactivity.

CONCLUSION

Future research should focus on science based evidence of lentinan to support our immune system, especially on a food product bases like using whole Shiitake mushrooms or food supplemented with isolated lentinan. These studies however should include standardized methods to verify the product bioactivity knowing the biological variation that can be caused by strain, storage, purification, process and probably still other factors which should be unraveled in order to develop reliable functional food products.

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A NEW COLORIMETRIC METHOD TO QUANTIFY β-1,3-1,6-GLUCANS IN COMPARISON WITH TOTAL β-1,3-GLUCANS AND A METHOD TO QUANTIFY CHITIN IN EDIBLE MUSHROOMS

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ABSTRACT

Mushrooms contain high amounts of substances like chitin and glucans, which can be used for medical purposes and nutrient matter. Therefore, an easy quantification method for chitin and for glucans, with high precision, is a matter of particular importance.
For this purpose, we developed an easy and unique photometric method for the determination of β-1,3-1,6 glucans in mushrooms. This method is specific for β-glucans with a triple-helix tertiary structure. The used dye, congo red, forms a complex with the triple-helix, which causes a specific bathochromic shift of the absorption maximum from 483 nm to 523 nm.
For the specific determination of total β-1,3 glucans a fluorimetric method was adapted, too, which easily can be performed. By the combination of both methods, it was possible to compare the amount of β-1,3-1,6 glucans with the total β-glucan content.
Furthermore, we developed a new colorimetric method for the quantification of chitin and chitosan, which is based on the specific reaction of polyiodide anions and chitosan. After deacetylation, chitin can also be quantified by this method. With this new spot assay, the chitin content of mycelia and fruiting bodies from several basidiomycetes and an ascomycete was analysed. The presented method can be used for determination in other samples, too.

Keywords: chitin, chitosan, β-1,3 glucans, β-1,3-1,6 glucans

INTRODUCTION

Mushrooms of different species are well-known for their therapeutic effects in traditional chinese medicine since centuries. Their cell-wall β-glucans are ingredients with biological activity, thus these biological response modifiers [1-3] mainly activate the immune system, with the possibility to even having effects as an anticarcinogen [4, 5]. β-Glucans such as Lentinan and Grifolan are known as anticarcinogenic agents since the early 1970s [1, 6, 7]. The structure varies with the linkage degree: β-glucans with none or little β-1,6-linkages mainly have a single helix structure. β-Glucans with higher degrees of β-1,6-glycosidic bonds form a triple helix as their tertiary structure. Recent work describes that the triple helix structure, together with the molecular mass, affects the biological activity of the β-glucans [4-7].

A number of existing methods determine the total amount of β-1,3-glucans [8-13,] but do not make any difference between their important tertiary structure. Up to this day, a direct, fast and quantitative method to determinate the important β-1,3-1,6-glucans with a triple helical structure does not exist. Therefore, a precise method would be of great interest. The aim of our

research was to establish a new method to quantify β-1,3-1,6-glucans in mushrooms and to use it for comparison with the total β-1,3-glucan content in various fungi. Our research can survey which mushroom is more useful for glucan preparation i.e. for functional purposes. Therefore, we would like to present a new colorimetric method for β-1,3-1,6-glucan quantification using congo red dye.

An incorporation of congo red into the triple helix leads to a bathocromic shift. The absorption maximum is moved from 493 to 523 nm [14]. Therefore, congo red can be used for characterisation of glucan tertiary structures, only interacting with the triple helix of β-1,3-1,6-glucans [14,15] and not reacting with other polysaccharides [16]. β-Glucans of selected basidomycetes and an ascomycete were isolated by a sequence of alkali and acid treatment and analysed with the new method and the fluorimetric method. In this work a wide variety of different mushrooms are compared among themselves and their mycelia are compared with some selected fruiting bodies. Therefore, the received data can be used to make comparisons between different and same genus, family and division and between mycelia and fruiting bodies.

Besides β-glucans, chitin is an important component of the fungal cell wall. The polymer is characterized by β-(1,4)-branched N-acetylglucosamine units. Partial deacetylation of this biopolymer yields chitosan. The wide variety of possible applications of chitosan in food industry includes, for example, functional foods, use as packaging material and filtration devices. Thus, the cell wall of mushrooms could be an important source for chitosan and glucan production. Basidiomycetes like *Lentinula edodes* or *Grifola frondosa* are well known for their application in various medical domains. β-Glucans, in particular, have been proposed as active immunostimulating agents. After isolation of the glucans, the remaining fibres contain mainly chitin and can be used for chitosan preparation as well. As chitin is insoluble in most solvents, a direct detection is difficult, but it is possible to quantify it as chitosan or N-acetylglucosamine indirectly. Therefore, we developed a modification of a method by Tsuji *et al.* [17] that relies on the formation of a coloured complex of chitosan with Lugol's solution [18]. Generally, Lugol's solution is used in microscopy as a specific dye for chitin in cell walls. Thus, chitin was converted to chitosan with concentrated potassium hydroxide and the hydrolyzed chitosan reacts to glucosamine. With this simple method, the chitin content of a number of mushrooms was determined. As the complex of Lugol's solution and chitosan is insoluble, a spot assay was developed. After pipetting the samples on plates, the optical density of the chitin–polyiodide complex was measured by a colorimetric technique which is commonly used for quantitative analysis of TLC plates as well.

MATERIAL AND METHODS

Chemicals. Chitin and chitosan with a purity of 99 % were obtained from Sigma–Aldrich (Seelze, Germany) for the calibration standard and model reactions. Schizophyllan (β-1,3-1,6-glucan) from S*chizophyllum communue* was used for the photometric quantification method as a calibration standard (Selco Wirkstoffe Vertriebs GmbH, Wald-Michelbach, Germany). Curdlan was used as the calibration standard for the fluorimetric determination of β-1,3-glucans, congo red and anilin-blue diammonium salt were products of Sigma-Aldrich (Seelze, Germany).

Mushroom Samples. The fruiting bodies of the analysed mushrooms were kindly provided by a local breeder. The following mushroom species were chosen for further analysis and mycelia cultivation on a malt-yeast extract media:

Lentinula edodes (Shiitake), *Pleurotus ostreatus* (Oyster Mushroom), *Pleurotus eryngii* (King Trumpet Mushroom), *Hypsizygus tessulatus* (Shimeji Mushroom), *Flammulina velutipes* (Enokitake), *Agaricus bisporus* (Button Mushroom), *Grifola frondosa* (Maitake), *Pleurotus*

pulmonarius (Lung Oyster Mushroom), *Trametes versicolor* (Turkey Tail), *Morchella esculenta* (True Morel).

Cell Preparation. Cell preparation and the preparing of dry cell material was accomplished according to [19, 20].

Determination of Chitin and Chitosan. A chitosan standard was used for calibration in a concentration range of 0.5–5 mg/mL of chitosan. The standard solution or the sample solution (2 lL) was pipetted onto the desired TLC plates or papers. The spots were detected with 1% Lugol's solution, which was sprayed on the plates evenly. Afterwards the optical density of the spots was analysed with a Bio-Rad Geldoc2000 with the contour tool of the Bio-Rad Quantity One quantitation software v.4.2. All results are reported in optical density (OD) units per mm^2. All analyses were performed in triplicate [19, 20].

Colorimetric Determination of β-1,3-1,6-Glucans with Congo Red. A α-Helios photometer was used at a wavelength of 523 nm for the photometric determinations for the standard solution with schizophyllan and the tested samples. A direct measurement of the bathochromic shift is used. Because of the light brownish colour of some fractions a measurement of the background absorbtion at 523 nm is necessary in the range of 50 – 150 µg/ml. All samples were also analysed in triplicate [19, 20].

Fluorimetric Determination of the Total-β-1,3-Glucans with Aniline Blue. The total β-1,3-glucan content was measured according to the method described by Ko and Lin [21]. All fluorimetric measurements were carried out on a Kontron Instrument SFM 25. The excitation wavelength was 392 nm, the emission wavelength was 502 nm and the spectral bandwidth was 20 nm. All samples were analysed in triplicate again.

RESULTS

Chitin Determination: Calibration. Commercial chitin was used to test whether chitin can be completely converted to chitosan. Therefore, we heated the chitin for 3 h with concentrated potassium hydroxide. The residue was then washed several times with distilled water and dissolved in 10% acetic acid. Then the resulting chitosan content was determined with chitosan standards and applicated in the spot test with Lugol´s solution (Fig. 1). The detection limit according to DIN 32 645 was 107 µg/mL. Mandel linearity test showed that a linear calibration in the concentration range from 0.5 to 5 mg/mL can be arranged (Fig. 2). The recovery rate was also determined. We used commercial chitin and chitosan samples and samples of mushroom chitin preparations together to determine the recovery rate. Repetitions (20) with three different mushroom samples were used for the recovery rate. It was 101 ± 8%. This proves the applicability of this method for mushroom samples.

Determination of chitin and chitosan in edible mushrooms. The chitin content of various mushroom mycelia is summarized in Table 1. It ranges from 0.8 wt % (*P. ostreatus)* to 9.6 wt % (*A. bisporus)* and the average chitin content of the mushroom mycelia is 2.5%.

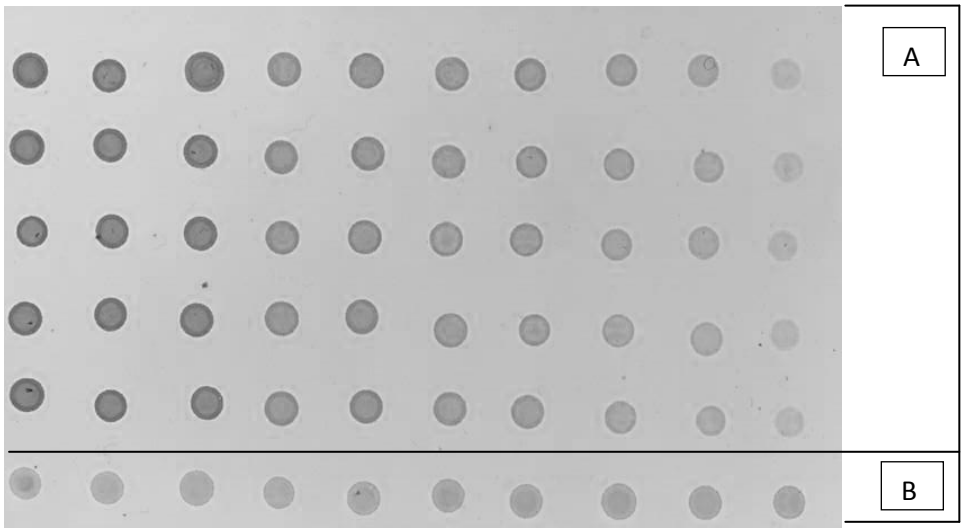

Figure 1: Picture of the spot assay: (A) chitosan standards in a concentration rangeof 5–0.5 mg/mL of chitosan (from left to right); (B) determination of the chitosan from *Agaricus bisporus* mycelia.

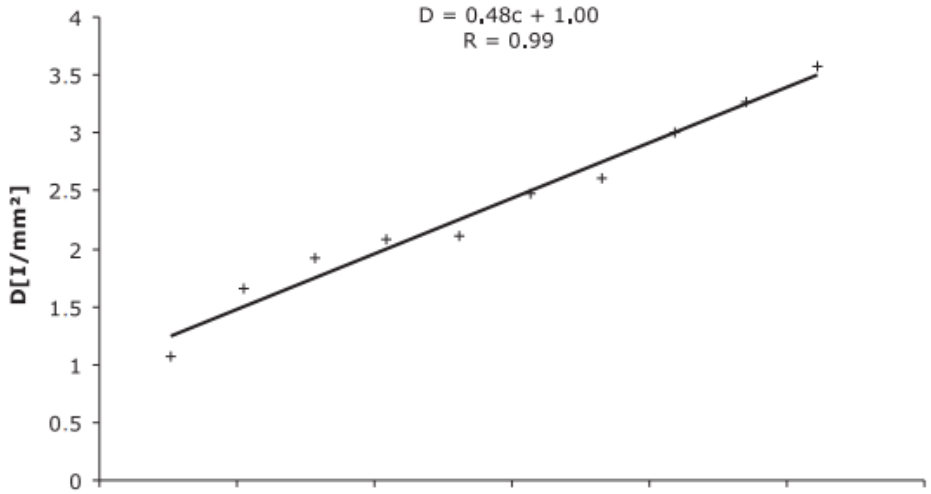

Figure 2: Calibration curve in a concentration range of 0.5–5 mg/mL of chitosan.

Table 1: Chitin content with standard derivations of various mushroom mycelia expressed as g chitosan per 100 g of dry mass (DM)

Mushroom mycelia	Chitin content [g/100 g DM]
Agaricus bisporus	9.60 ± 0.15
Pleurotus eryngii	3.56 ± 0.35
Lentinula edodes	2.49 ± 0.19
Morchella esculenta	1.70 ± 0.40
Grifola frondosa	1.67 ± 0.23
Pleurotus pulmonarius	1.64 ± 0.15
Hypsizygus tessulatus	1.57 ± 0.24
Trametes versicolor	1.35 ± 0.06
Flammulina velutipes	1.21 ± 0.18
Pleurotus ostreatus	0.82 ± 0.08

The chitin content of the fruiting bodies is summarized in Table 2. It varies in a range of 0.8–9.8 g/100 g dry mass. It becomes obvious that *A. bisporus* contains less chitin in its fruiting body in comparison to the mycelia. In contrast, *F. velutipes* has a significantly higher content in the fruiting body than in the mycelia. The other species have comparable amounts in their fruiting bodies and mycelia.

Table 2: Chitin content with standard derivations of various mushroom fruiting bodies expressed as g chitosan per 100 g of dry mass (DM).

Mushroom fruiting bodies	Chitin content [g/100 g DM]
Flammulina velutipes	9.83 ± 0.45
Agaricus bisporus	4.69 ± 0.90
Pleurotus eryngii	3.16 ± 0.40
Lentinula edodes	1.87 ± 0.20
Pleurotus ostreatus	0.76 ± 0.22
Hypsizygus tessulatus	0.39 ± 0.01

Chitosan could not be detected in all samples, an indicator that most of the amino groups of the glucosamine units are acetylated.

Glucan Determination: Calibration. Colorimetric Determination of Triple helical β-1,3-1,6-glucans with Congo Red

The bathochromic shift from the reaction of β-1,3-1,6-glucans with congo Red is effected by the β-1,3-1,6-glucan and dye concentration. For the calibration schizophyllan solutions in a concentration between 50 and 700 µg/ml were used.

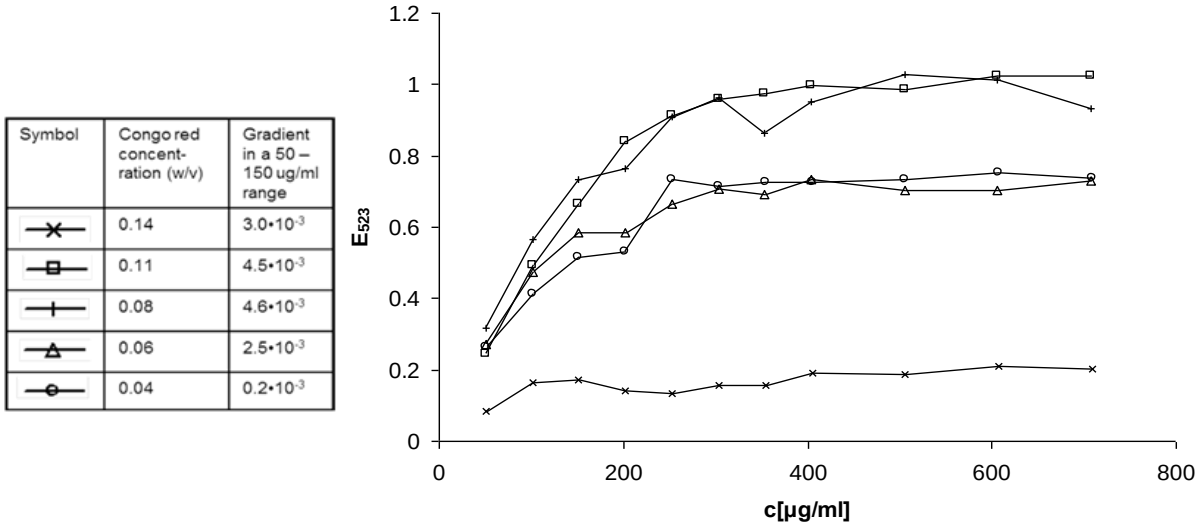

Figure 3: Correlation of different schizophyllan concentrations to the extinction of congo red and the calibration curves in the range of 50 – 150 µg/ml schizophyllan.

Figure 3 shows the dependence of different schizophyllan concentration to the extinction and compares the gradient of the calibration curves in the range of 50 – 150 µg/ml schizophyllan. The results show that a congo red concentration of 0.08 % (w/v) is most sensitive.

Therefore, this concentration is used for further analysis. A calibration curve in the range of 50 – 150 µg/ml schizophyllan is shown in Figure 4.

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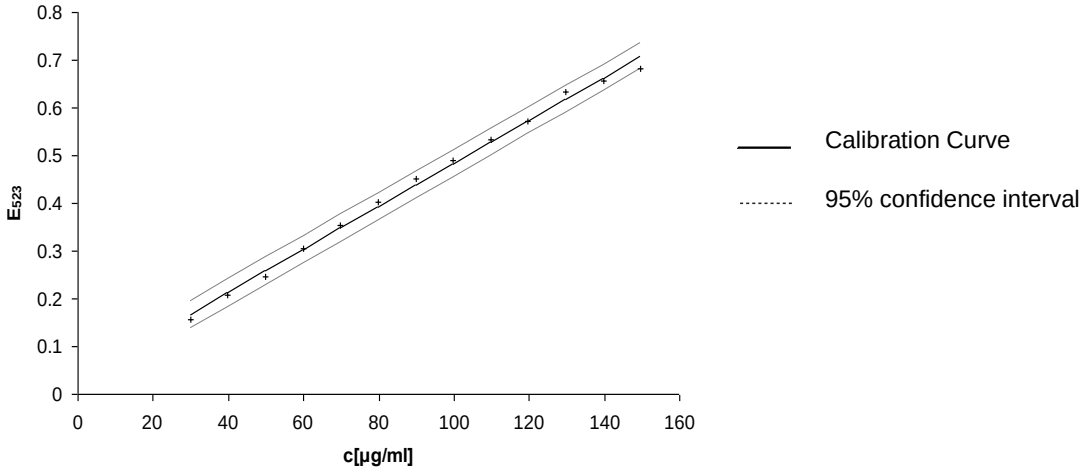

Figure 4: Calibration curve of congo red with schizophyllan with 95% confidence in the range of 50 – 150 µg/ml.

A method validation proved the applicability of the procedure, as the linearity of the calibration curve in the a spired working range of 50 – 150 µg/ml glucan was confirmed by the Mandel linearity test. The limit of detection was determined according to DIN 32 645. Afterwards the matrix influences by using all three glucan fractions of several mushrooms were analysed. The determined average recovery rate of 95.15 % showed that this method can be used for nearly all mushroom extracts. Absorption spectra which show the bathochromic shift are presented in Figure 5.

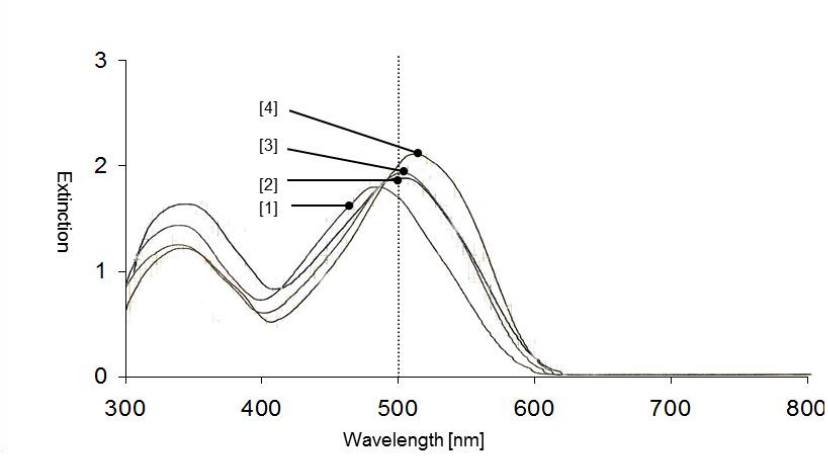

Figure 5: Absorption spectra of: [1] congo red without glucan, [2] with the sodium hydroxide extract of *Pleurotus ostreatus*, [3] 100µg/ml and [4] 200 µg/ml schizophyllan

There are two exceptions when using the KOH-fraction of *H. tessulatus* and *P. eryngii* mycelia, as no bathochromic shift could be observed. This method is appropriable for mushrooms extracts, because of recovery rates >95%. β-Glucans can be detected with good precision and sensibility, as the gradient of the calibration curve shows.

Glucane Determination: β-1,3-1,6-Glucan and β-1,3-Glucan Content of Various Basidomycetes. The total amount of congo red positive β-1,3-1,6-glucans of the mycelia from different basidomycetes, as presented in Table 3, differ from 0.4 – 4,0 g per 100 g for the mycelia and 1.9 – 12.9 g per 100 g dry mass for the fruiting bodies (Table 4)

Table 3: Total-β-glucan and triplehelicale-β-glucan content of mycelia from various mushrooms

Mushroom (Mycelium)	Total-β-Glucan [g/100 g DM]	Triplehelical-β-Glucan (β-1,3-1,6-glucans) [g/100 g DM]
Lentinula edodes	4,2 ±0.1	2,6 ±0.1
Hypsizygus tessulatus	4.2 ±0.1	2.1 ±0.1
Pleurotus pulmonarius	2.5 ±0.1	0.4 ±0.0
Grifola frondosa	4.9 ±0.3	1.8 ±0.1
Pleurotus ostreatus	4.6 ±0.1	3.0 ±0.1
Trametes versicolor	6.8 ±0.2	3.3 ±0.1
Agaricus bisporus	3.8 ±0.2	3.4 ±0.1
Morchella esculenta	4.0 ±0.2	4.0 ±0.2

The maximal difference between the various species is 87.5% for mycelia and 85 % for fruiting bodies. Furthermore, the β-1,3-glucan contents of the mycelium samples, as indicated in Table 3, too, vary between 2.5 g - 4.2 g in mycelia and between 3.8 – 13.5 g per 100 g dry mass for the fruiting bodies (Table 4). *Pleurotus pulmonarius* has the lowest content. In contrast, the Polyporales mushroom *T. versicolor* has the highest content of β-glucans. The contents in fruiting bodies are as mentioned notably higher, however an exception occurs. *Agaricus bisporus* has a lower content than the other analysed fruiting bodies. It is the only mushroom that has a higher content in its mycelia than in its fruiting body. *Agaricus bisporus* is the only secondary decomposer which we analysed. The other mushrooms are primary decomposer. The nutrient uptake may play an important role in cell wall synthesis and therefore in the glucan content and composition. Furthermore, the content of *G. frondosa* is not higher than the contents of *A. bisporus*. *M. esculenta*, the analysed ascomycete, has equal contents as the analysed Basidomycetes. That means that there is no correlation between family, genus or even division and total-β-1,3-glucan content.

Table 4: Total-β-glucan and triplehelical-β-glucan content of fruiting bodies from various mushrooms

Mushrooms (frutiing bodies)	**Total-β-Glucan [g/100 g DM]**	**Triplehelicale-β-Glucan [g/100 g DM]**
Lentinula edodes	9.5 ±0.1	9.5 ±0.1
Flammulina velutipes	9.0 ±0.1	7.8 ±0.1
Pleurotus eryngii	13.5 ±0.1	12.9 ±0.4
Hypsizygus tessulatus	9.1 ±0.3	7.0 ±0.3
Pleurotus ostreatus	9.1 ±0.2	8.3 ±0.3
Agaricus bisporus	3.8 ±0.2	1.9 ±0.0

Table 3 and 4 show that the amount of the important triple helical β-1,3-1,6-glucans on the total-β-1,3-glucan content is different in mycelia and fruiting bodies. The amount of β-1,3-1,6-glucans comports a maximum of 64.0 % and is in average 45 % in mycelia, while in some fruiting bodies like *L. edodes* and *P. eryngii* almost all β-1,3-glucans have a triple helix structure. The other fruiting bodies also have higher amounts of β-1,3-1,6-glucans than the mycelia. The average β-1,3-1,6-glucan amount is 88 %.

DISCUSSION AND CONCLUSION

A reliable specific colorimetric determination for triple helical β-glucans offers the first possibility to analyse β-1,3-1,6-glucans with high precision, fastly and without extensive clean up. Together with an fluorimetric method, both the triple helix β-1,3-1,6-glucans and the total-β-1,3-glucan content of basidomycetes can be compared. The used fluorimetric determination is also fast and our work proves that it is applicable for all mushroom samples. The results are reasonable and can be compared to existing data. The isolation process used by our group is quite similar to the processes described in several publications, so the obtained results can be compared with results of recent research.

Manzi et al [8, 9] quantified β-1,3-glucan contents of various fruiting bodies with an enzymatic hydrolysis of isolated mushroom cell walls and then analysed the released free reduced sugars. With this method 0.58 g for *P. pulmonarius*, 0.38 g for *P. eryngii* and 0.22 g/100 g for *L. edodes* on a dry mass basis were determined. The limitation of this direct enzymatic hydrolysis has been discussed previously [12]. Consequently, other groups first extracted the polysaccharides and then digested the glucans by β-glucanases [11, 13]. Their results show that the fruiting bodies of *Inotus obliquus* contain 8.3 g and of *Agaricus brasiliensis* 10.1 g β-1,3-glucans per 100 g dry mass. Even though these species were not analysed in our present work the contents are comparable with those of our work for related species.

Recent works showed that most of fungal β-glucans are highly branched β-1,3-1,6-glucans with a triplehelical structure [4, 12]. The presented analyses support that a triple helix is the dominant form of β-glucans in most mushrooms.

There is less information on the quantity of glucans in mushrooms that can be compared with our results. With an ELISA method, the content of β-glucans of the fruiting bodies from *L. edodes* and *G. frondosa* were analysed by Zhang [4]. Their results showed that *G. frondos*a contains 2.4 g, *L. edodes* 3.4 g and *F. velutipes* fruiting bodies 6.4 g β-glucans per 100 g dry mass. These values are comparable to our analyses with the direct determination with congo red of these mushrooms.

Several fruiting bodies and mycelia were examined and in summary the results support that the fruiting bodies have the most β-glucans and the highest amount of β-1,3-1,6-glucans, too. The high amount of triplehelical β-glucans in fruiting bodies corresponds to current structure examinations. They show that β-glucans of *P. ostreatus* [11] and *L. edodes* [4] mainly consist of highly branched β-1,3-1,6 -glucans with a triplehelical structure.

The high amounts of β-1,3-1,6 glucans in *L. edodes* and *P. ostreatus* manifest their role as important medical mushrooms. But the analyses support that other edible mushrooms such as *M. esculenta*, *H. tessulatus* and *P. eryngii* have comparable β-glucan quantities. Comparisons between different families, genus and division show that there are no real correlations.

Because of the notable higher amounts of β-glucans in fruiting bodies, a technical isolation of glucans from fruiting bodies seems preferable from this point of view. On the contrary the mycelium is a fast growing raw material, which grows with less effort. For fructification an induction period is necessary, which is expensive and time-consuming, while the mycelium is a renewable resource for β-glucan isolation.

A reliable and specific determination for chitin and chitosan is also achieved. The average chitin content in the analysed mushroom species is about 2.5 g/100 g for the mycelia and 3.5 g/ 100g for the fruiting bodies. Some exceptions occur: the fruiting bodies of *A. bisporus*, *F. velutipes and P. eryngii* have notably higher chitin content and the mycel of *A. bisporus* and *P. eryngii* contain the highest amounts of chitin. As the culture conditions for all mycelia are similar, this seems to be a characteristic for the particular species.

A correlation between chitin content and mushroom systematics like species, family or order could not be observed. The results can be compared with data recently published: Chen [22] has determined high concentrations of chitin in basidiomycetes with the Ride and Drysdale method [23] for quantification and found a content of 21.8 % for the mycelium of *L. edodes.* They did not look at the proportion of other aminohexoses that could additionally be detected with this method. Manzi et al. [8, 9] also used the Ride and Drysdale method [23] to quantify the chitin content as N-acetylglucosamine after hydrolysis with hydrochloric acid. For *A. bisporus* they measured 0.32 % and for *P. ostreatus* 0.43 % on a fresh weight basis. Because of the variation in water content of the mushrooms, comparison with our data is not possible. Vetter *et al.* [24] applied the Smith and Gilkerson method [25] to determine chitin in several wild growing and cultivated mushrooms by measuring the N-acetylglucosamine released by hydrolysis. They quantified chitin in a range from 0.1 to 9.7 g per 100 g of dry mass, including *P. ostreatus* with 2–3 g of chitin. The quantifications by our new method, where the optical density of the insoluble polyiodide–chitosan complex is measured, are comparable with these data.

Vetter later [26] quantified 6–8%, 2–5% and 5–6% chitin for different variations of *A. bisporus*, *P. ostreatus* and *L. edodes* fruiting bodies on a dry-mass basis. The variation in chitin content even in different varieties is remarkable. However, the magnitude of the chitin contents is again comparable to our data. Chen and Johnson [27] measured a chitin content for *Schizophyllum commune* fruiting body of 9.6% on a dry-mass basis. We did not analyse this specific mushrooms, but we measured equal amounts in related mushrooms such as *A. bisporus* or *F. velutipes*. Ofenbeher-Miletic´ *et al.* [28] determined chitin in wild-growing mushrooms. They quantified the N content in purified dietary fibre from fruiting bodies and then calculated the chitin content. Even though our work concentrates mainly on commercially available mushrooms, their measured amounts are comparable with our analyses, with chitin contents in a range of 1.9–13.6% on a dry mass basis. Furthermore, Dikeman *et al*. [29] quantified 7.7% chitin in the dry mass of the fruiting bodies of *F. velutipes* by using HPLC to determine the N-acetylglucosamine after digestion with chitinase. We determined a comparable chitin content of 9.8 % of dry mass.

These comparisons of known methods reveal similarity to our results, but in contrast to the other methods, we have established a method to analyse both chitin and chitosan directly with high precision. Chitosan should be dissolved in acetic acid, after a cleanup with sodium hydroxide, whereas chitin needs deacetylation to chitosan with highly concentrated alkaline solutions. Though the conversion of chitin to chitosan takes up to 3 h, it could be proven that the conversion was complete. We also proved that this assay is specific for cell extracts from mushrooms. Cross-reactions were not observed. We added up to 5 mg/mL of bovine serum albumin to various chitosan standards, but it did not affect the determination. We used an enzymatic starch assay to prove that our samples are starch free. As there are no starch and starch derivates in our mushroom samples, no other colour formation can occur.

The high amount of β-1,3-1,6-glucans could result in a more solid cell wall. This could be necessary for the compact fruiting bodies. On the other hand the high amount of single helix glucans could result in a more flexible cell wall, which is important for the mycelia for nutrient-uptake purposes.

ACKNOWLEDGEMENTS

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PROPERTIES OF GLUTAMATE DECARBOXYLASE (GAD) FROM EDIBLE MUSHROOM

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ABSTRACT

Enzymatic properties of glutamate decarboxylase (GAD) [EC4.1.1.15] obtained from the fruiting body of *Flammulina velutipes* were evaluated via protein isolation, purification, and characterization. To investigate the anatomical localization of GAD, crushed fruiting bodies were centrifuged; the supernatant and precipitate were subsequently subjected to enzyme reaction. GAD activity of the precipitate was much stronger than that of the supernatant. Although Nonidet P40 slightly solubilized GAD protein from cell wall fractions, most of the activity remained in the cell debris. A nearly homogeneous protein band was observed in SDS-PAGE analysis after 10 treatments of solubilization and subsequent purification with ammonium sulfate precipitation and ultrafiltration. In cell wall-binding enzyme experiments, formation of γ-amino butyric acid (GABA) was observed between pH 4 and 6, and the maximum GAD activity was observed at pH 6. However, GAD activity was lost after overnight dialysis against buffering of pH 6-10. The enzyme activity was optimum at 28°C and stable below 50°C. GAD of *F. velutipes* was specific for L-glutamate.

Key words: glutamate decarboxylase (GAD), *Flammulina velutipes*, γ-amino-butyric acid (GABA)

INTRODUCTION

Glutamate decarboxylase (GAD; EC4.1.1.15) is a pyridoxal enzyme and produces γ-amino-butyric acid (GABA) from glutamate. GABA and GAD are widely distributed in mammals [1, 2], plants [3], and microorganisms [4-9]. GABA has several physiological effects on the human body, including neurotransmitting, hypotensive, and diuretic effects. Several attempts to enrich GABA in functional foods have been reported: GABA-rich green tea produced by anaerobic treatment of green tea [10], GABA accumulation in red mold rice [11], GABA accumulation in rice germ by soaking in water [12], GABA enrichment in brown rice by high-pressure treatment [13] and GABA production by lactic acid bacteria [14].

The major enzymatic properties of GAD have been previously described [1-3, 5, 6, 9, 15]. Although many edible mushrooms accumulate GABA in their fruiting body, only a few studies have reported mushroom-derived enzyme.

In this study, we investigated the purification and enzymatic properties of *Flammulina velutipes* GAD.

MATERIALS AND METHODS

Materials. Fruiting bodies of *F. velutipes* used in this study were purchased in local supermarkets in Nara prefecture, Japan and stored frozen until use.

Crude enzyme preparation. Frozen fruiting bodies were cut into small pieces (ca. $1cm^3$) and crushed with 50 mM phosphate buffer (pH 5.5) containing 1 mM pyridoxal-5'-phosphate (PLP) and 1mM phenylmethylsulfonyl fluoride (PMSF). This homogenate was centrifuged for 30 min at 15,000 × *g* at 4°C. The resultant supernatant and cell debris were used as a crude enzyme source of GAD after dialysis.

Enzyme assay. Enzyme solution (0.1 ml) was mixed with 0.9 ml of substrate solution (50 mM phosphate buffer, pH 5.0, containing 10 mM sodium glutamate and 50 □M PLP) and incubated overnight at 28°C. The enzyme reaction was stopped by eliminating the cells from solution. Then, the formation of GABA was checked by thin-layer chromatography. The activity of GAD was estimated semi-quantitatively by color spot intensity.

Solubilized GAD enzyme preparation. Cell wall debris was obtained as precipitate of crushed and centrifuged fruiting bodies. The precipitate was washed 3 times with twice the volume of 50 mM phosphate buffer (pH 5.5) containing 1 mM PLP and 2% Nonidet P40. GAD enzyme was solubilized by washing 5 times with twice the volume of the same buffer. Solubilized enzyme solutions were combined and subsequently precipitated with ammonium sulfate precipitation at 70% saturation. Isolated proteins were dissolved in a small volume of 20 mM phosphate buffer (pH 5.5) and subjected to concentration by UF membrane.

SDS-PAGE analysis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [16] using 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS. Molecular weight standards used were SDS-PAGE standards (Bio-Rad Laboratories), and protein bands were detected by Coomassie Blue G-250 staining.

pH and temperature analysis. The optimum pH for enzyme activity was determined with 0.05 M acetate buffer (pH 4.0 and 5.0), 0.05 M phosphate buffer (pH 6.0 to 7.0), 0.05 M Tris-HCl buffer (pH 8.0 to 9.0) and 0.05 M borate buffer (pH 10.0). The effect of pH on enzyme stability was elucidated by dialysing the enzyme overnight at 4°C in the same buffers described above. The effects of temperature on GAD activity were examined under standard enzyme assay conditions except that the temperature was varied from 4°C to 60°C and the reaction time was overnight. To estimate thermal stability, the enzyme was incubated for 30 min at various temperatures (4°C to 70°C), and the residual enzyme activity was assayed under the standard assay conditions.

RESULTS AND DISCUSSION

Localization and solubilization of GAD. To clarify enzyme localization within the fruiting bodies, GAD activity of both supernatant and cell debris was assayed. When cell debris was used as an enzyme source, strong GAD activity was observed (Fig. 1). In contrast, supernatant fraction did not show apparent GABA spots. This result demonstrated that *F. verutipes* GAD is a cell wall-binding enzyme. A similar cell wall binding type of GAD was reported in *Aspergillus oryzae* [6]. In the case of *Aspergillus*, cell wall-degrading enzyme mixtures (Yatalase and lysozyme) effectively solubilized the GAD

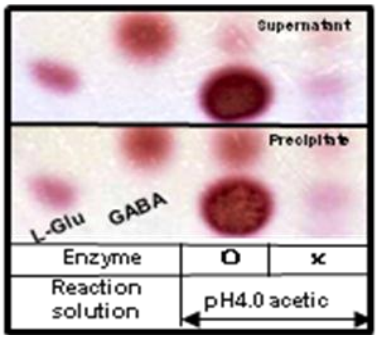

Figure 1. Localization of GAD activity.
The reaction was carried out in 50 mM acetate buffer (pH4.0) with 10 mM sodium glutamate and 50 μM pyridoxal-5'-phosphate (PLP)) at 37°C for overnight.

enzyme from cell walls. However, cell wall-degrading enzyme treatments with Yatalase were not effective for *F. velutipes* GAD because apparent GAD activity was not detected in Yatalase-treated supernatant.

In order to solubilize GAD activity, several detergents and cell-degrading enzymes were tested. Although Nonidet P40 (Sigma) was the best reagent for solubilization, its efficiency was not robust. However, Nonidet P40 was utilized because it was the most effective solubilizer at the time of this study.

For GAD enzyme solubilization, cell debris was treated with 50 mM phosphate buffer (pH 5.5) containing 1 mM PLP and 2% Nonidet P40. This procedure was repeated 5 times, and resulting supernatants were pooled as enzyme solution. A large part of GAD activity remained in cell wall fractions after solubilization treatment procedures. Crude enzyme proteins were precipitated from combined solution by ammonium sulfate with 30–70% saturation. The precipitated protein pellet was dissolved in a small amount of 20 mM phosphate buffer (pH 5.5) and subjected to concentration by UF membrane.

Enzyme purity and molecular weight of GAD. To check the purity of solubilized GAD, UF-concentrated samples were subjected to SDS-PAGE analysis. A single major protein band and several minor bands were observed (Fig. 2). The molecular weight of this major band, presumably GAD, was estimated to be approximately 30 kDa.

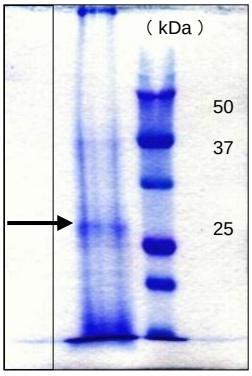

Figure 2. SDS-PAGE analysis.
The arrow shows the band of presumed GAD.

Enzymatic properties of GAD. GAD protein was the most abundant protein bound to cell wall fractions after treatment with Nonidet P40. This result led to the selection of cell debris as an enzyme source for pH, temperature, and substrate specificity evaluation.

Figure 3 depicts the effect of pH on GAD activity. GAD activity was detected at pH 4 to pH 6, and GABA spot color intensity was not different within this range. However, GAD activity was lost after dialysis in the buffer at pH 6. Effects of temperature are shown in Figure 4. GAD activity was detected between 4-60°C. Color spot intensity was highest at 28°C, stable below 37°C, and slightly decreased at 60°C.

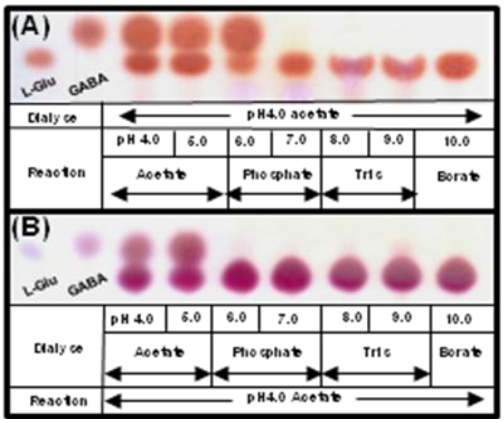

Figure 3. Effect of pH on enzyme activity.
The optimum pH (A) of the enzyme was determined with each indicated buffer. The effect of pH on enzyme stability (B) was elucidated by dialysing the enzyme overnight at 4°C in the indicated buffers.

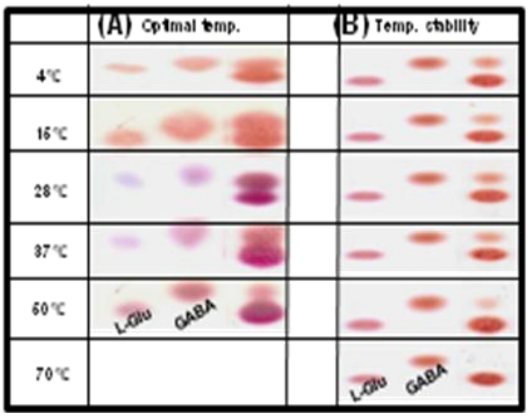

Figure 4. Effect of temperature on enzyme activity.
The effects of temperature (A) on the GAD activity were examined under the standard enzyme assay conditions at indicated temperatures. For thermal stability (B), the enzyme was incubated for 30 min at indicated temperatures and the residual enzyme activity was assayed under the standard assay conditions.

To elucidate enzyme substrate specificity, several α-amino acids listed in Figure 5 were added to the reaction mixture. If α-decarboxylation occurred, correspondent products would be detected. However, L-glutamate was a specific substrate for GAD; therefore, no other substrate was used (Fig. 5)

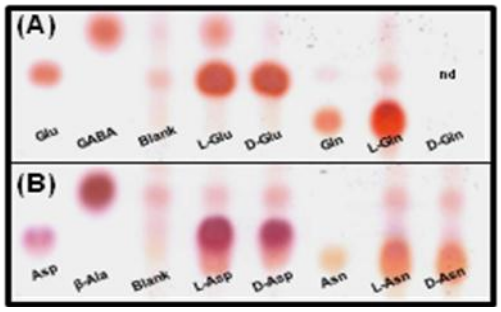

Fig. 5. Substrate specificity.
The enzyme was incubated with the indicated amino acid in 50 mM acetate buffer at 28°C

Table 1. Comparative properties of GADs derived from several origins.

	MW (KDa)	Subunit MW (KDa)	Opt. pH	Opt. Temp. (°C)	Spec. Act. (U/mg)	*K*m for L-glu (mM)	Ref
Flammulina verutipes	nd	30	4 - 6	28	nd	nd	This study
Aspergillus oryzae	300	48	5.5	60	48.2	13.3	[6]
Lactobacillus brevis	120	60	4.2	30	6.0	9.3	[5]
Escherichia coli	300	-	3.8	-	-	0.8	[4]
Escherichia coli	310	50	4.5	67.9	-	1.0	[7]
Squash	340	58	5.8	60	25.8	8.3	[3]
Human brain	140	67	6.8	-	1.0	1.3	[1]

Table 1 shows comparisons between GADs partially purified from *F. velutipes* and those from other organisms. At optimum pH and temperature, GAD from *F. velutipes* was more similar to that from *L. brevis.* The molecular weight of *L. brevis* GAD lesser than that of all GAD proteins listed in Table 1.

CONCLUSION

GAD was partially purified from *F. vertipes*, and its enzymatic properties were elucidated. *F. vertipes* GAD was strongly bound to cell walls and difficult to solubilize. Although Nonidet P40 could slightly solubilize GAD protein from cell wall fractions, most of the activity remained in the cell debris. A nearly single protein band was observed in SDS-PAGE analysis after 10 solubilization treatments and subsequent purification procedure with ammonium sulfate precipitation and ultrafiltration. Cell wall-binding enzyme experiments revealed GABA formation between pH 4 and 6, with the maximum GAD activity occurring at pH 6. However, GAD activity was lost after overnight dialysis against buffered at pH ranging from 6–10. The enzyme activity was optimum at 28°C and stable below 50°C. GAD of *F. velutipes* was specific for L-glutamate.

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GC-MS AND GC-OLFACTOMETRY ANALYSIS OF AROMA COMPOUNDS EXTRACTED FROM CULTURE FLUIDS OF *ANTRODIA CAMPHORATA*

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ABSTRACT

A comprehensive inventory of the organic components and aroma-active compounds produced by *Antrodia camphorata* during growth in submerged culture has been established by extracting culture fluids using three different organic solvent systems and subjecting the extracts to gas chromatography–mass spectrometry (GC–MS) and gas chromatography–olfactometry (GC-O). Forty-three organic components, of which esters, alcohols, acids and ketones were the most prevalent, were identified in pentane/ether (1/1, v/v) extracts. The most representative of *A. camphorata* aroma-active compounds were detected in pentane/ether and ether extracts (eleven and nine aroma-active compounds, respectively) by GC-O. Of these, ethyl acetate, γ-undecalactone, linalool and 3-hydroxy-2-butanone were assessed to be present at the highest intensity.

Keywords: Aroma analysis; Representative aroma extract; Sensory evaluation; Submerged culture.

INTRODUCTION

The basidiomycete, *Antrodia camphorata*, is a rare and expensive mushroom assigned to the family Polyporaceae (Aphyllophorales). The fungus is native to Taiwan, where it grows in the walls of the inner heartwood of *Cinnamomum kanehirai* Hay [1, 2], and has been ascribed various medicinal properties [3, 4. Our research has also revealed that submerged cultures of *A. camphorata* are highly odoriferous, suggesting that the mushroom might serve as an important source of natural aroma compounds for the food and cosmetic industries. Therefore, in the present study, we have first determined the effectiveness of three solvent systems for extracting a comprehensive inventory of organic compounds from culture fluids generated after submerged culture of *A. camphorata* mycelium under specified growth conditions. We have then used gas chromatography–mass spectrometry (GC–MS) combined with gas chromatography–olfactometry (GC-O) to identify the most important aroma active compounds.

MATERIAL AND METHODS

Fungal strain and culture conditions. *Antrodia camphorata* was obtained from the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences and grown on potato dextrose agar (PDA) slopes at 26^{O}C for 2 weeks and stored at 4^{O}C. Discs (0.5 cm) of agar containing fungal mycelium grown on PDA plates at 26 ^{O}C for 2 weeks were used as inocula for submerged cultures.

Submerged cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml basal medium consisting of (w/v): 4.0% glucose, 0.6% soybean, 0.1% K_2HPO_4, 0.05% $MgSO_4$ and 0.01% vitamin B_1. Mycelium was removed after incubation at 25 ^{O}C for 5 days and the culture fluid was retained.

Extraction of volatile compounds. Culture filtrates (100 ml) were extracted four times using three different organic solvents: diethyl ether (Extract E), a mixture (1:1, v/v) of pentane/diethyl ether (Extract P/E), and a mixture (2:1, v/v) of pentane/dichloromethane (Extract P/D). After extraction, the upper organic phases were separated, dried over anhydrous sodium sulphate, concentrated at 42 ^{O}C to 1 ml, and stored at –20 ^{O}C prior to GC–MS and GC-O analyses. All extractions were performed in triplicate.

Gas chromatography–mass spectrometry (GC–MS). Volatile components were identified by GC-MS using a Finnigan TRACE GC-MS (Thermo Quest Finnigan Co., USA) equipped with a DB-Wax capillary column (60 m × 0.32 mm). Helium (flow rate, 1.0 ml/min) was used as the carrier gas, and injection volumes were 1 µl. The column temperature was maintained initially at 40 ^{O}C for 3.5 min, followed by increases to 60 ^{O}C at a rate of 5 ^{O}C/min, from 60 to 120 ^{O}C at a rate of 6 ^{O}C/min, and from 120 to 230 ^{O}C at a rate of 8 ^{O}C/min, and then this temperature was held constant for 12 min. The electron impact energy was 70 eV and the ion source temperature was set at 230 ^{O}C. Electron impact (EI) mass spectra were recorded in the 33-450 aMU range at 1 s intervals.

Sensory evaluation of extracts. Sensory evaluation of aroma extracts obtained with the three organic solvent systems was performed to select extracts representative of the odour of *A. camphorata* culture filtrates. Samples were prepared by placing 1 ml of *A. camphorata* culture filtrate or an aroma extract in a brown flask, eliminating the solvent from the latter under a nitrogen flux, and hermetically sealing the flask prior to evaluation. The evaluation was carried out using triangular tests by a panel of 15 assessors (seven females and eight males) selected at random from university students with natural olfaction. The significance of the test was evaluated by binomial distribution using published tables [5] (Stone & Sidel, 1985).

GC–Olfactometry (GC-O). GC–O analyses were conducted using a Finnigan TRACE GC (Thermo Quest Finnigan Co., USA) equipped with a sniffing port. Column type and analysis conditions were as described above, and the temperature of the sniffing port was 250 ^{O}C. Sniffing tests on *A. camphorata* E and P/E extracts in combination with reference compounds were performed by two trained and experienced testers in paired alternate chromatographic runs conducted at 15 min intervals. Effluent from the GC containing the separated compounds was diluted with humidified air, and qualitative and semi-quantitative odour evaluation was carried out for each analyte leaving the chromatographic column during the entire GC analysis [6] (Pollien *et al*. 1997). Data were recorded only in cases where the testers assigned the same aroma attribute.

Table 1. Volatile compounds detected in aroma extracts from *Antrodia camphorata* obtained using different organic solvents

Compound	E	P/E	P/D	Compound	E	P/E	P/D
Esters				4-Hydroxybenzene ethanol	+		
Ethyl acetate	+	+	+	***Acids***			
2-Hydroxypentanoic acid ethyl ester	+	+		Acetic acid		+	
Carbitolacetate		+		4-*tert*-Butylcyclohexyl acetate		+	
2, 6, 10, 14-Tetramethyl pentadecanoic acid methyl ester		+		4-Methyl hexanoic acid		+	
Octadecyl acetate		+	+	2-Hydroxy-2-methylbutyric acid		+	
Hexadecanoic acid-1-methyl ethyl ester	+	+		Neodecanoic acid	+	+	+
γ-Undecalactone	+	+	+	3-Mercaptopropionic acid			+
Methyl formate	+			Hexadecanoic acid	+		
Phthalic acid diisobutyl ester		+	+	Caprylic acid	+		
4-Decanoic acid methyl ester			+	Tetradecanoic acid	+		
γ-Decalactone			+	2-Methylene-4-hydroxybutyric acid	+		
2, 2-Dimethyl-3-oxobutyric acid methyl ester			+	***Ketones***			
Acetic acid 3, 7, 11, 15-tetramethyl-hexadecyl ester			+	3-Hydroxy-2-butanone	+	+	+
Octadecanoic acid 2-2-hydroxyethoxy) ethyl ester			+	2 (1-Methyheptyl) cyclopentanone		+	
Alcohols				2, 5-Furandione		+	
1, 2-Propanediol		+		2-Butanone		+	
2-Butanol	+		+	1-(4-Methylphenyl)-1-pentanone	+		
3-Methyl-3-butylene-1-ol	+			4-Hydroxy-2-butanone	+		
1, 3-Butanediol	+			2-Furyl methyl ketone	+		
3-Methyl-2-pentanol		+		***Aldehydes***			
4-Methyl-2-pentanol	+			Furfural	+	+	+
3-Methyl buten-1-ol	+	+		5-Hydroxymethyl-2-furaldehyde	+	+	
3-Methyl-3-octanol	+			2, 4-Dimethyl benzaldehyde	+		

Table 1- Continue

2, 3-Butanediol	+			***Hydrocarbons***			
2-Phenylethyl alcohol	+			Heneicosane	+	+	+
Linalool	+	+	+	Undecane		+	
α-Terpineol	+	+		Docosane		+	
[E]-Nerolidol		+		1, 3, 5-Trioxacycloheptane	+	+	+
T-Cadinol	+	+		***Heterocyclic***			
Cubenol	+			2, 4, 5-Trimethyl-1, 3 dioxolane	+	+	
4-Methyl-5-thiazole ethanol		+	+	2-Methyl-1, 3 dioxolane		+	
8-Hydroxylinalool	+	+	+	4, 4-Dimethylcyclooctene		+	
7-Methyl-3-propyl-2, 6-decadien-1-ol	+	+		6-Methyl-3, 5-dihydroxy-2, 3-dihydro-4H-pyran	+	+	+
3-Methyl-2-butanol		+		Hydroquinone	+		
2-Hexyl-1-decanol		+		3, 4-Dimethoxyphenol	+		
[E] 9-Hexadecen-1-ol		+		1-Hydroxy-2-acetyl-4-methylbenzene	+		
2-Tetradecyl alcohol		+		Butylated hydroxytoluene	+		
Isobutyl alcohol		+					

(E) Ether extract, (P/E) Pentane/ether extract, (P/D) Pentane/dichloromethane extract. +, detected.

RESULTS AND DISCUSSION

Clear differences were observed in the organic components extracted from *A. camphorata* culture fluids using the three different solvent systems, and these probably reflected the different solvent polarities. The highest number of components (43) was detected in P/E extracts, while solvent E contained 40 and solvent P/D contained only 20 (Table 1). Organic components identified in P/E extracts consisted of 8 esters, 16 alcohols, 5 acids, 2 aldehydes, 5 heterocyclic compounds, 4 hydrocarbons and 5 ketenes. Solvent E extracts contained 5 esters, 15 alcohols, 5 acids, 3 aldehydes, 6 heterocyclic compounds, 2 hydrocarbons and 4 ketones.

Esters and alcohols that were predominant in both P/E and E extracts were 2-hydroxypentanoic acid ethyl ester, hexadecanoic acid-1-methylethyl ester, 3-methyl buten-1-ol, α-terpineol, T-cadinol and 7-methyl-3-propyl-2,6-decadien-1-ol. These compounds along with 5-hydroxymethyl-2-furaldehyde and 2, 4, 5-trimethyl-1, 3-dioxolane were identified in E and P/E extracts but not in the P/D extract. Octadecyl acetate, phthalic acid diisobutyl ester and 4-methyl-5-thiazole ethanol were identified in the P/E and P/D extracts, but not in the E extract. Ethyl acetate, γ-undecalactone, linalool, 8-hydroxylinalool, neodecanoic acid, 3-hydroxy-2-butanone, furfural, heneicosane, 1, 3, 5-trioxacycloheptane and 6-methyl-3, 5-dihydroxy-2, 3-dihydro-4H-pyran were identified in all three extracts. All the above compounds have hitherto not been reported in *A. camphorata*.

Sensory evaluation involving 15 assessors using triangular tests detected no differences in the aroma of E and P/E extracts compared with the original culture filtrates ($p < 0.05$). Accordingly, E and P/E extracts were chosen for GC-O analysis and quantification of the aroma-active compounds. Testers perceived thirteen aroma active compounds in these extracts. Seven of these were recorded in both extracts: ethyl acetate (sweet), γ-undecalactone (peach, sweet), linalool (citrus-like, fresh floral) and 3-hydroxy-2-butanone (buttery, milky) at strong intensities, α-terpineol (deal, clove) and furfural (almond, spicy) at medium intensities, and T-cadinol (spicy) at weak intensity. 2-Phenylethyl alcohol (soft rose, floral) and cubenol (spicy) were found only in extract E at medium and weak intensities, respectively. Isobutyl alcohol (fresh, spicy), *[E]*-nerolidol (mild floral), 4-methyl-5-thiazole ethanol (meaty, spicy) and 1,2-propanediol (floral, pollen) were all identified only in extract P/E, the first three at medium and the latter at weak intensities. [7] Chang *et al*. (2001) reported that *A. camphorata* mycelium contained high concentrations of soluble sugars and the flavour 5'-nucleotides, 5'-guanosine monophosphate (5'-GMP) and 5'-xanthosine monophosphate. Soluble sugars contribute a sweet taste and 5'-GMP a meaty flavour to mushrooms [8] (Litchfield, 1967). The related *Antrodia* species, *A. malicola* and *A. xantha*, were previously reported to have 'faintly fragrant' and 'lemon-like' aromas, respectively but the chemical components responsible were not identified [9] (Jong & Birmingham, 1993).

Although distinctive odours have long been used as taxonomic markers for mushroom identification, the potential of higher fungi for the industrial production of natural aroma compounds has remained largely unexploited. Ethyl acetate, γ-undecalactone, linalool and 3-hydroxy-2-butanone were adjudged to make a major contribution to the special aroma character of *A. camphorata* culture fluids while, interestingly, none of the C-8 alcohols (e.g.1-octen-3-ol) commonly found in other mushrooms were detected. Although γ-undecalactone has been identified earlier in fruits, to our knowledge this is the first time the compound has been reported in fungi.

Several key volatile compounds identified by GC-MS in all the extracts, e.g. 8-hydroxylinalool, neodecanoic acid and heneicosane, were not detected in the GC-O analysis. However, some of these odorants appeared in short, complex sections of the chromatogram thereby making detection and assessment of aroma characteristics more difficult. Furthermore, GC-O does not take into account matrix effects, which can have a large impact on odorant volatility and perception [10] (Ferreira *et al*. 2002).

In view of the range of compounds present in culture fluids of *A. camphorata*, the fungus has potential value as a source of food flavors in cases where floral-fresh-fruity-milky aromas are required such as chewing gums, sweets, teas, soft and energy drinks and milk products. It could also provide fragrances essential to the cosmetic industry for the manufacture of shampoos, soaps, shower gels, body lotions, deodorants and toothpastes.

This present study was conducted on extracts of culture fluids generated after submerged culture of fungal mycelium under specified growth conditions because of their highly odiferous nature and due to difficulties in obtaining sufficient quantities of mushroom fruit bodies. Future research will study the effects of different culture conditions, including the addition of precursors, on the production of flavour compounds by *A. camphorata*.

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SAFETY EVALUATION OF *AGARICUS SUBRUFESCENS* VARIETIES AND THEIR PRODUCTS OF THERARPEUTIC INTEREST OR FOR DISEASE PREVENTION

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ABSTRACT

Mushrooms are food traditionally consumed in Europe, Asia and America. They are being studied for medicinal benefits. Extensive studies have shown that *Agaricus subrufescens* (*A. blazei* Murrill or *A. brasiliensis*) has anticancer properties. A comparative study of *Agaricus subrufescens* strains (from Brazil and France) is presented herein using *Agaricus bisporus* (champignon de Paris) as control. *In vivo* OCDE test were performed to evaluate either tolerance and/or acute and sub chronic toxicity in rats and mice.

Our data reveal that all *A. subrufescens* strains are not toxic, either *in vivo* or *in vitro*, except some locomotor hypoactivity. All show a preventing effect against carcinogenesis, including *A. bisporus.* This is the first time that this mushroom is shown to be effective, even thought it is clearly less effective than *A. subrufescens*. However no anti tumour effect is found using Balb-c mice implanted with leukaemia cells. Furthermore they elicit slight cell growth stimulation at the concentrations tested in vitro, in Hep G2 (human hepatoma cells) and Neuro 2a (mouse neuroblastoma cells). The most active is *A. subrufescens* from Brazil.

These mushrooms do have many bioactive compounds, different from the polysaccharides that need to be isolated and characterised for their curative properties following accurate evaluation of toxicological effects. Indeed there is clearly a lake of information on toxicological assessment (acute and chronic toxicity) of compound such as agaritine, blazein among others and of the whole mushroom *A. subrufescens* itself, and overall on epidemiological data linking the consumption of *Agaricus* sp and eventual prevention and/or pathologies.

Keywords: *Agaricus subrufescens*, *Agaricus bisporus*, Safety, Prevention, Rodents

INTRODUCTION

Agaricus subrufescens Peck is also known as *Agaricus blazei* Murrill and/or *Agaricus brasiliensis* Wasser [1, 2] and has other common names, such as "piedade mushroom" or "Cogumelo do sol" in Brazil, "Himematsutake" in Japan and "Almond mushroom" in United States of America. It is named as ABM in several articles. This *Agaricus* is rich in substances that are expected to modulate biological functions in mammals. Various active compounds that can potentially be used to treat or prevent different diseases have been reported [3-5]. This mushroom has been used as a medicinal food for the prevention of cancer, diabetes, hyperlipidemia, arteriosclerosis, and chronic hepatitis and is known to impact putatively the immune system [4]. Potential effective compounds of this mushroom can be isolated either from their fruiting bodies, or even from pure culture of mycelia and culture broth filtrate.

Consumption of cultivated mushrooms or their extracts is suggested to have several benefits. Paradoxically no real safety study on wild *A. subrefescens* or *A. bisporus* has been performed. ABM is currently studied and commercialised as a potential functional food or beverage (tea). Several studies on many substances of this mushroom such as polysaccharides, sterol, sodium pyroglutamate, lectins and RNA-protein complex have been reported as potential bioactive substances [1-4]. Among those molecules the most extensively studied are the polysaccharides for their anti tumour effect. One has to admit that polysaccharide structures from isolated fractions are poorly resolved and not forceful. These enriched fractions of polysaccharide could be contaminated by undetected compounds (possibly strongly cytotoxic ones). Many questions arise that fully justify the proposed studies aiming to evaluate the safe use of this mushroom and its eventual pure substances of interest.

What do we learn from the literature on *A. subrufescens* Peck, *A. blazei* Murrill and/or *A. brasiliensis* Wasser?

Table 1: Bioactive compounds from *Agaricus subrufescens*

Bioactive Compounds	Main Potential Effects	References
Polysaccharides		[3], [6], [7], [8]
β-(1,6)-glucan	Immuno-active involved in	[6]
α-(1,4)-; β-(1,6)-glucan	anti tumour effect and/or	[9], [10]
α-(1,6)-; α-(1,4)-glucan	prevention; antimicrobial	[11]
β-(1,6)-; β-(1,3)-glucan		[11]
β-(1,6)-; α-(1,3)-glucan		[11]
Glucomannan		[12]
β-(1,2)- β-(1,3)-glucomannan		[13], [14], [15]
Glucan-Protein complex	Oncogenesis prevention	[6], [9], [10]
Lectin	Anti-tumour	[17]
Riboglucan	Anti-tumour	[18]
Ergosterol	Anti-tumour	[4]
Sodium pyroglutamate	Anti-tumour	[19]
RNA-protein complex	Anti-tumour	[20]
Agaritine	Anti-tumour	[21]
Blazein	Anti-tumour	[23]

The average composition of mushrooms is normally 90% water, 2-40% protein, 2-8% fat, 1-55% carbohydrate, 3-32% fibber, yielding 8-10% ash [5]. Several categories of molecules are supposed to be involved in beneficial effects and most of the molecules categories found in *A. subrufescens (A. blazei* Murill) are common to the entire fungal kingdom. Active compounds found in *A. blazei* are listed in Table 1. The active compounds isolated from *A. subrufescens* are believed to be mainly polysaccharides [3], [6], [7], [8] β-glucan [9], [10], [11], [14] and glucomannan [12], [13], [14] or riboglucan [18] (Table 1). Additionally, in *A. blazei*, a protein (or glycoprotein), Glucan protein complex, a lectin [17], [18], originally found associated to β (1, 6) -glucan, were characterized and claimed to be anti-tumour (Table 1). Recently, it has been shown that *A. blazei* sp do contain aromatic hydrazines, especially agaritine [21] (Table 1) and its derivatives [22]. More recently, blazein a steroid derivative found in *A. blazei (A. subrufescens)*, was shown to kill Human lung cancer LU99 cells but not normal Human lymphocyte [23]. Agaritine extracted from *A. blazei* was also showed to kill leukemic cell in vitro. Its cytotoxicity is triggered cell apoptosis with an IC50 in the range of 2.7 to 16 µg /ml

depending on the cell line [24], [25]. Agartine is found at similar concentration in both *A. blazei* and *A. bisporus* (approximately 1.8 mg/g dry weight) [21].

Several authors found antibacterial properties of glucan against *Staphylococcus aureus* [25] rendering this compound an attractive immunotherapeutic agent even against diverse virus [27], [28], [29]. [30].

Further, these immune modulators properties were also described for several diverse mushrooms polysaccharides extracts [29]. This potential effect was also clearly reported for *Agaricus blazei* extracts by several authors such as for example Mizuno *et al.* [31]. Therefore it is believed that mushrooms glucans or protein-polysaccharides complex have host mediated immune-modulator properties that trigger anti-cancer and anti-microbial effects. Furthermore, the water-soluble extracts (proteo-glucans) and AndoSan from crude *A. blazei* up regulate the in vitro maturation of dentritic cells [32], [33]. Ex vivo experiment on total heparinised blood of volunteers and in vitro experiment show that the monocytes-derived dendritic cells from peripheral blood mononuclear cells produce an increase level of cytokine and chemokines. The most abundant cytokines after *A. blazei* Murill stimulation were mainly pro-inflammatory cytokine and chemocytokines IL-8, G-CSF, TNF-α, IL-1β, IL-6, IL 17 and MIP-1β. The synthesis of IL-2 and IFN γ is also reported but not of IL-12 as previously said. Therefore only some Th1-type and Th17 type cytokines were up regulated whereas no anti-inflammatory or Th2 cytokines were stimulated in vitro. Whereas ex vivo there is a release of different cytokines including Th-1, TH-2, pro inflammatory or anti-inflammatory as well as the chemokines and leukocyte growth factor. Therefore this *A. blazei* immunostimulatory extract proceed mainly by pro inflammatory chemocytokine and cytokines in vitro or ex vivo.

The inhibition of pro-inflammatory cytokines appears to be dose dependant [34]. This is in contradiction with most of the tests made in murine models with different *A. blazei* extracts. This contradiction can be explained only by the capacity of certain glucans to cross the murine intestinal barriers. Ex vivo experiment with healthy volunteers heparinised blood controversially showed stimulation of all pro-inflammatory cytokines tested as expected. More recently, a clinical test with patients suffering of inflammatory bowel disease (IBD) inflammatory disease of colon and intestine has been conducted [33]. Patients with Chron's disease (CD) widely regarded as an autoimmune disease, and ulcerative colitis (UC) were exposed orally to *A. blazei* enriched mixture (AndoSan). After 12 days ingestion, the *A blazei* extract promotes in these patients anti-inflammatory effects with no side effect.

There are opposite effects between *in vivo* and *ex vivo* assays with normal patients, pro-inflammatory cytokines and chemokines are controversially down regulated and up regulated respectively. This discrepancy are tentatively explained by the antioxidant potential of *A. blazei* [35], [36] and the limited absorption of large macromolecules complex or bioactive molecule such β-glucans across the intestinal mucosa to the reticulo-endothelial system and blood through the gastrointestinal tract. With CD and UC patients the same conclusive explanation is proposed, but the intestinal barrier is hypothesized to be presumably selectively permeable to certain β-glucans bioactive fragments to body fluids (blood and lymphoid system) as in murine [33]. This is yet too speculative. Does the presence of molecules that differ from polysaccharides or PAMPS (Pathogen Associated Molecular Patterns) exert immunosuppressive effect on the innate immune system?

It appears clearly that most of the studies were performed on mixtures of substances (even through purified) allowing these contradictory data in which the same substance does something and the contrary. Thus purification and specific studies are absolutely needed in each of the structural groups of substance.

All together it appears that *A. blazei* extracts do exert both immunostimulatory and immunosuppressive effects. Globally glucan, proteoglucan complex peptide-glucan appeared to be rather immunostimulating and promoting indirectly anti cancer benefits. Whether

immunosuppression is triggered by the same categories of molecules such as more or less branched polysaccharides or proteoglucans remains unclear and too speculative. However, because of the immunosuppressive potential, safety of chronic exposure to dietary edible commercial *A. blazei* should be evaluated.

The biological material to be studied consists of original Brazilian and European *A. subrufescens* cultivated on conventional compost using *A. bisporus* as reference material for comparison. The whole study has then been designed to investigate on mushrooms safety and new products of therapeutic interest or for disease prevention: fruitbody composition, nature of the metabolites produced by this species and their toxicological or pharmaceutical effects. Preliminary data are included herein.

MATERIALS AND METHODS

Production of mushrooms extracts. *A. subrufescens* CA454 (CGAB collection, INRA, France) formerly *A. blazei* (strain ATCC 76739) from Brazil and at the origin of commercial strains, *A. subrufescens* CA 487, European strain of French origin (CGAB collection, INRA, France) and *Agaricus bisporus* 30A (commercial strain, France Mycelium), were grown in the facilities of the research group MycSA, INRA France.

Fungal preparation for animals' treatment: A mass of 100 g of freeze-dried mushrooms was ground with a blender; powder was added to 100 mL of ultra-pure sterile water (Milli-Q plus, Millipore, France) at room temperature for 30 min with stirring to yield a suspension of mushroom (mother suspension) which was subsequently diluted to 0.5g and 0.25g/ml, used for gavages. When needed suspensions were centrifuged at 2000 g for 20 min to yield supernatants used for intra-peritoneal injections.

Fungal water extraction methods for in vitro alternative assays: Hot water hydrophilic extracts: A mass of 10 g of freeze-dried mushrooms was ground with a blender; powder was added to 100 mL of ultra-pure boiling water (100 °C) for 6 hours with stirring [37]. The suspension was then centrifuged at 2000*g* for 10 min at 20 °C. The supernatant was freeze dried and the resulting powder weighted. For *in vitro* alternative assay, a stock solution was prepared by re-suspending the powder directly in cell culture medium without foetal calf serum and dilution was made with the same medium.

Cold water hydrophilic extracts: A mass of 10 g freeze dried mushroom was ground and lyophilized. The matrix was put into a flask by adding 100 mL of sterile ultrapure water. The vial was placed 1 h at room temperature and then at 4 °C for 48 h with agitation. The suspension was then centrifuged at 2000xg for 10 min at 4 °C. The supernatant was recovered, lyophilized and the resulting powder weighted. For *in vitro* alternative assay, stock solution and diluted solutions were made as described above.

Animal testing. Male and female Swiss mice (22 ± 2 g) and male and female Wistar rats (200 ± 20g) were weighed and stall feeding one week before the test. They were divided into two groups consisting of four subgroups of five animals.

Group 1, "Evaluation of tolerance and safety": Groups of 10 males and females Wistar rats separately caged received each day for a week, 1 gram of mushroom suspension per kilogram of body weight. They were kept under observation for an additional 15 days after the end of treatment for any signs of toxicity or behavioural modification. They were weighed daily and at the end of the experiment, 21 days (n = 10). Similarly, mice have been treated to evaluate the tolerance.

Group 2, Evaluation of acute oral dose "(limit test)": Animals were divided into groups of 5 males and females separately caged and received mushroom suspension either 1 g / kg of body weight or 2 g / kg of body weight in a single intra gastric tube.
"Evaluation of acute oral dose (limit test)": The animals received by gavages a dose of either 2g/kg or 5g/kg body weight and kept under observation for two weeks. The OECD test 423 was conducted with female rats of the Sprague Dawley strain F344 (SPF Caw) of about 8 to 12 weeks at the beginning of the test. These tests were performed in GLP conditions at the Phycher-Biodevelopment Laboratory in Cestas (France).
In vivo Model to assess anti carcinogenesis effect: The rat gut carcinogenesis model of methylimidazol and phenylimidazol which are known to promote cancer has been used. F344 rats were treated with aromatic amine derivatives (methylimidazol and phenylimidazol) for one week. Then they received crude extracts of *Agaricus sp* or suspension of mushrooms powder, or extracts daily by oral route (0.25, 0.5 and 1g/kg body weight) for three months. Intestines from all animals were examined following staining with methylene blue (1% in NaCl 0.9%) to assess the pre-neoplasic changes (different foci following histological observation under microscope; magnification 40).
Preliminary test in cancer model mice: Animals, Balb-c male and female used to develop the mouse model of cancer were treated (n = 5) orally and / or by the intra peritoneal route (supernatant of mushroom suspension following centrifugation at 2000g for 20min) with a dose of extract corresponding to 0.5, 1 and 2 g of fresh mushrooms each day for 15 days. They were observed daily before treatment and kept under observation for 15 days after the end of treatment. Then 4 groups of 10 mice were treated following transplant of L1210 and/or SP2-O leukaemia cells in the peritoneal cavity. After 10 days of incubation, animals were treated daily with either pure sterile saline (NaCl, 0.9%) or extracts of *A. subrufescens* CA454, *A. subrufescens* CA487, and/or *A. bisporus* 30A. They were kept under observation until death.

In vitro assays. Cell culture: The HepG2 human hepatocarcinoma cell line (ATCC HB8065) was grown in MEM-Glutamax containing 1 g/l glucose supplemented with 10% foetal calf serum (FCS), 50 U/ml penicillin and 50 µg/ml streptomycin and 1% non-essential amino acids. The Neuro2a mouse neuroblastoma cell line was cultured in RPMI 1640 containing 2g/l glucose, 2% L-glutamine and supplemented with 10% foetal calf serum, 1 mM sodium pyruvate, 50 U/ml penicillin and 50 µg/ml streptomycin. All cells were routinely grown in 75cm^2 and incubated in humidified atmosphere consisting of 5% CO2, 95% air mixture at 37° C.
Cytotoxicity assay by MTT test: Before treatment, cells (10^5 cells/ml) were cultured (100 µl) in 96-well plates for 24 h. The medium was removed from the wells and cells were exposed (triplicates) during 48 h to different concentrations of fungal extracts amended into the medium without foetal calf serum (100µl/well). Plate design omitted marginal rows and columns and 6 wells were used for cell viability control. The cell viability was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. After treatment, the medium was replaced by a FCS-free medium containing 0.5 mg/ml MTT (Sigma) for 2 h at 37°C. The medium was discarded prior the addition of DMSO to dissolve the formazan. The absorbance was read at 495 nm with a microplate reader (LT4000 Labtech, France). Viability was expressed as the percentage of mean absorbance of the cell viability in control (100% viability).

Statistical analysis. Data were expressed as mean ± SEM or absolute value where needed and always compared to controls by using Analysis of Variance and Tukey Honestly Significant Difference tests. Statistical analysis was done by using the R software (v2.3.1, 2006) and *P* values less than 0.05 was considered to be significant.

RESULTS AND DISCUSSION

First we found during the grinding and preparation of diverse solutions of the mushrooms that the strains of *A. subrufescens* showed different colours: CA487 yielded a yellowish white suspension in cold extracts while those of CA 454 were yellow curry. Comparatively suspensions of *A. bisporus* 30A were light brown. The colour differences between the suspension of strains of *A. subrufescens* (CA 454 and CA 487) and *A. bisporus* A30 might be related to a difference in composition including that of the active substances.

Evaluation of Tolerance and Acute Oral Dose "(Limit Test)". Animals, rats and mice (group 1) showed no toxic effect during treatment or within the 15 days of surveillance following treatment, excepted, those treated with the suspension of powder of *A. subrufescens* strains at a dose of 2 g / kg. In these animals a decrease in locomotor activity and a soothing effect were observed. The gains in body weight did not change significantly compared with controls who received only water. The relative weights of major organs (liver, kidney, heart, brain, genitals) have not changed either.

Group 2 animals (rats and mice) showed no acute toxic effects with the exception of animals receiving 2 g / kg body weight of *A. subrufescens* CA454. Similarly, decrease in locomotor activity and a soothing effect were observed within 24 to 72 hours. As before there were no other changes.

The OECD 423 test conducted under the conditions of COFRAC GLP accreditation revealed in the rat a LD50> 5g/kg of body weight.

Treatment resulted in no significant effect either by the intra peritoneal route (supernatant of suspension) or by the oral route. The product is well tolerated and does not lead to toxic effect except locomotor hypoactivity with all the suspensions *of A. subrufescens* CA454 and CA487. Neither weight gain nor the relative mass of the organs were affected. Safety testing (12 weeks) and oral acute toxicity tests show a difference between CA454 and CA487. The first one involved an unexpected and currently unexplained locomotor hypoactivity. We were unable to connect this hypoactivity (during the first 2-3days) with the swelling of foot pads as described by Chan *et al.* [38] in connection with a pro-inflammatory (TNF-alpha) and hypersensitivity phenomena. Altogether these data suggest that the extraction protocol using water (room temperature, and centrifugation) is not sufficient to extract all substances of the mushrooms. Hence extraction following boiling is subsequently proposed. Indeed, Chan *et al*. [38] have performed their extract preparation in hot water (80-85°C) and finished by sterilization at 105°C for 10 min. They have certainly extracted additional substances as compared to our extracts. The confirmation of this hypothesis is underway with further solid state NMR studies of our recent extracts at high temperature.

The dose of 5 g of fresh mushrooms per kilogram body weight would make 300 g of fresh mushrooms for a person of 60 kg, without acute toxic effects. However, one cannot exclude chronic or sub chronic effects, following daily or frequent ingestion of such a dose of mushrooms or the corresponding extract, for several weeks or months.

Such a chronic toxic effect (tumour-promoting activities) has been revealed in rodents fed with feeds containing *A. blazei* Murill in Japan [23]. This finding led to a severe restriction of commercialisation of *A. blazei* Murill, whatever the form, for human consumption by the Ministry of Health, Labour and Welfare of Japan in 2006.

This apparent lack of toxicity does not exclude the presence of active substances. Indeed many situations can be considered. It can there be a substance having an effect on immunity as described in the literature, immuno stimulant [38] or immunosuppressant [33,34].

In Vivo Model to Assess Anti Carcinogenesis Effect and Preliminary Testing of Anti Cancer Properties. The administration of the *Agaricus* powder suspension or fungal extracts (0.25, 0.5 and 1g/kg Body Weight) elicits positive effect on intestinal carcinogenesis. All mushrooms were effective in preventing gut-induced carcinogenesis (Tables 2-4). The relative preventions were respectively of 72%, 64% and 56% for CA454, CA487 and A30 (Table 5). For the later this preventive effect is unexpected and indeed demonstrated for the first time. This justifies the use of *Agaricus sp* in cancer prevention by millions of people all over the world.

Table 2: Number of histological foci observed in intestinal tissues of rats treated by *A. subrufescens* suspension and/or abstracts (0.25g/kg BW) following induction of carcinogenesis process with phenylimidazolic compounds. Each number represents the sum of foci counted in 4 different fields by slice.

Treatments	**Pre neoplastic lesions observed by slices of intestinal tissue**			
	Type 1 lesions (Big size foci)	**Type 2 lesions (Foci with thick margin)**	**Type 3 lesions (Extended Foci)**	**Type 4 lesions (Foci with peripheral zones)**
Untreated Controls (n=5)	1	0	0	0
A. subrufescens CA 487 (n=5)	3	3	2	3
A. subrufescens CA 454 (n=5)	1	2	2	4
A. bisporus A30 (n=5)	4	4	2	4
Imidazol-Treated Controls (n=5)	6	9	5	8

Table 3: Number of histological foci observed in intestinal tissues of rats treated by *A. subrufescens* suspension and/or abstracts (0.5g/kg BW) following induction of carcinogenesis process with phenylimidazolic compounds. Each number represents the sum of foci counted in 4 different fields by slice.

Treatments	**Pre neoplastic lesions observed by slices of intestinal tissue**			
	Type 1 lesions (Big size foci)	**Type 2 lesions (Foci with thick margin)**	**Type 3 lesions (Extended Foci)**	**Type 4 lesions (Foci with peripheral zones)**
Untreated Controls (n=5)	1	0	0	1
A. subrufescens CA 487 (n=5)	2	3	1	5
A. subrufescens CA 454 (n=5)	2	2	1	3
A. bisporus A30 (n=5)	3	5	1	3
Imidazol-Treated Controls (n=5)	4	6	4	10

Table 4: Number of histological foci observed in intestinal tissues of rats treated by *A. subrufescens* suspension and/or abstracts (1g/kg BW) following induction of carcinogenesis process with phenylimidazolic compounds. Each number represents the sum of foci counted in 4 different fields by slice.

Treatments	Pre neoplastic lesions observed by slices of intestinal tissue			
	Type 1 lesions (Big size foci)	**Type 2 lesions (Foci with thick margin)**	**Type 3 lesions (Extended Foci)**	**Type 4 lesions (Foci with peripheral zones)**
Untreated Controls (n=5)	0	1	0	1
A. subrufescens CA 487 (n=5)	4	3	2	2
A. subrufescens CA 454 (n=5)	3	2	3	2
A. bisporus A30 (n=5)	4	5	3	1
Imidazol-Treated Controls (n=5)	8	10	6	7

Table 5: Sum of all types of foci for each mushroom and percent of prevention following normalisation of values, taking into account that 5 foci/group of 5 animals is not significant.

Treatment	Total pre-neoplasic foci	% of prevention
Untreated Controls (n=15)	5	100
A. subrufescens CA 487 (n=15) (0.25-1g/kg BW)	33	64
A. subrufescens CA 454 (n=15) (0.25-1g/kg BW)	27	72
A. bisporus A30 (n=15) (0.25-1g/kg BW)	39	56
Imidazol-Trémate Controls (n=15)	83	0

The doses used in our experiments were fixed at respectively 1/20 to 1/5 of the maximum dose experimentally ingested since LD50≥ 5g/kg BW (as obtained in a previous work, BRG national project in 2007) that is 0.25, 0.5 and 1 g/kg BW. From the lower dose of 0.25 g/kg BW to the rat, we already observed almost the maximum protective effect. It appears that there is no dose-effect relationship in this protective effect. Interestingly Johnson *et al.* [33] and Forland *et al.* [34] have given to human a dose of 60 ml per day of a solution containing 340g/L that makes 0.34 g/kg BW for a person of 60 kg. These doses are quite similar to those given to the rats in the present experiments, however in our case and having the end-point of pre neoplasic changes, we found a very significant protective effect in the rats whereas in [33] and [34] immunosuppressant effects in humans were observed. If the preventive effect observed is triggered by immune stimulation as forecasted or hypothesised by several authors [25, 38], our data appear incongruent with those of Johnson *et al.* and Forland *et al.* [33, 34]. But, if the tumour preventing effects are triggered by cytotoxicity against modified cells bearing rapid growth rate [39, 40], then our data are congruent with the above studies [33, 34], since the immunosuppressant effect observed could be triggered by cytotoxicity.

Based on the present data our preliminary conclusion is that *A. subrufescens* CA454, CA 487 and *A. bisporus* 30A might have tumour preventing properties. Then further step was investigation on anti tumour properties. This has been done using the mouse transplanted-tumour model. Balb/c mice have been treated with similar doses of mushroom extracts by both intra

peritoneal and oral routes after having been injected with L1210-SP2O leukaemia cells. We were unable to prevent death of animals. Thus no anti-tumour effect can be claimed for *A. subrufescens* CA 454, CA 487 and *A. bisporus* A30, at least with these extracts.

In Vitro Assays. After obtaining cold and hot hydrophilic extracts they were tested in vitro on two cell lines Hep-G2 and Neuro-2A. The concentrations used in vitro were chosen from our in vivo data, considering that we should not put more than 1000 µg/ml when 1 g/kg BW is effective in vivo. Further we have seen that most of the research groups never exceeded 600 µg/ml of extracts or polysaccharides in their culture medium. Our *in vitro* data revealed no effect until concentrations of 200 µg/ml, then slight mitogenic effect, when cell growth was stimulated somehow between 200 and 500 µg/ml and then no clear inhibitory effect was observed above these concentrations (Fig. 1, a,b,c and d). Hep-G2 cells were more sensitive to these extract than Neuro-2a cells. Since Hep-G2 cells originate from the liver, it could be that liver cells are more sensitive to *A. subrufescens* than other cells. Indeed several authors found some liver damages (fulminant hepatitis) with extract of *A. subrufescens* sp [5, 41], *A. subrufescens* strains were more active than *A. bisporus*, with CA454 being the more active.

These data are congruent with the stimulatory effects observed on immune cells both *in vitro* and *in vivo*. Purification and characterization of active substances could help finding in the future any curative effect borne by these mushrooms. At present our *in vitro* data show some growth stimulatory effects at the tested concentrations. It is noticeable that the cold extracts are more effective than the hot extracts (Fig. 1 a, b, c and d). This observation could be due to the partial destruction of some active mitogenic substances by heating or differential extraction.

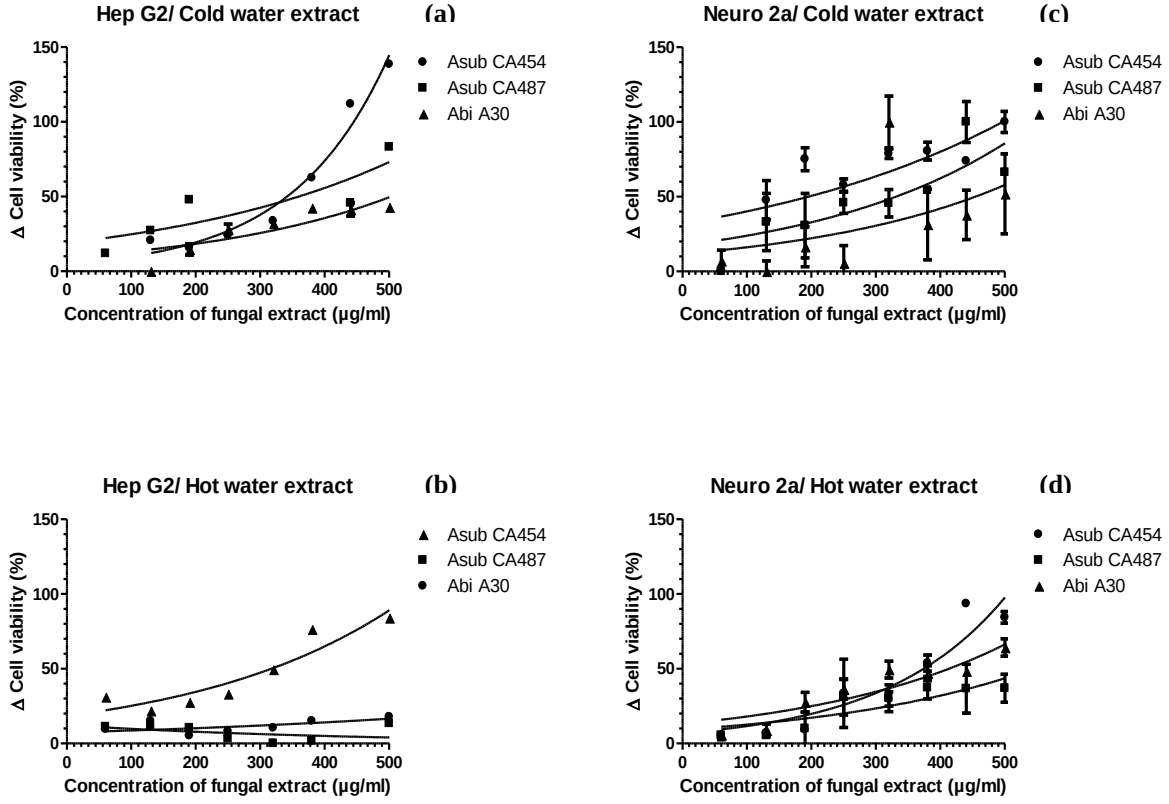

Figure 1: Effects of hydrophilic extracts of *A. subrufescens* sp (Asub) and *A bisporus* (Abi) on the viability of Hep G2 and Neuro-2a cells; (a) and (c) = cold water extracts of total mushrooms; (b) and (d) = hot water extracts of total mushrooms.

CONCLUSION

The final goal of the toxicological and chemical studies is to give safety information and to promote any of these mushroom strains which will prove to be of interest either as food or as drug to be used for curative or preventive purposes so that implementation of its culture at a large scale will proceed of sustainable and profitable agriculture.

Agaricus subrufescens or *A. blazei, A. brasiliensis* has been consumed by human for decades. Due to the popularity, it is increasingly cultivated in many countries for its putative medicinal properties. Many scientific studies reported various medicinal benefits i.e. anti-cancer, anti-microbial anti oxidant and anti inflammatory activities. Although these mushrooms have many bioactive compounds that could be developed for curative properties toxicological problems are still discussed. There is clearly a lake of information on toxicological assessment (acute and chronic toxicity) of compounds such as agaritine, blazein and of the whole mushroom *A. subrufescens* itself, and overall on epidemiological data linking the consumption of *Agaricus* sp and eventual prevention and/or pathologies.

Our data reveal that *A. subrufescens* strains of different origins are not toxic either *in vivo* or *in vitro* except some locomotor hypoactivity. Additionally they elicit slight cell growth stimulation at the concentrations tested *in vitro*. The most active is *A. subrufescens* (*A. blazei* Murrill) from Brazil whereas the most productive is the European species [42].

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PURIFICATION AND IMMUNE ACTIVITY OF SMALL-MOLECULE POLYSACCHARIDES FROM *GRIFOLA FRONDOSA*

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ABSTRACT

A water-soluble crude polysaccharide preparation (GFP) was obtained from *Grifola frondosa* fruit bodies by hot water (100 °C) extraction, followed by ethanol (75%) precipitation. Purified polysaccharides, GFP75-2D was obtained from the crude material by successive fractionation using DEAE-Sepharose Fast Flow ion exchange chromatography and Sephacryl S gel filtration chromatography. GFP75-2D mainly contained fucose, galactose, and mannose by UV spectroscopy scan. Moreover it showed the activities to stimulate macrophages and enhanced the release of NO from RAW264.7 macrophages. Furthermore, the concentration of Interleukin-1β (IL-1β) and TNF-α were significantly stimulated after exposed to GFP75-2D whereas modest increase was detected in IL-6.

Keywords: *Grifola frondosa*; Polysaccharide; Purification; Immune activity; Macrophage

INTRODUCTION

Maitake (*Grifola frondosa*) is a polypore mushroom that grows in clusters at the base of trees in temperate forests. The fungus is native to many provinces such as Hebei, Zhejiang, Sichuan, Jilin and Fujian in China. Great attentions have been paid to artificial cultivation of it for over 30 years in Zhejiang and Hebei province due to its delicious tastes and unique aroma. It is also prized as a medicinal mushroom rich in a variety of active substances. *Grifola frondosa* polysaccharides, main active ingredients, have been identified to have significant effects in anti-tumor [1], anti-hypertensive [2], anti-diabetic [3], immunity-improving [4], as well as many other pharmacological activities.

In this study, crude polysaccharides were purified, and then their activities were tested. In addition, structures of purified polysaccharides were analyzed using chromatographic and spectroscopic methods. Further tests were also implemented to evaluate the immune activity of these polysaccharides.

MATERIALS AND METHODS

Maitake (fruit bodies) was obtained from Zhejiang Province, China. Bacterial lipopolysaccharide (LPS) from Sigma; double-antibody (penicillin, streptomycin) from Amresco Inc., DMEM medium, RPMI-1640 medium, fetal bovine serum (FBS) for the Gibco products; ELISA kit is the product of Beijing Zheng Si Bo Ltd., and other reagents were analytical grade domestically. RAW264.7 macrophage cell line was purchased from cells institute of Chinese Academy.

Purification of small-molecule polysaccharides from GFP. Crude polysaccharides were obtained from *Grifola frondosa* by hot water extraction and ethanol precipitation, and were sequentially purified by ion exchange chromatography on DEAE-Sepharose Fast Flow column and gel permeation chromatography on Sephacryl S column, and the fraction GFP75-2D was obtained.

Identification to initial structure of GFP75-2D. Qualitative detection of sugar was determined with phenol - sulfuric acid assay [6]. GFP75-2D was prepared to1mg/mL solution for the 200 ~ 400nm UV scanning.

Analysis of monosaccharide composition. To 2mg of GFP75-2D, 3ml 2M trifluoroacetic acid (TFA) were added, and hydrolyzed for 3h at 110 °C, and after addition of methanol, the residual TFA was evaporated in vacuum. The sample was dissolved with 1mL pure water for ion chromatography analysis. The chromatographic system was: Dionex ICS2500, column for the Carbon Pac PA-20; flowing phase, A phase: deionized water, B phase: 0.25M NaOH, C phase: 1M NaAc; flow rate 0.45mL/min; elution program shown in Table 1.

Table 1: Ion chromatographic elution procedure

Time Minutes	A phase, deionized water %	B phase, 0.25M NaOH %	C phase, 1M NaAc; %
0	99.2	0.8	0
30.0	99.2	0.8	0
40.0	79.2	0.8	20
40.1	20.0	80.0	0
60.0	99.2	0.8	0

Measurement of nitric oxide (NO). Production of nitric oxide (NO) was estimated by measuring nitrite levels by the Griess reaction [5]. To each well of a 96-well microplate, 180 µl of the macrophage suspension (1×106 cells/ml) and 20 µl of various test substances were added. After incubation 37 °C in a 5% CO_2 atmosphere for 48 h, 100 µl of cell-free supernatants was mixed with 50 µl of Griess reagent (1% sulfanilamide,0.1% naphthylethyylene-diamine dihydrochloride, 2.5% phosphoricacid) and incubated for 10 min at room temperature. The optical densities (O.D.) of samples were measured at 543 nm. The nitrite concentration was determined with a standard curve of linear sodium nitrite from 0.1 to 100 µM.

Quantification of interleukin-1ß (IL-1ß), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α). Production of the cytokines IL-1ß, IL-6 and TNF-α by RAW264.7 macrophages were quantified by ELISA according to the manufacturer's instructions. RAW264.7 macrophages were adjusted to a concentration of 1×10^5 cells/ml. To each well of a 96-well microplate, 180 µl of the cell suspension and 20 µl of various test substances were added. After incubation at 37 °C

in a 5% CO_2 atmosphere for 24 h, 50 µl of cell-free supernatant from each well was used for the IL1 ß, IL-6 and TNF- α ELISA-assay.

RESULTS

Crude GFP75 fractions by ion chromatography. Six components including a component of aqueous phase and five salt phase components, named GFP75-1, GFP75-2, GFP75-3, GFP75-4, GFP75-5 and GFP75-6 respectively，were obtained by DEAE FF column chromatography(see Fig.1). Among the six components, GFP75-2, possessing immune activity and being light-colored, was ready for the further separation.

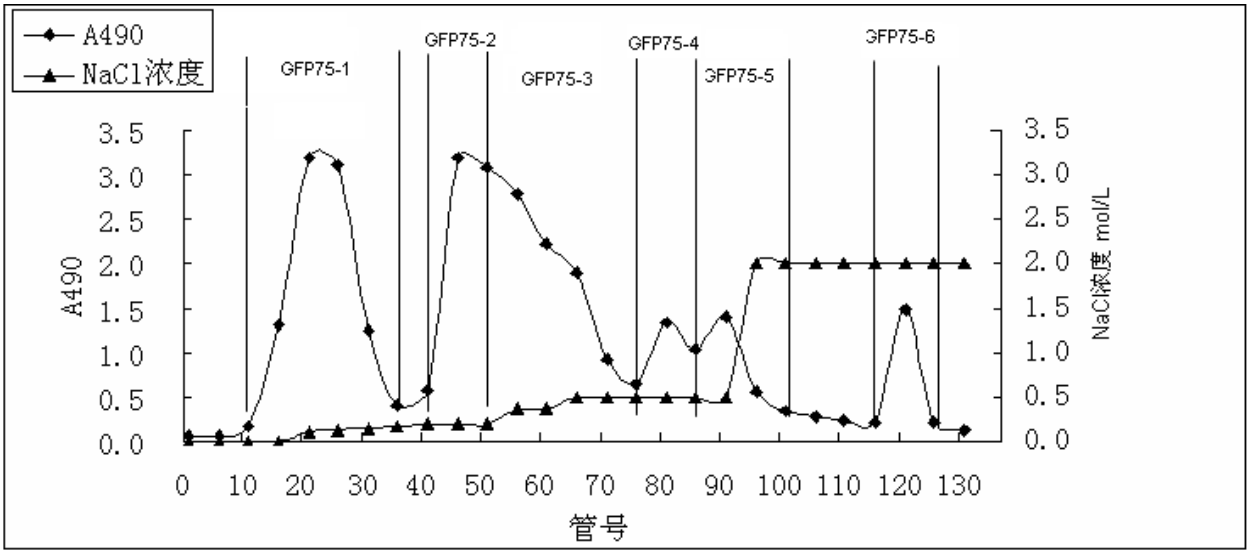


Figure 1: DEAE-Sepharose Fast Flow column chromatography of fraction GFP75

Gel column chromatography of eluted fractions of GFP75-2. Four components, named GFP75-2A, GFP75-2B, GFP75-2C and GFP75-2D, were obtained as fractions of GFP75-2 separated by Sephacryl S-400 (see Fig. 2). Parts of continued collection of small molecular weight were further separated by Sephacryl S-100 to achieve GFP75-2C and GFP75-2D of complete separation (see Fig. 3). We obtained two symmetrical single peaks. GFP75-2D was tested as a single symmetrical peak (Figure 4). Its molecular weight was about 2600Da by HPLC.

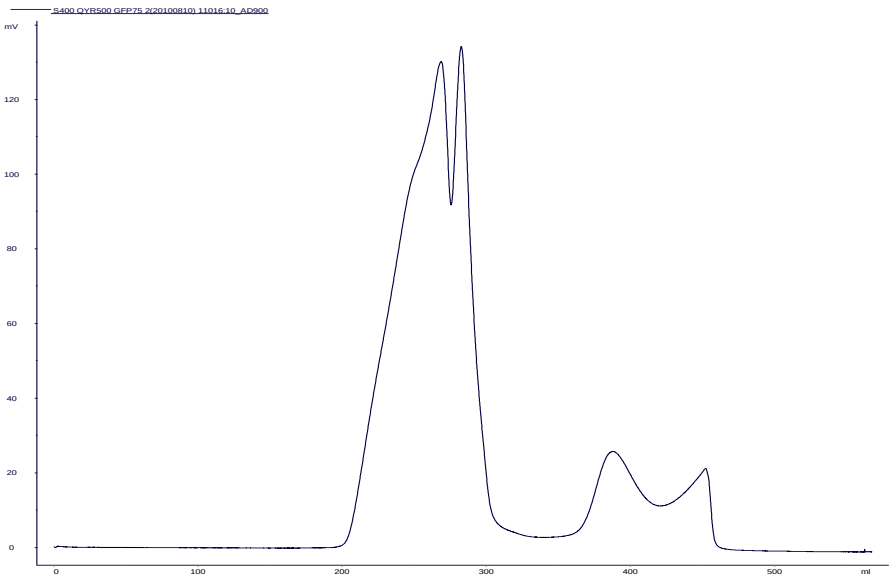


Figure 2: Sephacryl S-400 column (26×100) chromatography of fraction GFP75-2

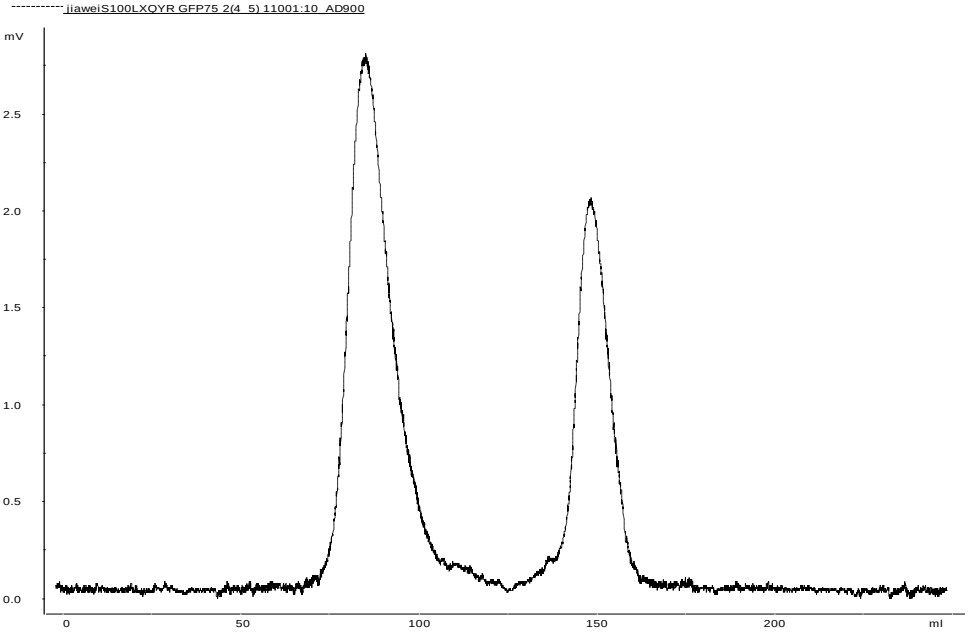


Figure 3: Sephacryl S-100 column (16×100) chromatography of low molecular weight fraction GFP75-2

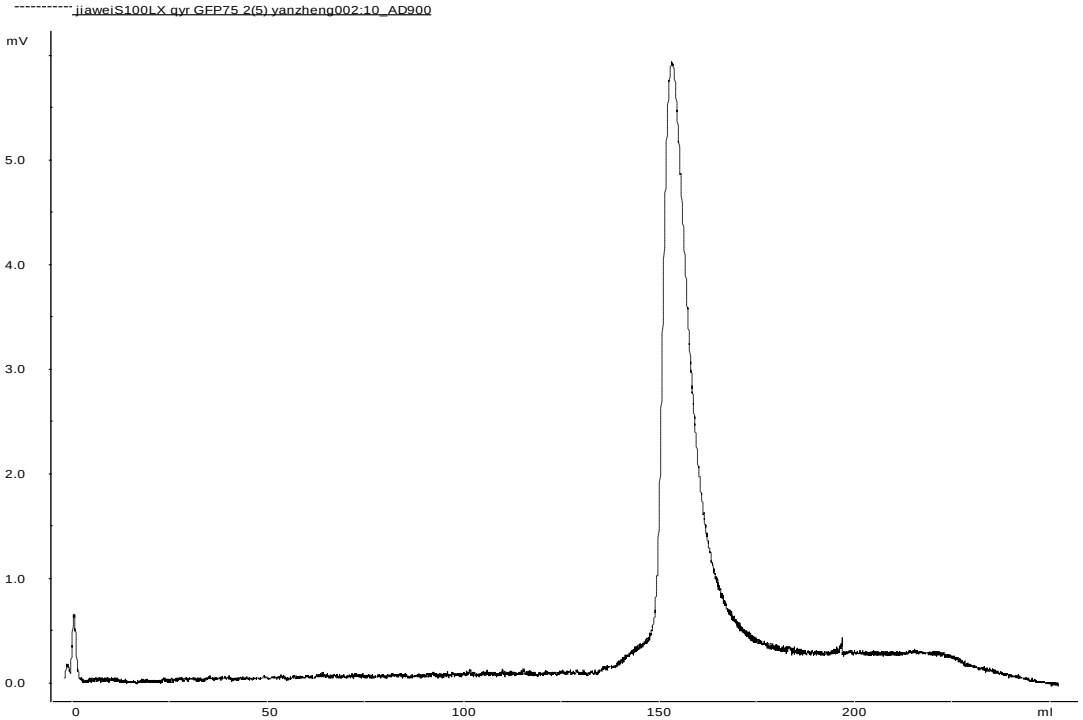


Figure 4: Sephacryl S-100column (16×100) chromatography of fraction GFP75-2D

Preliminary identification of the structure. GFP75-2D showed a characteristic color reaction of sugar by the phenol - sulfuric acid assay. There was no characteristic absorption peak in the vicinity of 280 nm and 254 nm, indicating no proteins and nucleic acids. 200 ~ 400nm in the UV scan shows that there was no-phenolic components in GFP75-2D (Fig. 5).

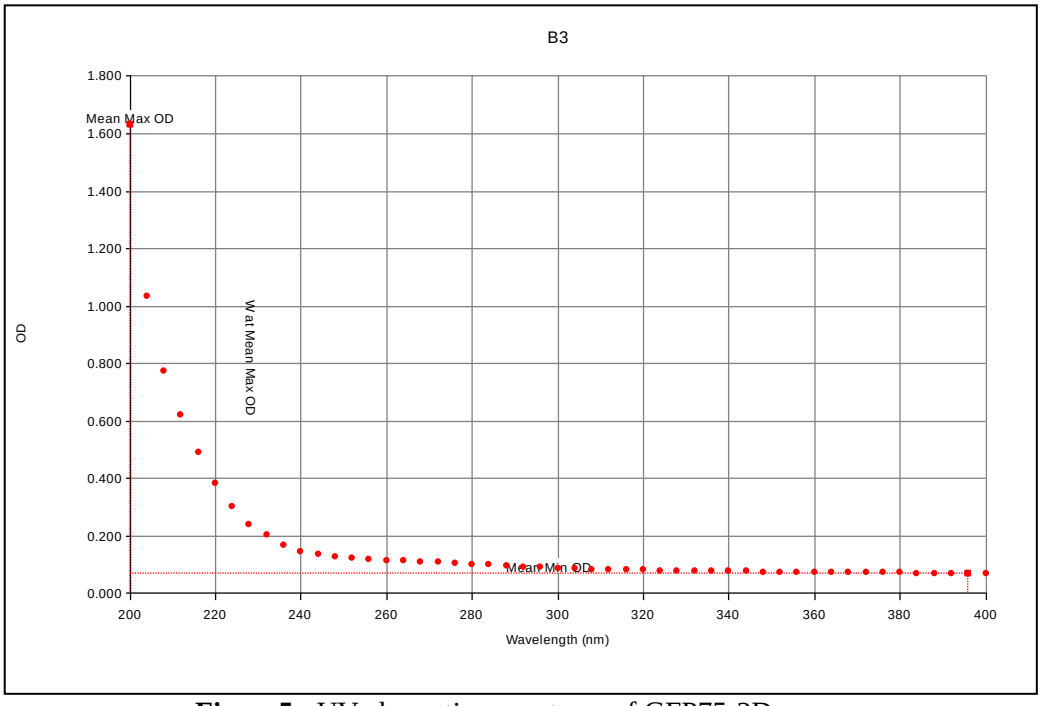


Figure5: UV absorption spectrum of GFP75-2D

Six peaks were displayed in GFP75-2D ion chromatogram results (Fig. 6). We could draw that GFP75-2D contains mainly fucose, rhamnose, galactose, glucose and mannose with the molar ratio of 1:1.3:3.6:4.0:4.5 by reference to standard single-sugar control standard.

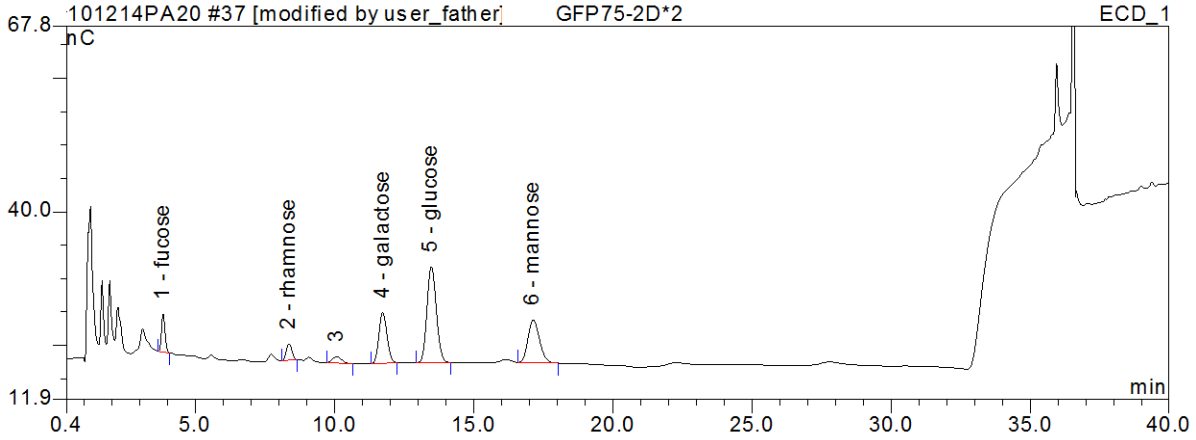


Figure 6: Ion chromatogram of the monosaccharide in GFP75-2D

NO production of the macrophage cell line RAW264.7 was stimulated with eluted fractions by ion-exchange chromatography. The water phase elution fractions couldn't stimulate macrophages to produce NO (Fig.7). Relatively, salt elution fractions could stimulate macrophages to produce NO. The results indicated that immune activity of GFP75 obtained by ion chromatography was strengthened. The salt phase elution fractions, GFP75-2 and GFP75-3 showed higher immune activity than the others. In addition, these two fractions contained more active ingredients due to gradually increased immune activity with higher sample concentration. GFP75-2 possessing good immune activity and light color was further purified.

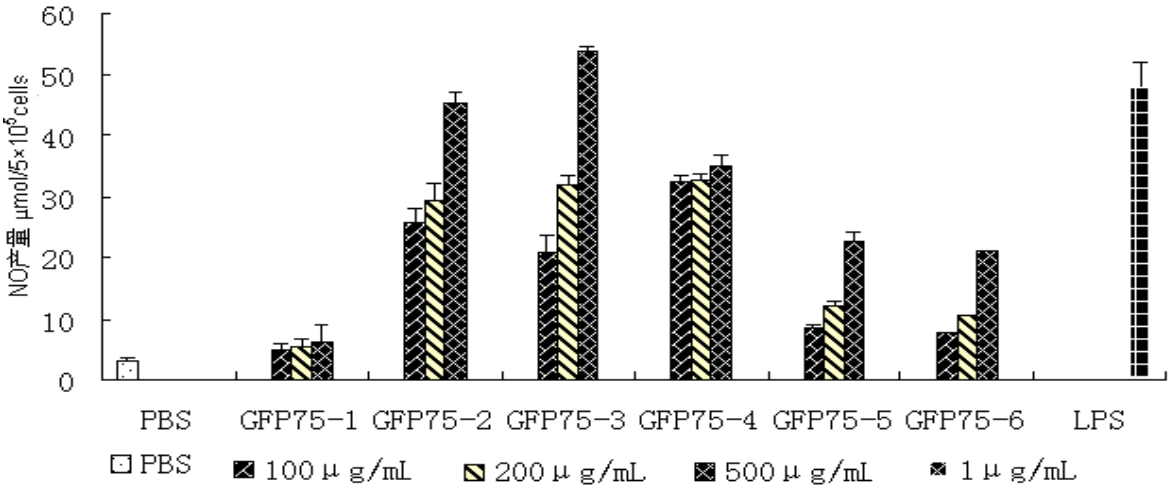


Figure7: NO production of fractions from anion exchange chromatography of GFP75

The NO production of RAW264.7 after stimulation by the purified GFP75-2D significantly increased (Fig.8). At low concentrations 10 μg/mL, GFP75-2D already showed a good stimulation. The concentration of 100 μg/mL，the activity of GFP75-2D was higher than the positive control LPS.

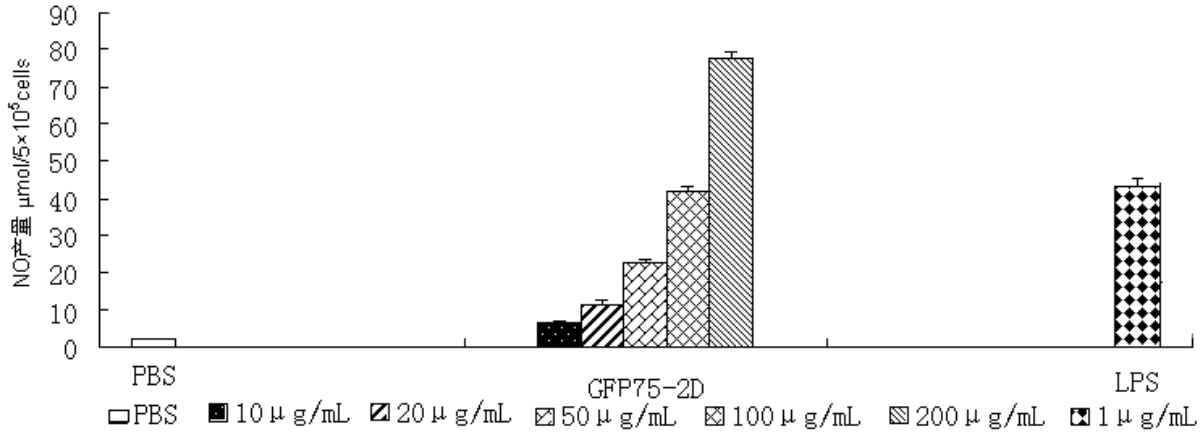


Figure8: Effect of GFP75-2D on NO release from RAW264.7 cells

Change in cytokine TNF-*α*, IL-1β, IL-6 production of RAW264.7 after stimulation by GFP75-2D *in vitro*. The release of TNF-α (Fig.9) after stimulation by GFP75-2D were significantly increased in the cell culture supernatant, at the same time, the sample stimulus group didn't release TNF-α. Tthe production of TNF-α was the highest when GFP75-2D concentration reached 100 μg/mL, slightly higher than the positive control LPS. The release of IL-1β (Fig.10) and GFP75-2D concentrations was positively correlated. When GFP75-2D concentration increased into 100 μg/mL, the release of IL-1β was the highest too, but slightly lower than the positive control LPS. The release of IL-6 (see Fig.11) and GFP75-2D concentrations were positively correlated too, and achieved the highest at 100 μg /mL, but far in the positive control LPS.

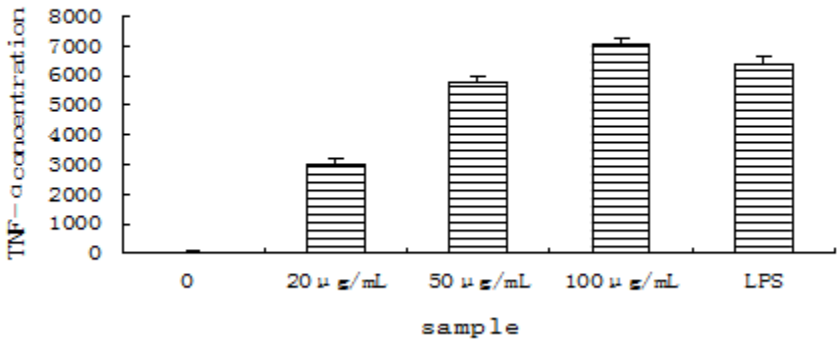
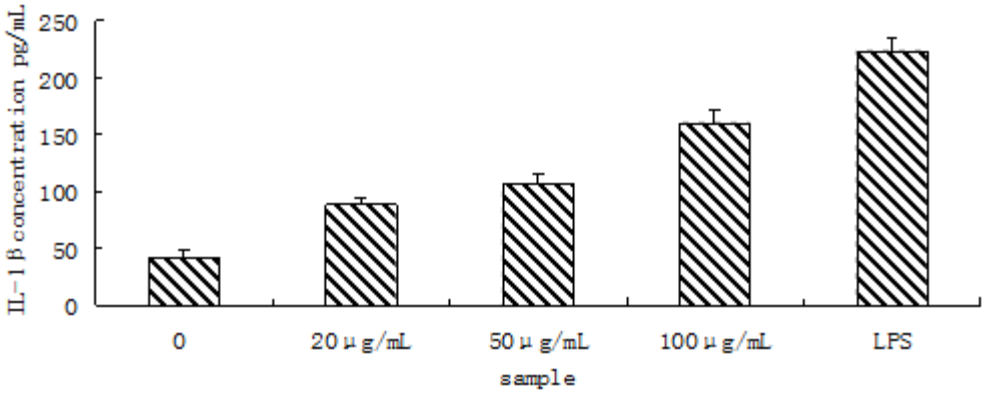


Figure 9: Effect of GFP75-2D on TNF-α production in RAW264.7



Fiure10: Effect of GFP75-2D on IL-1β production in RAW264.7

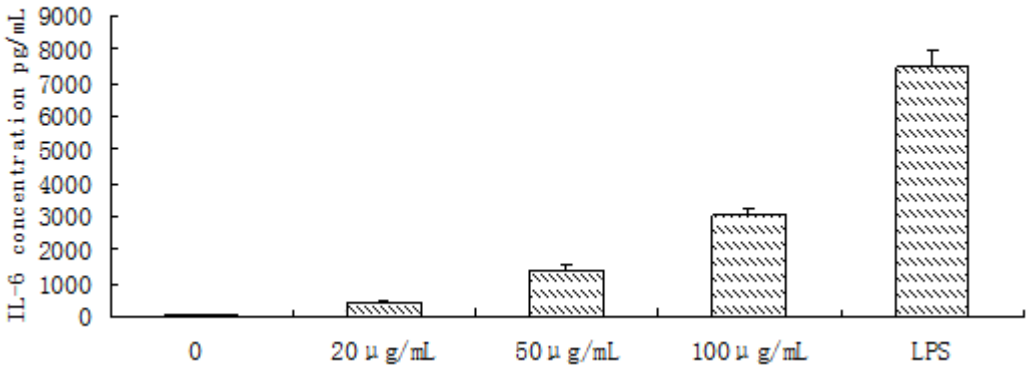


Figure 11: Effect of GFP75-2D on IL-6 production in RAW264.7

DISCUSSION

Generally we believed that the biological activity of polysaccharides depended on molecular size. The small molecular weightpolymers of sugar obtained from *Grifola frondosa* fruiting bodies in this study has shown a good immune activity, while the small molecular weight GFP and their immune activity was rarely reported. The *Grifola frondosa* polysaccharides stimulated macrophage cell line RAW264.7 into the activated state, and also made contribution to increase in cellular NO secretion, cytokine TNF-α, IL-1β and IL-6 secretion, and concentration-dependent with the purified polysaccharide GFP75-2D. This suggested that GFP enhance the body's immune system related to activation of macrophages to promote TNF-α, IL-1β and IL-6 secretion of cytokines. It also showed that GFP activated macrophages through a variety of ways to play a role in immune response. It's worth further investigating at the reason whether the existance of receptor-binding specificity of the fragments or not that lead to GFP75-2D possesses a good immune activity with low molecular weight.

ACKNOWLEDGEMENTS

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THE INFLUENCE OF CULINARY-MEDICINAL MUSHROOMS: *AGARICUS BISPORUS, LENTINULA EDODES* AND *PLEUROTUS OSTREATUS* ON INJURIES OF GASTRIC MUCOSA IN RATS EVOKED BY STRESS.

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ABSTRACT

Agaricus bisporus, Lentinula edodes and *Pleurotus ostreatus*, are widely used culinary-medicinal mushrooms that have been traditionally consumed in many countries to prevent cancer, diabetes, hyperlipidemia, arteriosclerosis, and chronic hepatitis. Many components of these mushrooms have potentially significant biologic activity and perspective for development of new medicines. The aim of this study was to evaluate effects of edible mushrooms in model of stress-induced gastric ulcerogenesis in rats. Rats were randomly assigned to one of four groups; each group consisted of six animals. Jelly starch (control group) and jelly starch powder suspensions from dry fruiting body of *A. bisporus*, *L. edodes* and *P. ostreatus* (200 mg/kg, i.g.), were administered in 0.5ml volumes, daily, during a 7-day period to different groups. The animals were exposed to acute immobilization stress combined with water immersion on fifth day, and were sacrificed on eighth day after starting experiment. Gastric wounds were classified as erosions - small lesions on the sub-mucosa, and ulcers with hemorrhagic borders own areal and deep mucus invasion.

Our research has shown that treatment with *P. ostreatus* and *L. edodes* inhibited ulcer formation by 76.2% and 87.1% respectively, compared to the control group ($P<0.05$). Daily administration of powder suspensions of *L. edodes* inhibited erosion formation by 71.5% ($P<0.05$). *Pleurotus ostreatus* and *A.bisporus*, presented only unproven activity.

We conclude that using *P. ostreatus* and *L.s edodes* by gastric ulcer disease patients might help in the clinical as a prophylactic agent, and in healthy persons as stress-protective agents.

Keywords: *Agaricus bisporus* (brown strain), *Lentinula edodes, Pleurotus ostreatus*, stress, gastric ulcers.

INTRODUCTION

Higher Basidiomycetes represent an important group of fungi for industrial production of a nutritionally functional food and different kinds of new pharmaceutical products [1]. Healthy nutrition and diet are gaining importance, not only in the everyday life of human beings, but also in the treatment of chronic diseases. Mushrooms have recently become attractive as a functional food and source for the development of new drugs. Edible mushrooms are a valuable source of biologically active compounds. Some are used in the prophylaxis and therapy of such diseases as cancer and cardiovascular diseases. The biologically active substances in mushrooms decrease DNA damage, reduce carcinogen concentrations and their activation, inhibit the growth of cancer cells by scavenging free radicals, stimulate the immune system, and induce tumor cell apoptosis. The stimulation of the immune system by the biologically active compounds in edible mushrooms

protects against cold, flu, infections, well as AIDS by inhibition of viral replication. Mushrooms contain effective substances which decrease the LDL fraction of cholesterol in blood. They also prevent the accumulation of serum triacylglycerols, thus decreasing the risk of developing cardiovascular disease [2-4].

Stressful environment leads to development of many chronic and acute diseases globally in the modern world. Dietary mushrooms contain a diverse array of biologically active molecules rendering them potentially protective against stress. In fact, dietary mushrooms have been shown to improve cardiovascular health, stimulate immune function, contribute to glucose homeostasis, and to modulate detoxification, as well as exert anti-allergic, anti-tumor, anti-viral, antibacterial, antifungal, and anti-inflammatory activities [5, 6]. As a result, both cellular components and secondary metabolites of myriad dietary mushrooms have been used in treatment for a variety of diseases [7]. Some of the therapeutic properties of mushrooms result from the specific polysaccharides, such as beta-glucans and chitosans that are present in the fructification of fungi [8].

The basidiomycete fungus *Pleurotus ostreatus, Agaricus bisporus* and *Lentinula edodes* are widely used culinary-medicinal mushroom that have been traditionally used as a health food source in many countries for the prevention of cancer, diabetes, hyperlipidemia, arteriosclerosis, and chronic hepatitis. Nowadays, these mushrooms are produced on an industrial scale. Many of the components of these mushrooms potentially act as biological response modifiers, meaning that they are able to affect physiological responses, thus attracting attention to the development of new medicines, as a cure or for prophylactic uses [9]. Some authors have also described activity in mushrooms and/or mushroom extracts as dietary supplements based on theories that they enhance immune function and promote health. To some extent, selected mushrooms have been shown to have stimulatory action on immune responsiveness, particularly when studied in vitro [6, 9-13]. However, despite their widespread use for potential health benefits, there is a surprising paucity of epidemiologic and experimental studies that address the biological activities of mushrooms after oral administration to animals or humans.

Considering all the effects found for the biological activity of commonly used culinary medicinal mushrooms and its relation to important physiological processes, the aim of this study was to evaluate protection effects of edible mushrooms in model of stress-induced gastric ulcerogenesis in rats.

MATERIALS AND METHODS

Air dry fruiting bodies of cultivated culinary-medicinal mushrooms *L. edodes* and *P. ostreatus* were obtained from a local market. Fruiting body samples of *A. bisporus* were collected for this study in a mushroom house during first flush and then air dried.

The crude dried fruiting body of *A. bisporus, L. edodes* and *P. ostreatus* was powdered and aqueous suspensions were prepared by mixing dry powder of mushrooms in starch jelly as vehicle (300 mg starch add to 30 ml of distilled water and hit at 90°C in a water bath for 3 minutes).

Male rats *(Rattus norvegicus,* albino, nonlinear), weighing 150-200 g, n=24 were acquired from the vivarium of the National Taras Shevchenko University of Kyiv, Ukraine. The animals were kept in small groups in polyethylene boxes in a standard vivarium environment and with food and water *ad libitum*, for at least 30 days before the experiments. The animals were provided only water *ad libitum* for the 24 hours before stress procedure. This study was conducted according to internationally accepted principles of laboratory animal use [14, 15].

Animals were exposed to acute immobilization stress combined with water immersion [16, 17] for 180 minutes. Restrained in metal perforated containers rats were immersed in the water bath (22° C) in such a way that the head of the animal was outside of water. After the exposure to stress the rats were dried and all rats were returned to the home cage.

Rats were randomly assigned to one of four groups, all groups were exposed to stress. Each group consisted of six animals. Jelly starch (control group) and jelly starch powder suspensions of *P. ostreatus*, *A. bisporus* and *L. edodes* (200 mg/kg, i.g.) were administered in 0.5-ml volumes, daily, during a 7-day period to different groups. The animals were stressed on fifth day, and they were sacrificed on eighth day after starting experiment. Stomachs were removed and opened at the small curvature for analysis. Gastric wounds were classified in different levels, as follows: Type I wounds present erosions, small lesions on the sub- mucosa, and Type II wounds present ulcers with hemorrhagic borders own areal and deep mucus invasion. Lesions were observed using an optical lens (X5) and quantified by its length for erosions and area for ulcers.

The statistical analyses were done using t-test for independent samples analysis followed by the Shapiro-Wilk's W tests of normality. Results with $P < 0.05$ were considered to be significant. Data are expressed as Mean ± SD values.

RESULTS AND DISCUSSION

It was observed that stress evokes wide-spread effect and leads to various injuries on gastric mucosa: ulcers, erosions. The inflammatory reaction in the non-treated group (control) was more intense than in the treated groups. The control group presented an ulcer area of 22.6 mm^2 per animal. These values were defined as 100%. The treatment with *P. ostreatus* and *L. edodes* inhibited ulcers formation by 76.2% and 87.1%, respectively, compared to the control group ($P < 0.05$). The other treatment with *A. bisporus* did not show inhibition of ulcer formation. *Lentinula edodes*, commonly known as Shiitake mushroom has been used as medicinal food in Asian countries, especially in China and Japan and is believed to possess strong immunomodulatory properties [18], showed the best performance, inhibiting ulcers formation in rats after treatment. These results are shown in Fig. 1.

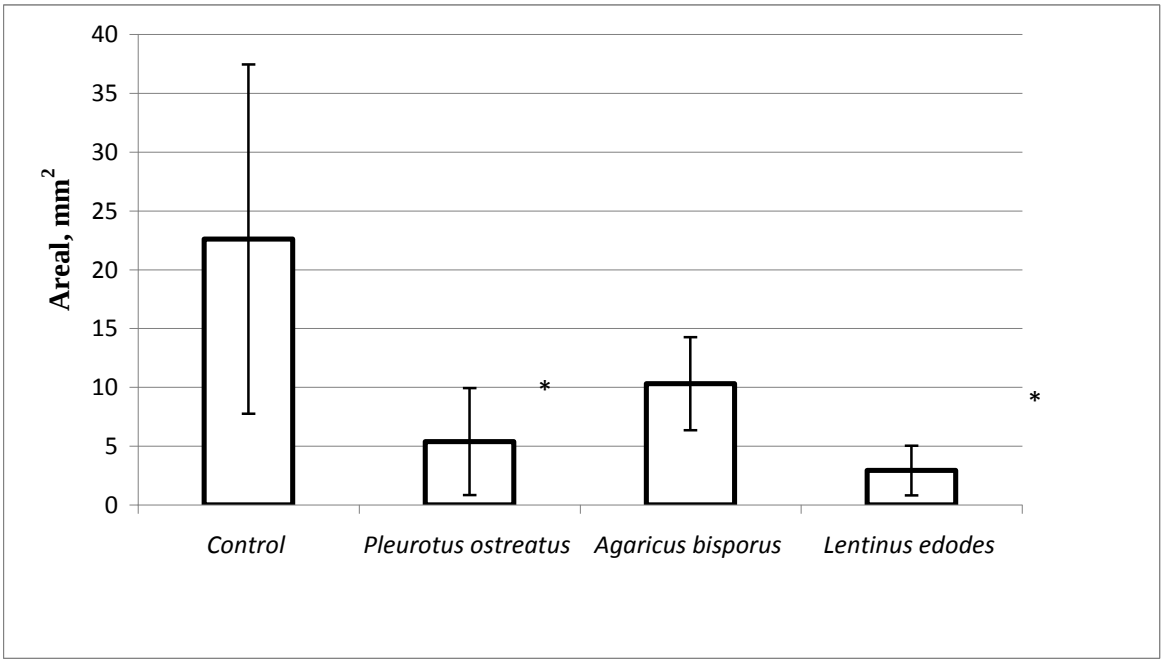

Figure 1: Stress-induced gastric ulcers in rats treated with powder suspensions of *Pleurotus ostreatus*, *Agaricus bisporus* and *Lentinula edodes* (200 mg/kg, i.g.). Data are mean ± SD
* - $P < 0.05$, compared to control group of animals.

Daily administration of powder suspensions of *L. edodes* (200 mg/kg, i.g.) for a 7-day period inhibited erosion formation by 71.5% ($P < 0.05$). The *P. ostreatus* and *A. bisporus* presented only unproven activity, inhibiting this formation, as shown in Fig. 2.

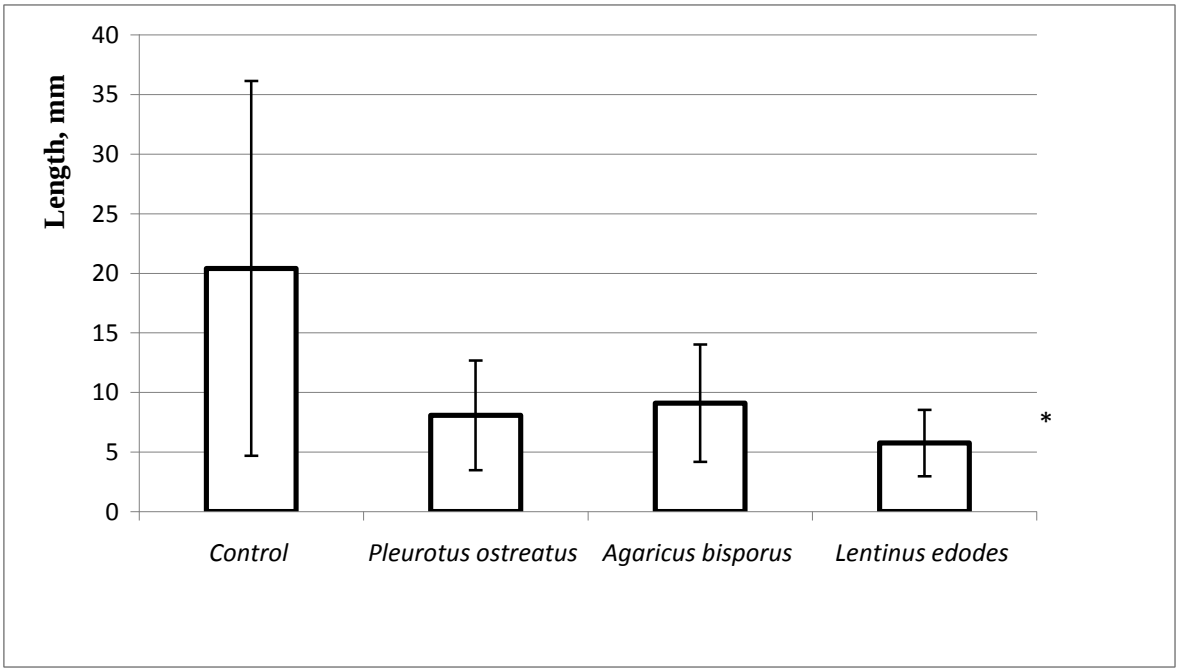

Figure 2: Stress-induced gastric erosions in rats treated with powder suspensions of *Pleurotus ostreatus*, *Agaricus bisporus* and *Lentinula edodes* (200 mg/kg, i.g.). Data are Mean ± SD
* - $P < 0.05$, compared to control group of animals.

The suspension crude powders of *P. ostreatus* and *L. edodes* were able to strongly inhibit the gastric wounds induced by combined stress, on day 3 after stress implying ($P < 0.05$).

Also it is possible to suggest a trend of protective effect of *A. bisporus* on the gastric damages induced by stress, unproven in this study possibly due to high variation. The treatment with suspension crude powder of *P. ostreatus* and *L. edodes* effectively protected stomach from more complicated lesions, such as ulcers when compared to the control group.

In the present study, our group evaluated the stress-protective activity of powder suspension of *P. ostreatus*, *A. bisporus* (brown strain) and *L. edodes*, in model of acute stress in rats.The investigated mushrooms are cultivated worldwide. The special importance of these mushrooms is determined not only by their high gastronomic value but also by the fact that they represent a source of valuable pharmaceuticals [19]. In our study it is generally considered that administration of mushrooms powder suspension for a 7-day period had a noticeable effect in inhibiting ulcers and erosions formation, probably due to immunomodulatory activity on the cell-mediated immunity.

The peripheral inflammation induced by stress might cause enhancing proinflammatory cytokine expression in the tissues. One possible mechanism for gastroprotection is through the release of pro-inflammatory cytokines, such as interleukin-1, tumor necrosis factor-α, and interleukin-6. Administration of *P. ostreatus* and *L. edodes* may inhibit this process, probably regulating cell migration and release of chemotaxic factors. Other studies suggest an immune modulatory action of the mushrooms extracts, it may inhibit the effect of stress by enhancing the anti-inflammatory activity in stress model [8, 10, 11, 13].

Anti-inflammatory drugs used in chronic diseases usually lead to gastric ulceration as a side effect. The development of new medicines that do not have this side effect would be a major aid to

patients with chronic inflammatory ailments, such as arthritis. *Pleurotus ostreatus*, and *L. edodes* were able to inhibit the ulceration process, protecting the gastric mucous wall.

It is noteworthy that mushrooms also contain high levels of bioactive agents including polyphenols and the novel antioxidant ergothioneine, which is produced exclusively in mushrooms and some bacteria [20]. It is possible that varying levels of these bioactive agents alone or in combination contributed to the beneficial effects observed.

CONCLUSIONS

Therefore, the use by gastric ulcer disease patients of *P. ostreatus*, and *L. edodes* as a health food may help in the clinical situation as a prophylactic agent, and in healthy persons as stress-protective agents.

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PRODUCTION OF THERAPEUTIC GLYCOPROTEINS IN MUSHROOMS

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ABSTRACT

The market for N-glycosylated therapeutic proteins represents multi-billion dollars in sales and is growing more than 3% each year. We investigated the potential of mushroom-forming basidiomycetes for the production of these drugs.

Keywords: N-glycosylation; Therapeutic proteins

INTRODUCTION

Proteins can be used as drugs. Examples include antibodies, insulin, erythropoietin (EPO). They are used for the treatment of many diseases, including cancer, cardiovascular and (auto) immune disorders. Protein therapeutics is currently the fastest growing class of human drugs. It is growing about threefold above the average growth rate for pharmaceuticals in general[1]. Over 160 protein products are marketed representing USD 50-60 billion [1,2] and over 500 proteins are still in the developmental pipeline. Most of these products are N-glycoproteins (60%).

N-glycoproteins are proteins decorated with sugar groups (glycans). The glycans determine behavior of the protein in the human body. When proteins are produced in another host cell than human or mammalian, the glycans that are added to the protein are different, e.g. plants add plant-specific glycans and yeasts add yeast-specific glycans. This changes the behavior of the protein and makes it difficult to control the specific activity of the drug. In some cases production in other hosts even makes the protein immunogenic; the human body will recognize the protein as foreign or non-human. It is desirable to have protein production systems with (1) no non-human glycosylation, and (2) homogeneous glycosylation.

Currently, most therapeutic glycoproteins are produced in mammalian cells. However, costs of mammalian cell fermentation technology are high, and also the strict testing needed as result of the risk for human viral contaminations of the product increase costs. In addition, stable and homogeneous glycosylation is difficult to achieve, especially upon changes in culture conditions, upscaling etc.

Transgenic animals and plants are alternative production platforms. The human therapeutic protein glucocerebrosidase produced in carrot cells (Protallix, Israel) and also, AtrynT derived from the milk of transgenic goats (GTC biotherapeutics, Massachusetts, USA) are approved human drugs.

Yeast and filamentous fungi are under investigation for production of glycoproteins, as these organisms have been extensively used for industrial protein production. One product has been approved so far that was produced in the yeast *Pichia pastoris* (Ecallantide; Dyax, Cambridge, USA).

TRENDS FOR PRODUCTION OF THERAPEUTIC GLYCOPROTEINS IN MUSHROOMS

Mushroom-forming basidiomycetes as cell factories. Mushroom-forming basidiomycetes may represent an interesting platform for protein production. They have a high natural protein-secretion capacity. The mycelium can be grown in liquid fermentations and the fruiting bodies can be formed on solid state fermentation. This latter yields interesting opportunities, e.g. fast and flexible scaling, and conservation of the principle production unit during up- or downscaling (i.e. the individual fruiting body). Mushrooms are grown at large scale for food purposes already. Technology for growing and also for stable storage of production strains is at hand. Spore-less varieties of *Agaricus* and *Pleurotus* could be used for production of drugs to prevent spread of GMO's, and production should be done on sterile synthetic media. Genetic modification of mushroom-forming fungi is relatively easy. Many suitable expression signals are also available.

Time to market of novel transgenic strains of mushroom-forming fungi is expectably relatively short. The gene of interest is transformed. After 16 h colonies can be selected using available selection markers. Within four days, colonies can be screened for protein production and selected for further propagation.

Therapeutic glycoprotein production in basidiomycetes. For the specific purpose of producing ***glyco***proteins mushrooms were shown to have a highly suitable starting situation[3]. This is caused by the following. As indicated, different species produce different glycans, which may change the behavior of a protein to be used in therapy. We have made the surprising observation that mushroom-forming basidiomycetes have an N-glycosylation profile much more similar to humans than yeasts and ascomycetes; in contrast to these species, mushrooms produce no non-human glycans. In addition, the glycans that they do produce can very easily be changed into all possibly desired glycans needed on therapeutic glycoproteins [3].

Humanization of N-glycosylation in mushrooms. By the deletion and introduction of genes involved in formation of glycans we were able to change the N-glycosylation of the model organism *Schizophyllum commune*. We have developed different strains that homogeneously produce specific glycans. For instance, we recently produced a strain that shows >80% of a glycan moiety required for some commercial products on produced proteins. We could also show that this glycan was indeed attached to a human therapeutic protein that we produced in this strain.

CONCLUSION

Mushrooms may be further developed to provide a novel alternative production platform for therapeutic glycoproteins. Intrinsic features of mushroom-forming fungi, longstanding mushroom culturing and processing, the suitable natural N-glycosylation and the possibility to adapt the glycan profile towards highly homogeneous production of human-like glycans strongly support further investigation into this field.

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CHARACTERIZATION OF ANTIHYPERTENSIVE PEPTIDES FROM *PLEUROTUS CYSTIDIOSUS* O.K. MILLER (ABALONE MUSHROOM)

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ABSTRACT

The antihypertensive activities of the crude water extracts from nine fruiting bodies of edible mushrooms were screened. The ACE inhibitory activities of the water extracts at a concentration of 10 mg/ml were in the range of 71.9% to 95.5%. Following this, proteins were precipitated from the crude water extracts using ammonium sulphate precipitation method from 10% to 100% salt saturation (S10-S100). The crude proteins were grouped according to the protein bands resolved by SDS PAGE. The ACE inhibitory activity of the protein fractions were tested at a concentration of 10 µg/ml protein. Fraction 1 of *P. cystidiosus* (S10-S20) had the highest activity where 96.2% of ACE was inhibited. This was followed by fraction 5 of *P. cystidiosus* (S90) where 86.7% of ACE was inhibited. Thus, fraction 1 and fraction 5 of *P. cystidiosus* were selected for further purification by reversed phase high performance liquid chromatography (RPHPLC). The proteins were eluted at an increasing gradient of acetonitrile in 0.05% TFA from 0 to 50% in 10 min. The protein fractions were grouped into sub-fractions. The ACE inhibitory activity of the RPHPLC fractions was tested at 1 µg/ml. Fraction F1a and F5b from fraction 1 and fraction 5 respectively showed the highest ACE inhibitory activity. These fractions were further characterized using SELDI-TOF-MS analysis. The result obtained showed the fractions contained several low molecular weight proteins where the most abundant protein had a molecular weight of 8300 Da.

Keywords: Low molecular weight proteins, ACE inhibitory peptide, SELDI

INTRODUCTION

Hypertension or high blood pressure has been on the rise in Malaysia over the past 10 years [1]. Hypertension is one of the risk factors for cardiovascular diseases, such as stroke, heart failure, cardiac arrhythmia and arteriosclerosis [2]. The most common blood pressure control has been associated with the renin-angiotensin system. Renin (protease synthesized by kidney) converts angiotensinogen from the liver to angiotensin I, which is a biologically inactive decapeptide. Later, angiotensin I is converted to an active octapeptide vasoconstrictor, angiotensin II, by the action of angiotensin I-converting enzyme (ACE). This reaction will cause the contraction of blood vessels and thereby raising blood pressure. Thus, blocking the production of angiotensin II with ACE inhibitors will prevent constriction of blood vessels, lowers blood pressure and lessens the energy of the heart to expand when beating [3]. Besides blood pressure lowering effect, ACE inhibitors have also shown improvement in the treatment of several clinical disorders such as memory dysfunction, cerebral blood flow and cerebroprotection, stress, depression, alcohol consumption, seizure, Alzheimer's and Parkinson's diseases and diabetes [4]. Various ACE inhibitors commercially available in the market, such as captopril may effectively help to regulate the blood pressure in hypertensive patients. However, these pharmaceutical products may have side effects such as cough, taste disturbances, skin rashes and allergic reactions.

Previous study has reported the occurrence of angioedema caused by the consumption of ACE inhibitors [5]. Thus, the interest to find ACE inhibitors from natural sources such as food has increased. Many ACE inhibitory peptides have been discovered, among which some are derived from food-proteins such as milk, buckwheat, potato and tuna [6-9].

Mushrooms have received increasing attention in recent years because of their nutrition with health-stimulating properties and medicinal effects. Beside nutritional value, the unique colour, taste and aroma of the mushrooms which can sometimes stimulate one's appetite are also the reasons that attract their consumption by humans [10]. It is not easy to separate edible and medicinal mushrooms because many of the common edible species are beneficial in the prevention and treatment of various human diseases and several medically related mushrooms are also eaten [11]. The most reported medicinal mushroom with blood pressure lowering effect is *Ganoderma lucidum* [12, 13]. Previous studies have also reported on the antihypertensive effect of some edible mushrooms. The low concentration of sodium and the presence of a high amount of potassium in mushrooms have suggested its utilisation as an antihypertensive diet [11]. Potassium has been proven to have blood pressure lowering effect [14]. *Pleurotus* species such as *P. cornucopiae, P. nebrodensis* and *P. sajor-caju* have been reported to possess hypotensive activity [15-17]. *Lentinula edodes* and *Flammulina velutipes* have also been reported to exert antihypertensive activity [18, 19]. Previous studies have showed successful purification of ACE inhibitors from fruiting bodies of edible mushroom, *Tricholoma giganteum* and *Grifola frondosa* [20, 21]. Excess production of reactive oxygen species (ROS) will contribute to hypertension. Antioxidant administration may help to prevent and treat hypertension [22]. *Pleurotus cystidiosus* has strong antioxidant effect [23]. Therefore, it may be a good source of an antihypertensive drug. Thus, the objective of the current study is to extract and characterize the ACE inhibitory peptide from the fruiting body of *P. cystidiosus*.

MATERIALS AND METHODS

Materials and Chemicals. Fresh mushroom fruiting bodies: *Agaricus bisporus* (button mushroom), *Flammulina velutipes* (golden needle), and *Lentinula edodes* (shiitake) were purchased from the local hypermarket, Jaya Jusco while *Hericium erinaceus* (monkey's head mushroom), *Pleurotus citrinopileatus* (yellow oyster mushroom), *P. cystidiosus* (abalone mushroom), *P. flabellatus* (pink oyster mushroom), *P. florida* (white oyster mushroom) and *P. sajor-caju* (grey oyster mushroom) were purchased from the local mushroom farm, Ganofarm Sdn Bhd. Captopril, a commercial antihypertensive drug and the α-cyano-4-hydroxy-cinnamic acid (CHCA) used for SELDI-TOF-MS was purchased from Sigma-Aldrich. Acetonitrile used for HPLC and the other chemicals such as ammonium sulphate, trifluoroacetic acid (TFA) and methanol were purchased from Merck.

Partial Purification of ACE Inhibitor. The fruiting bodies were homogenized with distilled water at a ratio of 1:2 (w/v). The mixture was filtered and centrifuged to remove the unwanted debris. Proteins were precipitated out from the water extracts using ammonium sulphate precipitation method. The concentration of the ammonium sulphate was increased stepwise from 10% to 100% salt saturation and the precipitated protein was recovered at each step by centrifuging at 10000 rpm for 15 min. Then, the crude protein was dialyzed to remove the salt from the sample. They were lyophilized and stored at -20^{o}C for further analysis.

Protein Estimation. Protein content was estimated using Pierce® Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific) according to the protocols provided by the manufacturer. Absorbance of the samples was measured with Sunrise^{TM} ELISA microplate reader (Tecan) at

562 nm. Protein content was determined by comparing the absorbance value of the samples with the standard curve of bovine serum albumin (BSA).

SDS Polyacrylamide Gel Electrophoresis (SDS PAGE). SDS PAGE was carried out in a vertical slab gel apparatus according to the modified method of Laemmli (1970) [24]. Sample buffer and crude protein were mixed at a ratio of 1:3 (v/v) and boiled for 5 minutes. The mixture and broad range SDS PAGE standard markers (Bio-Rad) were then loaded into the well. Electrophoresis was conducted at a constant current of 60V for stacking gel (4% polyacrylamide) and followed by 100V for separating gel (16% polyacrylamide). After electrophoresis, the gel was fixed with fixing solution which consists of 40% (v/v) methanol and 10% (v/v) acetic acid for 30 min. The protein bands were then stained by silver staining.

Assay of the ACE Inhibitory Activity. The protein fractions were tested using angiotensin converting enzyme inhibitory assay kit (ACE kit-WST, Dojindo Laboratories). The crude mushroom extracts and the protein fractions were tested at a concentration of 10 mg/ml and 10 µg/ml protein, respectively. The assay was carried out according to the protocol provided by the manufacturer. Absorbance of the samples was measured with Sunrise™ ELISA microplate reader (Tecan) at 450 nm. The ACE inhibitory activity of the samples tested was calculated using the formula given in the protocol.

Purification of ACE Inhibitor from *Pleurotus cystidiosus*. Purification of the protein fractions with active ACE inhibitory activity was carried out using HPLC system (Shimadzu, SPD-M10A UP). The column used in the current study was Chromolith® SemiPrep RP-18 column (100-10 mm, Merck KGaA, Darmstadt, Germany). First, the crude proteins were filtered through 4 mm X 0.45 µm syringe filters (Whatman) before injected into the column. The crude proteins were eluted with an increasing gradient of acetonitrile containing 0.05% TFA, from 0% to 50% in 10 min at a flow rate of 3.5 ml/min. The UV absorbance of the eluent was monitored at 220 nm. All the protein fractions were sub-fractioned according to the peaks obtained. They were concentrated by lyophilisation and the antihypertensive activity of the purified protein fractions was determined at a concentration of 1 µg/ml protein.

Surface-Enhanced-Laser-Desorption-Ionization-Time-of-Flight-Mass-Spectrometry (SELDI-TOF-MS) Analysis. Protein fractions of F1a and F5b of *P. cystidiosus* were analysed using hydrophobic H50 ProteinChip® arrays (Bio-Rad Laboratories, Inc). The arrays were first equilibrated with binding buffer, which consist of 0.5% TFA in 50% acetonitrile (v/v). Then, 5 µl of samples containing 0.5 µg of protein were spotted on the arrays. After the arrays were air-dried, 2 µl of CHCA in 0.5% TFA in 50% acetonitrile (v/v) was added onto the arrays. The arrays were left to air-dry. The analyses were carried out with the ProteinChip SELDI system (Bio-Rad Laboratories Inc., PSC 4000). Data collection was carried out in positive ion mode using the following acquisition settings: mass range of 0 to 20 kDa, focus mass of 10 kDa. Laser energy used for the shot sequences were based on the following settings: warming shots 1000 nJ and data shots 900 nJ. Spectra were calibrated using an external calibration against a mixture of standards consisting of somatostatin (1637.9 Da), arg-insulin (5969.0 Da) and cytochrome c (12263.3 Da).

RESULTS AND DISCUSSIONS

ACE Inhibitory Activities of Water Extracts from Fruiting Bodies of Edible Mushrooms. Water extracts from the fruiting bodies of the edible mushrooms were tested for their ACE

inhibitory activity at a concentration of 10 mg/ml. As shown in Table 1, the nine edible mushrooms tested showed high ACE inhibitory activity where 71.9 to 95.5% of ACE was inhibited. Among the mushrooms tested, *F. velutipes* and *L. edodes* showed the highest ACE inhibitory activity. This was followed by *H. erinaceus*, where 90.1% of ACE activity was inhibited. About 87.2% of ACE was inhibited by the water extract of *A. bisporus*. The five *Pleurotus* spp tested exhibited 87.6 to 71.9% of ACE inhibition activity. The result obtained in the current study was comparable with the results reported in the previous study. According to the report by Kim and co-workers, 50% of the ACE activity was inhibited by *F. velutipes* at a concentration of 7.4 mg/ml while the IC_{50} values for *L. edodes* were in the range of 18.4 to 39.3 mg/ml [19]. Meanwhile, Lee and co-workers reported 38.7% and 27.3% of ACE activity were inhibited by water extract from *P. sajor-caju* and *P. ostreatus*, respectively. The ACE inhibitory activities of *F. velutipes* were in the range of 13.7% to 32.9% while *A. bisporus* showed 27.3% of inhibition [20]. Thus, all the nine mushroom species tested in the current study are potential ACE inhibitor. Proteins were precipitated from the water extracts of the nine mushroom species for further study.

Table 1: Percentages of ACE inhibitory activity of water extracts from various edible mushrooms

Scientific name	ACE inhibitory activity (%)
A. bisporus	87.2 ± 0.8^{b}
F. velutipes	95.6 ± 0.3^{g}
H. erinaceus	90.1 ± 0.6^{f}
L. edodes	95.2 ± 0.3^{g}
P. citrinopileatus	71.9 ± 1.0^{d}
P. cystidiosus	81.3 ± 0.8^{a}
P. flabellatus	75.4 ± 1.3^{c}
P. florida	87.6 ± 1.4^{b}
P. sajor-caju	85.2 ± 0.6^{e}
Captopril	20.2 ± 3.2^{h}

Mushroom extracts were tested at 10 mg/ml. Captopril was tested at 10×10^{-8} mg/ml. Each inhibitory activity is expressed as mean ± standard deviation (n=3). Means with different alphabet letters within a column denotes the ACE inhibitory activity of the samples are significantly different ($P<0.05$).

Comparison of the Protein Concentrations among the Edible Mushrooms. Protein content in mushrooms has been ranked below animal meats but well above most other foods, such as vegetables, fruits and milk [10]. In the current study, proteins were precipitated from the crude water extracts using different salt saturation ranging from 10 to 100%. More hydrophobic proteins will precipitate out first at lower salt saturations. The concentrations of the precipitated protein were estimated using BCA protein assay kit. Referring to the results shown in Fig. 1, the graph obtained showed a binomial distribution, where the amount of protein precipitated increased as the percentage of the salt saturation increased. After it reached a maximum

percentage of salt saturation, the amount of protein collected were decreased until it reached 100% salt saturation. Most of the mushroom species in the current study had the highest concentration of protein precipitated in between 50 to 70% salt saturation, except for *A. bisporus* (40% salt saturation) and *P. sajor-caju* (80% salt saturation). Thus, it can be assumed that most of the proteins contained in the mushroom fruiting bodies tested in the current study have an intermediate hydrophobicity.

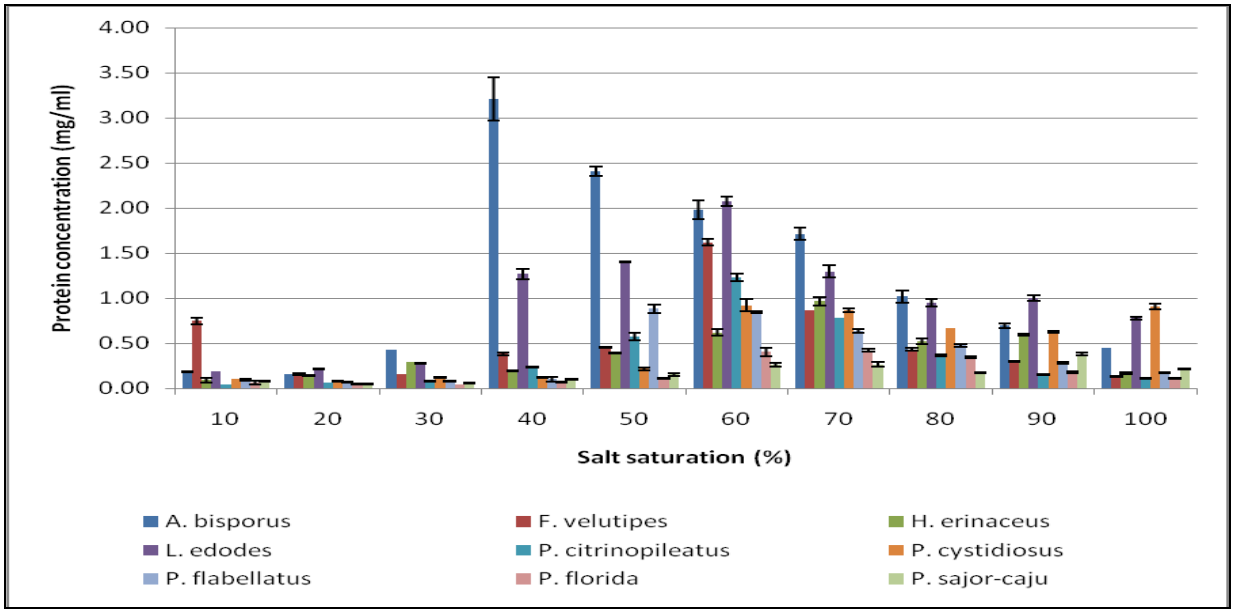

Figure 1: Protein concentration (mg/ml) of the nine selected species of edible mushrooms at ten ammonium sulfate salt saturations.

Comparison of the ACE Inhibitory Activity of the Partially Purified ACE Inhibitor. Proteins of the mushroom species precipitated at different salt saturation were separated by SDS PAGE according to their molecular weight. Some of the proteins precipitated at the neighboring salt saturation showed similar bands (Data not shown). Thus, proteins precipitated at different salt saturation that showed similar protein bands were pooled together. *Hericium erinaceus* and *P. citrinopileatus* have been pooled into four groups while *A. bisporus*, *P. flabellatus*, *P. florida* and *P. sajor-caju* were divided into five groups. *Flammulina velutipes*, *L. edodes* and *P. cystidiosus* were pooled into six groups.

The ACE inhibitory activity of the protein fractions were tested at 10 µg/ml protein. Even though all the mushroom water extracts showed good antihypertensive activity (Table 1), however not all of the protein fractions showed good activity (Table 2). For example, water extract of *H. erinaceus* managed to inhibit the ACE activity at 90.1%, but protein fractions of *H. erinaceus* showed less than 7% ACE inhibitory activity. Although the purified protein has low ACE inhibitory activity, the mushrooms may still be a good ACE inhibitor due to other compounds. According to a previous study, D-mannitol in *P. cornucopiae* has been reported to exhibit a blood pressure lowering activity by inhibiting the angiotensin I-converting enzyme activity [25]. Triterpenoids in *G. lucidum* was believed to have blood pressure lowering effect [12].

Referring to Table 2, about 50% of the mushroom species tested have higher ACE inhibitory activity exhibited by hydrophobic proteins from fraction 1 compared to the other

protein fractions for the same mushroom species. This is supported by the previous report indicating that high ACE inhibitory activity is due to more hydrophobic peptides [26, 27].

Among the protein fractions of the various mushrooms, *P. cystidiosus* showed the highest antihypertensive activity where 96.2% of ACE was inhibited by fraction 1. This was followed by fraction 5 where 86.7% of ACE was inhibited. Thus, the two protein fractions have been selected for further purification of ACE inhibitory peptide.

Table 2: Percentages of the ACE inhibitory activity of the protein fractions from the nine selected species of edible mushrooms.

Mushroom species	Protein fractions					
	1	2	3	4	5	6
A. bisporus	78.4 ± 4.4	8.3 ± 3.2	71.4 ± 0.9	39.3 ± 1.3	8.1 ± 2.8	-
F. velutipes	18.3 ± 3.5	15.8 ± 0.8	12.6 ± 2.2	13.7 ± 3.2	16.4 ± 3.7	19.8 ± 0.5
H. erinaceus	5.4 ± 3.4	5.9 ± 2.9	6.3 ± 1.8	6.9 ± 4.6	-	-
L. edodes	11.7 ± 2.1	16.6 ± 6.3	24.9 ± 2.8	5.7 ± 2.9	3.3 ± 1.9	4.6 ± 2.6
P. citrinopileatus	41.1 ± 4.4	20.9 ± 1.6	15.2 ± 4.9	20.2 ± 1.5	-	-
P. cystidiosus	96.2 ± 0.6	47.5 ± 2.7	31.6 ± 6.1	31.5 ± 2.3	86.7 ± 2.8	48.3 ± 2.6
P. flabellatus	28.4 ± 3.1	36.5 ± 4.1	21.8 ± 3.9	19.9 ± 3.0	22.0 ± 3.4	-
P. florida	25.5 ± 2.3	24.2 ± 8.5	17.0 ± 3.1	22.9 ± 4.7	4.5 ± 4.2	-
P. sajor-caju	41.2 ± 7.3	30.6 ± 0.8	35.9 ± 2.2	32.2 ± 4.2	5.5 ± 1.7	-

Protein fractions were tested at 10 µg/ml protein. Each inhibitory activity is expressed as mean ± standard deviation (n=3).

Purification of ACE Inhibitor from *P. cystidiosus* by RPHPLC. Fraction 1 and 5 of *P. cystidiosus* which showed the highest ACE inhibitory activity were selected for further purification by RPHPLC. The RPHPLC chromatogram for the two protein fractions are shown in Fig.2. The ACE inhibitory activity of the collected RPHPLC fractions was tested at a concentration of 1 µg/ml protein. The result obtained showed lower antihypertensive activity than expected. This indicates that there may be a synergistic action among the peptides when present together in the whole protein fractions. This synergistic effect has also been proposed for active ACE inhibitory peptides isolated from cheese and tilapia [28, 29]. RPHPLC peaks collected from fraction 1 inhibited the ACE activity in the range of 3.3 to 32.4%. The highest activity was exhibited by fraction F1a, which was eluted at 1.7 min. RPHPLC peaks collected from fraction 5 inhibited the ACE activity in the range of 17.4 to 36.9%. The highest activity was exhibited by fraction F5b, which was eluted at 2.1 min. Thus, the two protein sub-fractions have been selected for SELDI profiling.

SELDI Profiling of the Partially Purified ACE Inhibitor from *P. cystidiosus*. In the last few years, proteomics profiling experiments were performed using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled to mass spectrometry (MS). Previous study has reported on the protein profiling of mushroom using the 2D-PAGE MS technique [30]. However, this technique is laborious and limits the isolation of low-abundant proteins. Besides, it requires large

amounts of samples [31]. Thus, to overcome the limitations of 2D-PAGE MS, SELDI-TOF-MS has been used for the protein profiling analysis in the current study.

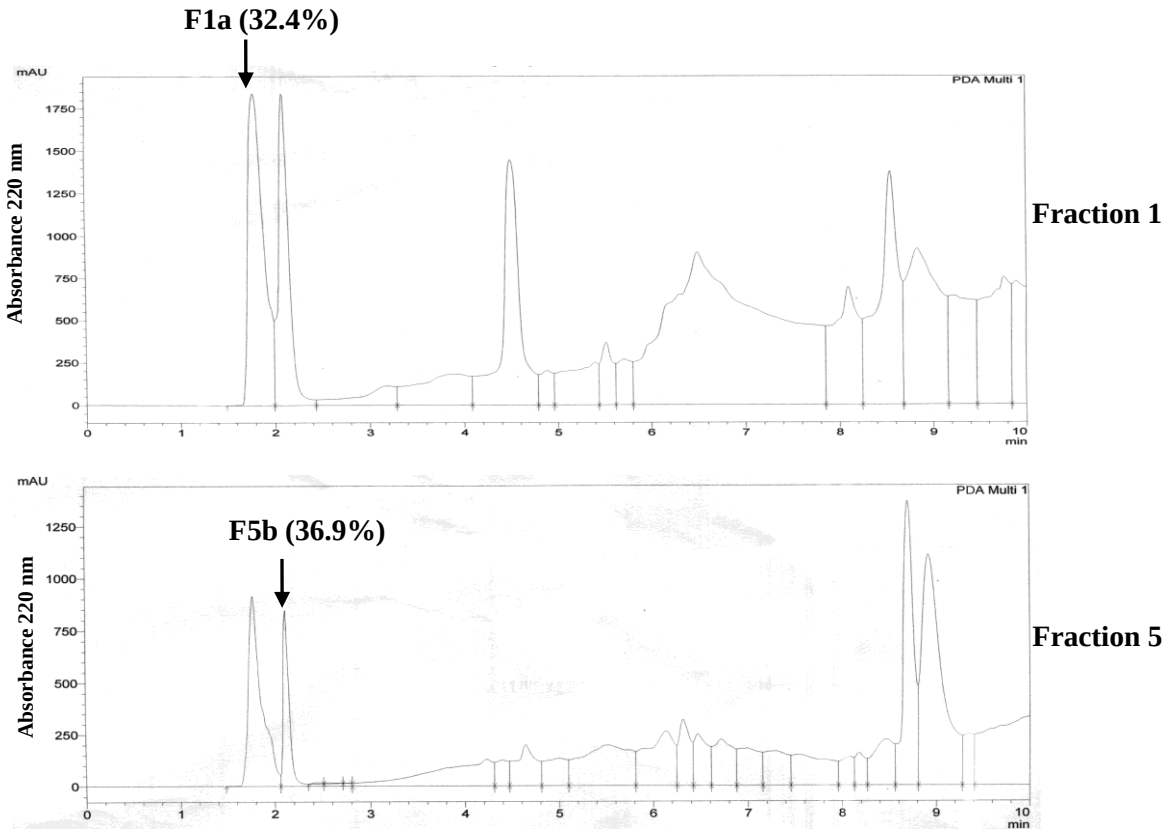

Figure 2: RPHPLC chromatogram of fraction 1 and fraction 5 of *P. cystidiosus*. Sub-fraction with the highest ACE inhibitory activity and the percentages of inhibition are presented.

Referring to Figure 3, there were five proteins observed in fraction F1a with the m/z of 4151.65, 8299.77, 12494.41, 48794.11 and 54060.12. Three proteins were observed in fraction F5b with m/z of 4153.63, 8303.48 and 10308.76. In both fractions, the peak with m/z of 8300 has the highest intensity followed by m/z of 4100. Hence, the ACE inhibitory activity of the two fractions might be due to these low molecular weight proteins. Previous studies had reported on the higher ACE inhibitory activity exhibited by lower molecular weight peptides [32, 33]. Protein with large molecular size was reported to have disadvantage of metabolic instability. Thus, it is not suitable to be used as cardiovascular drug [34]. Peptides with two or three amino acid residue lengths could be absorbed directly from the digestive tract into the blood circulatory system and be able to reach the action sites to exert physiological functions [35]. Hence, the low molecular weight protein from *P. cystidiosus* could be a good source of ACE inhibitor.

CONCLUSION

Edible mushrooms can be a good source of bioactive compounds with ACE inhibitory activity. Among the nine mushroom species tested in the current study, protein fractions from *P. cystidiosus* had the highest ACE inhibitory activity which may be due to low molecular weight protein. Although the ACE inhibition effect is lower compared to captopril, the inhibitor from *P. cystidiosus* is a peptide derived from a food source that can be eaten daily. Further studies have to be carried out to identify the peptide sequence of the ACE inhibitor.

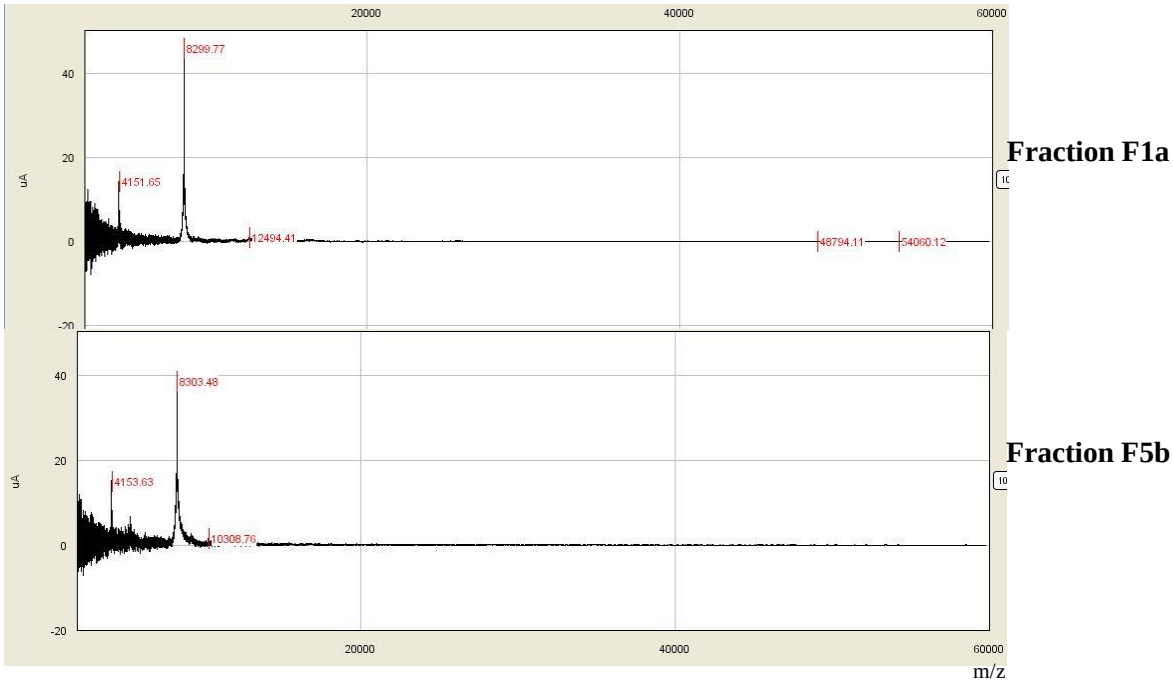

Figure 3: SELDI-TOF-MS spectra for protein sub-fractions F1a and F5b of *P. cystidiosus* generated using H50 ProteinChip array.

ACKNOWLEDGEMENTS

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IMMUNOMODULATING PROPERTIES OF *PLEUROTUS* SP. FRUITING BODIES POWDER ON CYCLOPHOSPHAMIDE TREATED MICE.

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ABSTRACT

Since the potentiation of host resistance is one of the most important objectives in cancer and AIDS therapy, the present study examined the immunomodulating effects of *Pleurotus* sp. fruiting bodies powder on cyclophosphamide (CY) treated mice. *Pleurotus* powder was administered during 7 days to Balb/c mice by oral route (1000 mg/kg) and the CY (100 mg/kg) was inoculated intraperitoneally, at the beginning of the experiment or at the fifth day. The influence of the supplement on CY immunosuppresion was evaluated on the eighth day. The prophylactic administration of *Pleurotus* powder increased the bone marrow cellularity (9.89 $x10^6$ *vs.* 4.94 x 10^6 per femur in therapeutically treated mice, p=0.035) and the white blood cell counts (7.8 $x10^9$ *vs.* 4.7 $x10^9$ cells/L, p=0.016). The *Pleurotus* supplement in the prophylactic treatment stimulated the liver protein synthesis; although no significant effects were found in serum protein concentrations. The effect of *Pleurotus* powder on cell-mediated immunity was determined by the delayed-type hypersensitivity reaction (DTH) in mice under the therapeutic schedule. The DTH response measured at 48 and 72 h after antigen challenge was similar that of normal control mice ($p< 0.05$). The induction of DTH reaction was associated with an increase in the mass index of popliteal lymph nodes ($p= 0.044$). An *in vitro* lymphoproliferative-stimulating response evaluated in mice spleen cells was also demonstrated with aqueous and ethanolic extracts obtained from *Pleurotus* powder. These effects suggest that *Pleurotus* supplement could potentiate the host defense mechanisms *in vivo* and should be promising for further pharmacological studies.

Keywords: *Pleurotus*; medicinal mushrooms; fruiting bodies; cyclophosphamide; immunomodulation.

INTRODUCTION

Medicinal mushrooms have an established history of use in traditional oriental therapies and modern clinical practice in several Asian countries continues to rely on mushroom-derived preparations [1, 2]. Medicinal effects have been demonstrated for many traditionally used mushrooms, including extracts of species from genera *Auricularia, Flammulina, Ganoderma, Grifola, Hericium, Lentinus* (*Lentinula*), *Pleurotus, Trametes* (*Coriolus*), *Schizophyllum,* and *Tremella* [3, 4].

The genus *Pleurotus* comprises some of the most popular *Basidiomycetes* edible mushrooms which cultivation has increased greatly throughout the world during the last few

decades [5]. Its popularity has been expanded due to its vigorous growth on a variety of agroforestry substrates and for the production of a high nutritional value-food [6] containing biologically active compounds with therapeutic effects [7].

Recent studies on various *Pleurotus* species have shown a number of pharmacological activities, such as anti-tumour, immunomodulatory, antigenotoxic, antioxidant, anti-inflammatory, hypocholesterolaemic, antihypertensive, antiplatelet-aggregating, antihyperglycaemic, antimicrobial and antiviral activities [8].

With the view of developing new therapeutic agents to potentiate host resistance to cancer and infectious disease, such as AIDS, there has been an upsurge of interest in immunomodulating substances from medicinal mushrooms [9-11]. *Pleurotus* species, like many edible and medicinal mushrooms, are a good source of immunomodulators and substances considered as "host defense potentiators" (HDPs) as judged by their immunostimulating properties. Several molecules able to augment or complement a desired immune response have been isolated from *Pleurotus* spp., such as: water-soluble polysaccharides from *P. citrinopileatus* fermentation broth [12]; glucans from *P. florida* fruiting bodies [13]; proteoglicans, polysaccharides and polysaccharopeptides from *P. ostreatus* mycelia [14-16] and DNA from *P. ostreatus* fruiting bodies [17]. These compounds stimulate different cell populations of the immune system, for instance, macrophages, Natural killer (NK) cells, T cells, and also modulate cytokine system [8].

Much research work has been reported for various extracts and isolated compounds, particularly polysaccharides. Therefore, the study of the synergy exerted by the vast structural diversity of biomolecules found in *Pleurotus* crude extracts, powders and other preparations on immune responses deserves special attention. In this context, dietetic supplements with a high therapeutic potential formulated from refined or partially refined mushroom extracts, or from dried mycelia/fruiting bodies biomass are referred as "mushroom nutriceuticals" [18, 19]. Those supplements with an immunostimulating effect are defined as "immunoceuticals" [20].

In Cuba, the implementation of technologies for the cultivation of *Pleurotus* spp. on agricultural substrates, in addition to food generation for human consumption [21, 22] opened new research activities towards mushroom immunoceuticals. *Pleurotus* fruiting bodies obtained under Good Manufacture Practices can be used in the formulation of biologically active products, such as, powders, capsules and tablets.

The present study examined the immunomodulating effects of *Pleurotus* sp. fruiting bodies powder on the immunosuppression caused by cyclophosphamide in mice. An extended knowledge of the immuno-enhancing activity of *Pleurotus* would be useful in understanding the potential applications of *Pleurotus*-derived preparations for immunotherapy.

MATERIALS AND METHODS

Preparation of *Pleurotus* sp. powder. *Pleurotus* sp. **(**strain CCEBI-3024) deposited at the Culture Collection of the Center for Studies of Industrial Biotechnology (CEBI) was used in this work. Cultivation was performed by solid-state fermentation of mushroom spawn on pasteurized coffee pulp used as substrate in plastic bags of 2 kg (30x40 cm) [21, 22]. The fruiting bodies were harvested, sliced into small pieces and dried at 45°C for 24 h. The dried material was milled and the resulting powder was preserved from light and humidity in plastic bags for further use.

The sugar and protein contents in the powder were determined by the method of Dubois et al. [23] and by Lowry's method [24] using glucose and bovine serum albumin (BSA) as standards, respectively.

The powder was extracted with hot water and ethanol to obtain both aqueous and ethanolic extracts for assessing the *in vitro* lymphoproliferative activity.

Animals and treatments. Pathogen-free male Balb/c mice were purchased from the National Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). The 20-25 g mice were fed a standard diet and acidified water *as libitum.* Fifty mice were divided into five groups (n= 10). *Pleurotus* powder was administered during 7 days to Balb/c mice by oral route (1000 mg/kg) and the cyclophosphamide USP 23 for injection (CY, JSLYP, China) (100 mg/kg) was given intraperitoneally (i.p.), at the beginning of the experiment (CY-*Pleurotus* group) or at the fifth day (*Pleurotus*-CY group). CY-Saline and Saline-CY groups were designed as controls replacing the *Pleurotus* powder by physiological saline solution. A non-administered control group of 10 mice (C) was also included in the study. The influence of treatments on CY immunosuppresion was evaluated on the eighth day. Blood was collected from the orbital vein of mice and then, the animals were killed.

All experiments were approved by the institutional Ethical Committee (University of Oriente) and have been performed in accordance with Cuban legislation and the National Research Council Guidelines for the Care and Use of Laboratory Animals.

Hematological methods. The blood specimens were analysed for hemoglobin with the Hemotest reagent (HELFA Diagnósticos, EPB Carlos J. Finlay, Havana, Cuba) and for white blood cell counts. Femoral bone marrow cells were withdrawn with Hanks´ solution and counted with a Neubauer chamber (Germany) under a binocular microscope. The spleen cell suspension was prepared by gently teasing the tissue with ice-cold Hanks' solution and passing it through antiseptic gauze (Johnson and Johnson Medical, TX, USA). The number was counted with a Neubauer chamber.

Liver and serum protein analysis. Liver samples were homogenized in ice-cold 0.01 mol/L phosphate buffer saline (PBS) pH 7.4 (1:3 w/v). Total protein was measured according to the Lowry´s method [24].

Serum was prepared from collected blood and stored at -20°C until required. Total serum proteins were measured by a Biuret colorimetric assay using BSA as standard, albumin by the colorimetric reaction with the bromocresol green reagent and globulins as the difference between total proteins and albumin.

Evaluation of cell immunity. In a parallel-conducted experiment, the cell-mediated immune response was assessed by the delayed-type hypersensitivity reaction (DTH) only in mice under the therapeutic schedule (CY-*Pleurotus* group) and its control (CY-Saline) group.

Animals (n=5) were immunized by an intradermal (i.d.) injection of 50 µL of 5 mg/mL bovine serum albumin (BSA) emulsified in Complete Freund Adjuvant (CFA) (Sigma, St. Louis, MO) at two sites on the abdomen. Eight days after immunization, the mice were rechallenged by injection of 20 µL of 5 mg/mL BSA into one rear foot pad, while the other rear foot pad received a comparable volume of phosphate buffered saline (PBS). Measurements of foot pad swelling were taken at 24, 48 and 72 h after challenge by use of micrometer (Mitutoyo, Tokyo, Japan). The magnitude of the DTH response was determined as the differences in foot pad thickness between the antigen and PBS injected foot pads [25].

The popliteal lymph nodes (right and left) of the antigen sensitized and rechallenged animals of DTH experiment were removed and washed with PBS pH 7.4. The excess of humidity was discarded with a filter paper and the lymph nodes were immediately weighed separately in an electronic analytical balance (Sartorius).The mass index was expressed as the

relation between the weight of the popliteal node belonging to BSA-injected foot pad with respect to that of PBS-injected pad [26].

***In vitro* lymphoproliferative test.** The assay was carried out according to a modification of the method described by Soto-Velazco et al. [27] using murine spleen lymphocytes instead of human lymphocytes. The suspension of splenocytes was obtained by the gentle teasing of spleens in RPMI-1640 (Sigma, St. Louis, MO) containing 8% fetal calf serum and supplemented with antibiotics. Viable cells estimated by the Trypan Blue exclusion method were counted with a Neubauer chamber and the cell concentration was adjusted to $2x10^6$ cells/mL.

Briefly, phytohemagglutinin (PHA) (200 µL) as a B-cells mitogen was added to conical tubes of 15 mL containing 5 mL of supplemented RPMI-1640 to a final concentration of 5 µg/tube, followed by the addition of $2x10^6$ cells and 100 µL of powder extracts (aqueous or ethanolic). The tubes were incubated at 37ºC for 27 h and then 500 µL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H tetrazolium bromide (MTT, 5 mg/mL) was added. After the incubation of the resulting mixture at 37ºC for 4 h, the tubes were centrifuged 10 min at 1500 rpm. The supernatants were discarded and 1 mL of isopropanol was added to each tube. The absorbance of the mixture was measured at 570 nm in a Genesys 10 UV/VIS spectrophotometer. The stimulation index was calculated considering the absorbance of control cultures without PHA as the unit.

Statistical analysis. The results were expressed as mean ± standard deviation (SD). The Kruskal-Wallis rank test followed by the Student-Newman-Keuls test was applied to determine the significance of differences between treatments. The Student's *t*-test was used to compare the two means in the experiment of popliteal lymph nodes mass index. Differences at $p< 0.05$ were accepted as significant. The software Statgraphics Plus version 5.1 (Statistical Graphics Corporation, 1994-2001) was used in all the analysis.

RESULTS AND DISCUSSION

Immune system is a very complex homeostatic system consisting of a network of interacting cells, tissues and organs. It allows the organism to exist within itself and maintains a surveillance to recognize components considered nonself. The body's immunity has been shown to be suppressed in several diseases, like AIDS and cancer. The chemotherapy and radiotherapy in cancer treatment contribute to further depression of the immune system [28]. Use of immunomodulating therapeutic agents can solve these problems largely and efforts to find new immunomodulators are on-going. Among higher fungi investigated for immunomodulating effects, several mushroom species demonstrate great potential and some of them are already commercially developed [29].

Dried *Pleurotus* mushroom would become an attractive alternative for the development of drugs and immunoceuticals preparations. The powder evaluated in this work contained 55% (w/w) carbohydrate and 25% (w/w) protein. Traditional preparations of medicinal species, used for thousands of years, might give some support to the idea that a heat-treatment might preserve at least part of the activity [30].

During the experimental period, no environmental factors other than the stated variables were thought to have affected the results of the study. No deaths occurred in either the control or the administered groups.

Cyclophosphamide is probably the most common antineoplastic used in cancer chemotherapy and is as essential component of several effective chemotherapeutic formulas. However, cyclophosphamide shows potent immunosuppressing properties, thus affecting the bone marrow cellular production as well as the B cells and the antibodies responses [31]. As

expected, cyclophosphamide severely impaired the mice hematopoietic tissue, but the *Pleurotus* powder was found to have an active protective effect in mice, particularly when administered before cytostatic.

The hematological parameters assayed on the eight day are shown in Fig. 1. The prophylactic administration of *Pleurotus* powder increased the bone marrow cellularity (9.89 $x10^6$ *vs.* 4.94 x 10^6 per femur in therapeutically treated mice, p=0.035). In mice receiving the therapeutic schedule no significant differences were found compared to CY-saline control groups. A significant contribution to the protective effect of *Pleurotus* product on hemopoiesis, may be in the production and differentiation of auto-regenerative bone marrow hemopoietic cells (precursors of both myeloid and lymphoid lines) and a more rapid recovery of these series of hemopoietic cells in animals protected by the immunoceutical supplement. We can infer that one of the mechanisms of increased cyclophosphamide tolerance of animals is the activation effect on hemopoiesis by the evaluated *Pleurotus* powder.

The white blood cell counts in peripheral blood were also higher in the prophylactic administered mice (7.8 $x10^9$ *vs.* 4.7 $x10^9$ cells/L in therapeutically treated mice, p=0.016). The observed significant improved recovery of leukocyte numbers against CY-induced leucopenia could be concerned with enhancement of hematopoietic factors such as colony-stimulating factors. Furthermore, increased white blood cell number would be an important contributing factor to reduce the risk of various infectious diseases in immunocompromised patients.

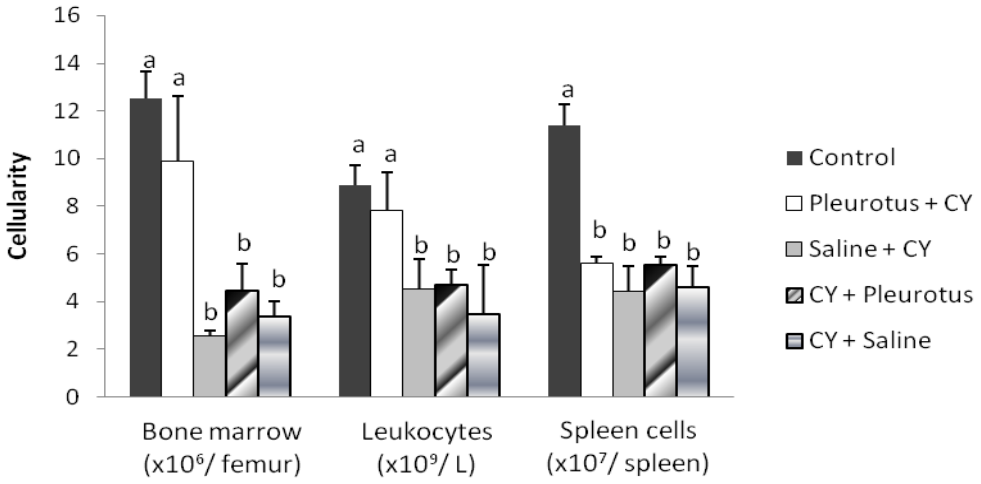

Figure 1: Effect of prophylactic or therapeutic administration of *Pleurotus* powder on the hemopoiesis of cyclophosphamide treated Balb/c mice.
All values are given as the arithmetic mean ± standard deviation of 10 mice. Different letters indicate significant differences among the groups (Kruskal-Wallis, Student-Newman-Keuls).

The stimulant effect on bone marrow cellularity and leukocyte counts exerted by a hot-water extract from *Pleurotus ostreatus* mycelium (obtained by solid state fermentation) administered in a prophylactic schedule to cyclophosphamide treated mice has been reported by Morris et al. [32]. On the other hand, a *P. ostreatus* mycelial extract inhibited the tumor growth in mice administered alone or concomitant with CY. The extract also decreased the severity of leucopenia caused by the cytostatic [33].

The spleen cellularity of mice treated with *Pleurotus* powder did not differ from their saline control groups. All the experimental treatments showed a significant decreasing in the spleen cell counts compared to the immunocompetent mice (p= 0.0175) (Fig. 1). One of the most

important functions of this lymphoid organ is the production of antibodies through the antigenic stimulation of B cells [28]. It has been reported that B cells are particularly sensitive to CY (more than T cells) and in some cases, the suppression of antibody response to an antigenic specific stimulus would be lasting [31].

No significant differences were found in hemoglobin levels of mice belonging to both prophylactic or therapeutic administered groups (139±16 *vs.* 108±9 g/L, respectively). However, only the animals treated with *Pleurotus* powder before CY administration reached hemoglobin concentrations similar to that of the control group (145±3 g/L, p= 0.0231). This favorable effect of *Pleurotus* supplementation would be an important aspect to consider during immunonutritional interventions of .patients with cancer or pathologies related with the immune system.

The results of liver and serum protein content in all groups are given in Table 1. The liver total protein concentration was significantly higher in animals administered with *Pleurotus* powder before CY compared to the therapeutic schedule (p= 0,0318), although the levels of control immunocompetent mice were not reached. The partial recovery observed in this parameter could be associated with more efficient nitrogen utilization as judged by the protein content of the powder and therefore, the stimulation of protein anabolism in the liver, including the synthesis of acute phase proteins.

There were non significant differences between groups with respect to the total serum protein concentration, albumin and globulin concentrations (Table 1). However, Llauradó et al. reported an increasing in total serum protein levels in malnourished Balb/c mice supplemented with a cold-water extract from the fruiting bodies of *Pleurotus* sp. [34].

In our study, the prophylactic treated mice showed the lowest values of the albumin-to-globulin (A/G) ratio (p=0.0351). This fact would sustain the stimulation of serum globulins synthesis.

Table 1: Effect of prophylactic or therapeutic administration of *Pleurotus* powder on liver and serum protein content of cyclophosphamide treated Balb/c mice.

	Control	*Pleurotus* + CY	*Saline* + CY	CY+ *Pleurotus*	CY + *Saline*
Liver protein (mg/g) (p = 0.0317)	112.04 ± 4.32^{a}	79.9 ± 8.8^{b}	59.2 ± 1.27^{c}	67.5 ± 0.14^{c}	69.25 ± 0.35^{c}
Serum total protein (g/dL)	6.49 ± 0.05	6.07 ± 0.8	5.29 ± 0.2	5.3 ± 0.35	5.5 ± 0.58
Albumin (g/dL)	3.61± 0.11	3.12 ± 0.62	3.99 ± 0.08	3.63 ± 0.37	4.02 ± 0.38
Globulins (g/dL)	2.88 ± 0.48	2.93 ± 1.37	1.8 ± 0.98	1.98 ± 0.57	1.47 ± 0.62
Albumin-to-Globulin ratio (p=0.0351)	1.25 ± 0.43^{abc}	1.3 ± 0.76^{c}	3.15 ± 0.74^{ab}	2.32 ± 0.74^{ab}	3.18 ± 175^{a}

All values are given as the arithmetic mean ± standard deviation of 10 mice. Different letters indicate significant differences among the groups (Kruskal-Wallis, Student-Newman-Keuls).

The immunostimulating properties of *Pleurotus* powder administered therapeutically to cyclophosphamide treated mice on cell-mediated immune response were assessed by the assay of induction of delayed-type hypersensitivity (DTH) response.

Mice supplemented with *Pleurotus* powder showed a higher DTH response as judged by the increasing of foot pad swelling compared to saline control group, particularly at 48 h and 72 h after antigen rechallenge (p <0,05) (Fig. 2). The DTH response mounted at these times by CY-

Pleurotus group was similar that of control mice. The reconstitution of DTH response reflected the induction of $CD4^+$ Th1 cells and the activation of macrophages by cytokines: tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) [25].

Paulik et al [35] evaluated the effects of two glucans obtained from *Pleurotus ostreatus* and yeast in different immunological functions of mice. Both glucans augmented the DTH response compared to control mice, but the induction was higher for *Pleurotus* glucan. A significant increase in the number of T cells (both $CD4^+$ and $CD8^+$) was found in mice administered with a water soluble polysaccharide extracted from the fermentation broth of *Pleurotus citrinopileatus* [36].

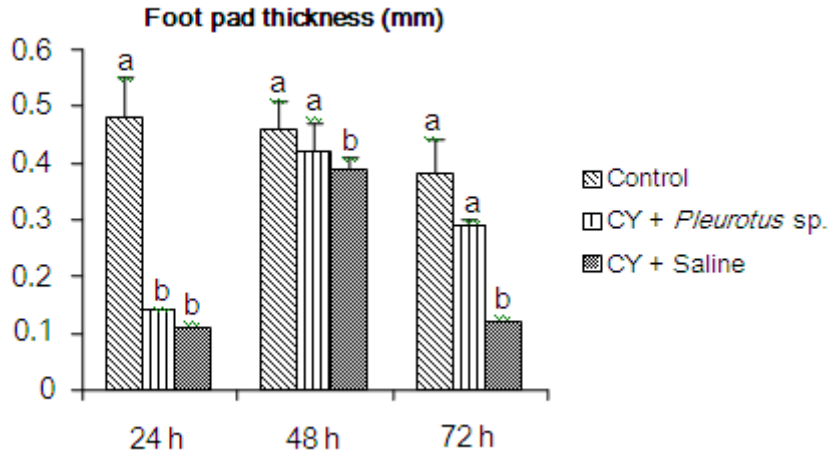

Figure 2: Effect of therapeutic administration of *Pleurotus* powder on the delayed-type hypersensitivity response (foot pad thickness) of cyclophosphamide treated Balb/c mice.
All values are given as the arithmetic mean ± standard deviation of 5 mice. Different letters indicate significant differences among the groups (Kruskal-Wallis, Student-Newman-Keuls, $p<0.05$).

These findings suggest that oral administration of edible mushrooms derived products with potential immunostimulating activities would stimulate the immune system after their absorption in the gastrointestinal tract and the activation of gut-associated lymphoid tissues, thus integrating different elements of the immune function.

However, the results of the administration of an aqueous extract of *P. florida* to female Balb/c mice showed that DTH responses were not affected by various doses of the product in different routes. This study indicated that the effects of *P. florida* on cellular responses depends on dose and route of administration [37].

The DTH reconstitution was associated with the increase observed in the mass index of popliteal lymph nodes of the *Pleurotus* supplemented animals (1.87 ± 0.27 *vs.* 1.34 ± 0.15 in CY-Saline group, $p=0.044$). Antigens are concentrated in the secondary lymphoid organs, including lymph nodes, where they are presented by mature dendritic cells, the most efficient type of antigen-presenting cell for initiating responses of naive T cells [28].

Cellular immune response was also evaluated by the *in vitro* lymphoproliferative response of murine splenocytes (B cells) through the colorimetric reaction with the MTT reagent. The incubation of splenocytes with aqueous and ethanolic extracts derived from *Pleurotus* powder for 72 h lead to stimulation indexes of 1.90 and 1.28, respectively (Fig. 3). The higher index obtained for aqueous extract could be related with the presence in this fraction of immunostimulating glucans. It has been reported that hydroalcoholic extracts are likely to have less activity, partly because the high-molecular weight compounds are precipitated by alcohol and may not go into solution [30].

Soto-Velazco et al. [27] reported the *in vitro* stimulation of lymphoproliferative response of human mononuclear cells incubated with different extracts from *Ganoderma lucidum*. On the other hand, glucans isolated from *P. florida* fruiting bodies significantly induced the proliferative response as well as phagocytic activity of fish leukocytes (*Catla catla*) *in vitro* [38].

The genotoxic effects of cyclophosphamide and the ability of several- both cold- and hot-water extracts from different mushrooms to protect against damage to cellular DNA [39], suggest that further research on the potential genoprotective activity of *Pleurotus* powder is needed.

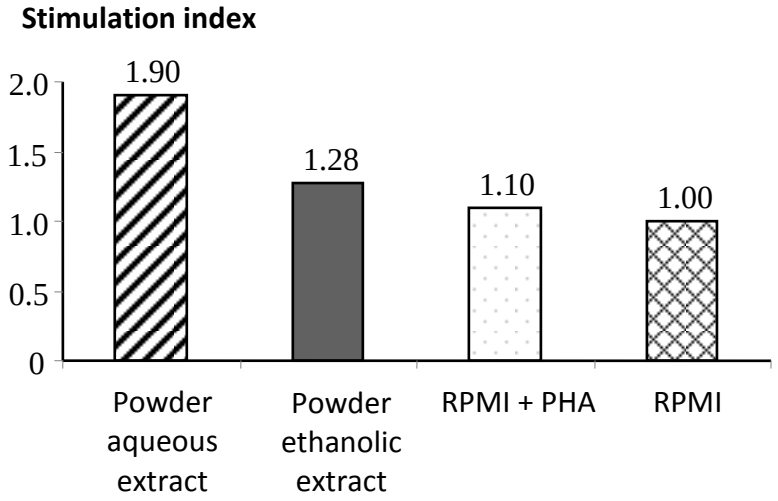

Figure 3: *In vitro* lymphoproliferative-stimulating response of aqueous and ethanolic extracts obtained from *Pleurotus* powder on murine spleen cells.

CONCLUSION

The results of this study evidence that *Pleurotus* supplement, administered orally to cyclophosphamide-treated mice, provide immunological benefits in terms of: (i) the recovery of bone marrow cellularity, (ii) the increase of white blood cell counts, and (iii) the stimulation of cell-mediated immune responses. *Pleurotus* powder could potentiate the host defense mechanisms *in vivo* and should be promising for further pharmacological studies. The effects on cell immunity are especially valuable in the prophylaxis of tumors, immunodeficiencies and as co-adjuvant in chemotherapy. Further studies are needed to address effective phytochemicals of this edible mushroom and their mechanisms.

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PRODUCTION OF LIGNOCELLULOLYTIC ENZYMES BY MUSHROOMS

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ABSTRACT

Biopolymers contained within or derived from plant biomass form by far the largest pool of soil carbon. The decomposition of lignocellulose in the soil environment thus attracts considerable attention. Lignocellulose is composed mainly of the polysaccharidic polymers cellulose and hemicelluloses, and the polyphenolic polymer lignin. During transformation in soils, humic substances (humus, humic and fulvic acids) are formed from both lignocellulose and structural components of microbial decomposers. This is achieved through the concerted action of lignocellulose-degrading enzymes, whose activity is regulated by soil properties, land use and the identity of their microbial producers. Soil fungi seem to be the most important players in lignocellulose transformation processes due to their ability to attack both polysaccharides and polyphenols in the soil organic matter. While some basic concepts of regulation of enzymatic activity have been outlined, questions regarding enzyme production and diversity at the molecular level are just recently being addressed. Moreover, current results show that the ability to decompose lignin and cellulose is not restricted to saprotrophic basidiomycetes and ascomycetes but that the contribution of ectomycorrhizal fungi can be important. From a practical viewpoint, the ability to produce lignocellulose-decomposing enzymes by mushrooms is important for their cultivation, efficient substrate use, high yield and production of value-added products from lignocellulosic agrowastes.

Keywords: Lignin; Cellulose; Decomposition; Enzymes

INTRODUCTION

Since the biopolymers contained within lignocellulose form by far the largest pool of soil carbon and represent the most important input of organic material into soils, its decomposition attracts considerable attention. Nowadays, lignocellulose transformation is in a focus of biotechnology efforts aiming at a cost-efficient production of bioethanol, edible or medicinal mushrooms or other value-added products.

The lignocellulose is composed mainly of the polysaccharides cellulose and hemicelluloses and the three dimensional polyphenolic polymer of lignin. During transformation, lignocellulose is incorporated into fungal biomass or serves as an energy source while its remains are transformed into humic substances. It is important to note that while polysaccharides are resources for both carbon and energy acquisition by soil microorganisms, the degradation of lignin and probably also humic substances does not provide enough energy to support its decomposition and it thus does not have the primary nutritional role. The degradation of lignin and cellulose has been the topic of several recent reviews [1-4] but the degradation of other lignocellulose components, namely the pectins and hemicellulose, has only recently attracted attention [5].

RESULTS AND DISCUSSION

Lignin-modifying enzymes. Lignin is a branched three dimensional polymer of phenylpropane units. Due to the variety of chemical bonds and the complexity of its structure, lignin represents the most recalcitrant component of lignocellulose. The ligninolytic systems of basidiomycetous fungi consist of oxidases, peroxidases and hydrogen peroxide-producing enzymes. Ligninolytic oxidase – laccase – oxidizes its substrates using molecular oxygen, while the peroxidases need hydrogen peroxide provided by auxilliary enzymes.

Lignin peroxidase (EC 1.11.1.14) and manganese peroxidase (MnP; EC 1.11.1.13) are able to cleave the lignin polymer and to ultimately perform lignin mineralization [3, 6] while laccase (phenoloxidase, polyphenol oxidase, EC 1.10.3.2) can oxidise phenolic compounds including lignin and its derivatives. However, although it might be involved in some lignin transformation pathways, the enzyme alone can not cleave or mineralize lignin or humic compounds [7, 8]. In addition, lignin can also be attacked by fungal peroxygenases reported from the mushroom *Agrocybe aegerita* and several others [9]. Recently, the evidence increases that nonenzymatic, oxidative pathways are also involved in the decomposition of lignin [2]. This is supported by the fact that there is only a weak relationship between lignin mineralization and production of ligninolytic oxidases and peroxidases [10-12].

Laccase is the most frequently measured oxidative enzyme in soils, a target of several past studies [13, 14]. However, laccase is the enzyme of multiple roles spanning from interspecific interactions over defence against the toxicity of phenols or heavy metals up to fruiting processes and morphogenesis [7, 15, 16]. However, due to the inability to transform lignin, its ecological role in lignocellulose decomposition turnover seems to be generally largely overestimated.

As mentioned above, ligninolytic enzymes also need for their action enzymes providing hydrogen peroxide – most typically the aryl alcohol oxidase or glyoxal oxidase. These enzymes have not been assayed in lignocellulosic substrates very often, but at least one of them, aryl alcohol oxidase, was detected in cultures of litter-decomposing basidiomycetes [17].

Since humic substances carry structural similarities to lignin, ligninolytic enzymes are probably the most important in the degradation of soil humic substances [6, 11, 12]. The producers of the most important enzyme, Mn-peroxidase, the litter-decomposing basidiomycetes, are thus thought to play a major role in the transformation of these compounds [18]. However, due to the heterogeneous nature of humic substances, also horseradish peroxidase, β-glucosidase and Mn^{3+} or H_2O_2 are able to cleave or decolorize them as well as the radical-producing systems involved in polysaccharide degradation [2, 19].

Polysaccharide hydrolases. Cellulose is the main polymeric component of most lignocellulosic materials and represents the most abundant polysaccharide on Earth. The chemical composition is simple: it consists of D-glucose residues linked by β-1,4-glycosidic bonds to form linear polymeric chains of several thousand glucose residues. Cellulose contains both highly crystalline regions where individual chains are linked to each other, and less-ordered amorphous regions. The degradation of crystalline regions is much slower than that of the amorphous ones and some microorganisms are able to attack only amorphous cellulose [2]. A typical system for efficient cellulose decomposition includes endo-type hydrolases (endo-1,4-β-glucanases, EC 3.2.1.4), exo-type hydrolases (cellobiohydrolases [CBH] EC 3.2.1.4) and 1,4-β-glucosidases (EC 3.2.1.21), the complementary activities acting synergistically. Typical cellulolytic systems of saprotrophic cellulose-degrading fungi (e.g. the cord-forming or wood-rotting basidiomycetes) consist of multiple enzymes of all the three above groups. Cellobiohydrolases are produced with specificity for either the reducing or non-reducing ends of cellulose polymer [2, 20].

Hemicelluloses are low molecular mass linear or branched polymers usually containing various sugar units including mannose, galactose, xylose and glucose [5]. Xylans, consisting of xylose units, and glucomannans, consisting of glucose and mannose units, are the main hemicelluloses of angiosperm and conifer trees, respectively, while other lignocellulosic materials may additionally contain considerable amounts of arabinogalactans and galactans [1]. Enzymatic decomposition of hemicelluloses requires a set of hydrolytic enzymes reflecting the structural variability of the substrate. Hemicellulose hydrolysis proceeds through the concerted action of endo-type enzymes, side-group cleaving enzymes and exotype enzymes as well as enzymes cleaving the main chain substituents, e.g. the acetyl groups in xylans. Cleavage ultimately results in the liberation of monomeric sugars and acetic acid.

The hemicellulases most typically found among fungi are endo-1,4-β-xylanase (EC 3.2.1.8) and 1,4-β-xylosidase (EC 3.2.1.37), but several other enzymes are known to be produced by saprotrophic soil fungi, including endomannanases, β-mannosidases, galactosidases, arabinosidases and acetyl esterases as well as debranching enzymes [11, 21-23]. The decomposition of hemicellulose is not limited by its physical structure but rather by the diversity of chemical composition and intramolecular bonding. Many cellulases and hemicellulases have been recently demonstrated to have a broad and overlapping substrate specificity, so that it is not always simple to link a specific enzyme with a target substrate [2]. Polysaccharides in wood or other lignocellulosic materials have also demonstrated to be degraded by non-enzymatic radical-producing systems based on cellobiohydrolase, quinone cycling or small glycopeptides. Their role in the soil is probably more limited in litter or straw than in wood, but cellobiose dehydrogenase has already been demonstrated in several soil or litter-associated fungi [2].

Genes encoding lignocellulose-degrading enzymes in the *Pleurotus ostreatus* genome. Recent efforts led to the analysis of full genome sequence of the lignocellulolytic fungus, *Pleurotus ostreatus*. The results confirm previous studies reporting the production of a complex complement of extracellular enzymes by this fungus [15, 24], showing that its genome contains genes encoding various enzymes active on cellulose, hemicelluloses and lignin (Table 1). Especially the ligninolytic system is highly functionally redundant in this white-rot fungus.

Table 1: Predicted gene models in the genome of the saprotrophic basidiomycete *Pleurotus ostreatus* PC15 (http://genome.jgi-psf.org).

EC number	Enzyme	Predicted gene models
3.2.1.1	α-amylase	6
3.2.1.4	cellobiohydrolase	1
3.2.1.15	polygalacturonase	3
3.2.1.20	α-glucosidase	9
3.2.1.21	β-glucosidase	13
3.2.1.23	β-galactosidase	3
3.2.1.24	α-mannosidase	1
3.2.1.25	β-mannosidase	3
3.2.1.26	β-fructofuranosidase	1
3.2.1.58	glucan 1,3-β-glucosidase	8
3.2.1.67	galacturan 1,4-α-galacturonidase	1
3.2.1.X	unspecified glycosylhydrolases	>5
1.10.3.2	laccase	12
1.11.1.X	haem peroxidases	9

CONCLUSIONS

Fungi seem to be the most important players in lignocellulose transformation processes under environmental conditions due to their ability to attack both polysaccharides and polyphenols in the soil organic matter. While some basic concepts of regulation of enzymatic activity have been outlined, questions regarding enzyme production and diversity at the molecular level are just recently being asked [25]. Moreover, current results show that the ability to decompose lignin and cellulose is not restricted to saprotrophic basidiomycetes and ascomycetes but that the contribution of ectomycorrhizal fungi can also be important [26]. From a practical viewpoint, the ability to produce lignocellulose-decomposing enzymes by mushrooms is important for their cultivation, efficient substrate use, high yield and production of value-added products from lignocellulosic agro-wastes, and future research will hopefully provide better insight into the relationships among fungal enzyme production, substrate transformation and production of desired products.

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AGRO-FOOD INDUSTRY WASTES AND AGRICULTURAL RESIDUES CONVERSION INTO HIGH VALUE PRODUCTS BY MUSHROOM CULTIVATION

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ABSTRACT

Several aspects of our research on the valorization of various agro-industrial residues through mushroom cultivation for the production of added-value products (mycelial biomass, mushrooms, enzymes and medicinal compounds) are presented. These comprise: (a) evaluation of lignocellulosic wastes for the production of the important edible and medicinal mushrooms genera *Pleurotus* and *Lentinula*, through examination of their growth rates and conversion efficacy to fruiting bodies, and (b) production of mycelial biomass and extracellular enzymes during solid-state fermentation (SSF) of agricultural residues by *L. edodes* and the impact of agro-residues properties on the bioconversion process. Firstly, mycelial growth rates of several *Pleurotus* and *Lentinula* strains cultivated on agro-food industry wastes and their bioconversion to fruiting bodies (monitored by biological efficiencies - BEs) are presented. *Pleurotus* species demonstrated high colonization rates on wheat straw (WS) and cotton waste (CW), while peanut shells (PS) furnished the poorest results. Recorded BEs on the former two substrates averaged between 75% and 100%. Regarding *L. edodes*, oak-wood sawdust (OS) and WS supported faster growth than corncobs (CC) and CW, while the highest average BEs ($\leq$ 100) were achieved with CC and WS. Secondly, experiments investigating the ability of several *L. edodes* strains to grow on reed grass (RG), bean stalks (BS) and WS residues are presented. Results showed the importance of simultaneous evaluation of mycelium growth rate, biomass yield and activities of hydrolytic and oxidative enzymes, along with analysis of constituents of the substrates. Data obtained support the potential effectiveness of RG and BS residues as *L. edodes* cultivation substrates.

Keywords: *Pleurotus* spp.; *Lentinula edodes*; Growth rate; Biomass; Enzymes; Fruiting bodies

INTRODUCTION

Agricultural production and the agro-food industry furnish large volumes of solid wastes, residues and by-products, produced either in the primary agro-forestry sector (crop-based) or by secondary processing industries (processing-based), the major part being lignocellulosic biomass. Recently, Zhang [1], reviewing the global world information about lignocellulose availability, estimated the production of lignocellulosic biomass to be more than 200×10^9 tons per year. Especially, the amount of crop residues produced annually in the world from 27 food crops is estimated at about 4×10^9 tons, from which 3 billion tons account per annum for lignocellulosic residues of cereals [2]. The majority of this organic matter becomes a source of environmental

problems. However, if residues are utilized, such as to enhance food production, they are not considered as wastes but new resources.

Residues such as cereals straw, corn cobs, cotton stalks, various grasses and reed stems, maize and sorghum stover, vine pruning, sugarcane and tequila bagasse, coconut and banana residues, corn husks, coffee pulp and coffee husk, cottonseed and sunflower seed hulls, peanut shells, rice husks, sunflower seed hulls, waste paper, wood sawdust and chips, are some examples of residues and by-products that can be recovered and upgraded to higher value and useful products. Current literature shows that lignocellulose degrading mushroom species are used in various SSF applications such as biodegradation of hazardous compounds and biological detoxification of toxic agro-industrial wastes [3-7], biotransformation of agro-industrial residues to mushroom food and animal feed [8-11], compost and product developments such as biologically active metabolites, enzymes, food flavour compounds and other added value compounds [12-14]. Moreover, recent research work indicates medicinal attributes in several species, such as antiviral, antibacterial, antiparasitic, antitumor, antihypertension, antiatherosclerosis, hepatoprotective, antidiabetic, anti-inflammatory, and immune modulating effects [15-17].

Commercial mushroom production, carried out in large or small scale, is an efficient and relatively short biological process of food protein recovery (regarded also as functional food) from low value lignocellulosic materials utilizing the degrading capabilities of mushroom fungi [11]. Among edible mushroom fungi, *L. edodes* and *Pleurotus* species have received considerable attention for their nutritional value, medicinal properties and biodegradation abilities [15, 17, 18]. They both are efficient colonizers and bioconverters of lignocellulosic agro-industrial residues into palatable human food with medicinal properties, with the productivity of the conversion being expressed by biological efficiency [11, 12, 18]. Their mycelium can produce significant quantities of a plethora of enzymes, which can degrade lignocellulosic residues and use them as nutrients for their growth and fructification [19]. However, the nature and the nutrient composition of the substrate affect mycelium growth, mushroom quality and crop yield of this value-added biotransformation process [20, 21].

The present paper addresses aspects of (a) lignocellulose and nitrogen composition of agro-residues and their effect on *L. edodes* and *Pleurotus* spp. growth and fructification, (b) evaluation of different residues for cultivation of *Pleurotus* mushrooms, emphasizing on their colonization and efficiency of conversion to fruiting bodies, (c) evaluation of selected residues for cultivation of *L. edodes* through monitoring mycelium growth rate, biomass yield and endoglucanase and laccase activities.

MATERIALS AND METHODS

Culture media and substrate analysis. The culture medium used for routine culture and storage purposes was Potato Dextrose Agar (PDA, Merck). Grain spawn was prepared as previously described [9]. All substrates (e.g. wheat straw-WS, mixture of reed grasses-RG or bean stalks-BS) were prepared in a ratio of 80% residue to 20% supplements (d.w), i.e. 12% wheat bran, 7% soybean flour and 1% $CaCO_3$ [20]. The residues mixture was left to soak in water for 12-24 h, and after the surplus water had been drained off, supplements were added and mixed. The moisture content of the sterilized substrates was 60-65% and the C:N ratio 50-55:1

For substrate analysis, samples were dried to constant weight in a 60 °C oven and milled to size <0.3 mm. Carbon and nitrogen concentrations were determined using a combustion-gas

chromatography technique, while cellulose, hemicellulose and lignin were determined by, ^{13}C cross-polarization CP magic angle spinning MAS NMR spectroscopy as previously described [21].

SSF in tubes, extension rate and biomass determination. Solid state fermentation was performed as previously described by Philippoussis et al. [9] in glass-tubes (200 x 30 mm) uniformly filled with the substrates to a 80 ml volume and sterilized twice for 1 h at 121 °C. The moisture content of the sterilized substrates was 62-65%. In a completely randomized design, fifteen replicate tubes per strain and substrate were inoculated with two agar plugs (6 mm diam.) cut from the periphery of actively growing mycelium on PDA, transferred onto the top of the substrate and incubated at 26 °C in the dark.

The growth rate of mycelium (mm day^{-1}), was recorded daily in a set of three test tubes by measuring the visible penetration of mycelia into the substrate in two perpendicular directions and the extension rate K_r (mm day^{-1}) was calculated after the mycelium front has reached more than 30 mm.

Twelve replicate tubes per substrate and strain were used for biomass estimation and enzymes assays. At predetermined percentages of substrate volume colonization (i.e. intervals of 4-7 days, depending on the strain and substrate used) two replicate tubes per substrate and strain were withdrawn for biomass and enzymes activity determinations. Samples, comprising the entire solid fermented medium (substrate and mycelium), were frozen (-20 °C, 48 h) and dried by a Heto LyoLab 3000 freeze-dryer (Heto-Holten Als, Denmark), milled and sieved.

The glucosamine content of the fungal cell wall was used to monitor mycelial biomass. At the beginning, a glucosamine standard curve was obtained. Moreover, the glucosamine content of mycelium for each individual strain were determined through liquid state fermentation (LSF) for 35 d, in 100 ml Erlenmeyer flasks with 50 ml Malt Extract Broth (MEB; Merck, Germany), at 26 °C under static conditions. The biomass was determined by the method of the fungal chitin hydrolysis into N-acetylglucosamine, as described by Rigas et al. [6], glucosamine was quantified spectrophotometrically (Jasco V-530 UV/VIS) and results were expressed as mg fungal biomass per g of dry substrate.

Determination of enzymes activities. For enzymes extraction, frozen colonized substrate of each replicate (equivalent to 2 g of dry sample) and 20 ml 0.05 M sodium acetate buffer (pH 5.0) were transferred to 100 ml Erlenmeyer flasks and extracellular enzymes were extracted after agitation at 100 rpm for 1 h at room temperature in an orbital shaker [11]. Endoglucanase (cellulase, EC 3.2.1.4) activity was determined using as substrate 0.5 ml of 1% (w/v) carboxymethylcellulose sodium salt (CMC; Sigma, Germany), in 0.05 M sodium citrate buffer (Merck, Germany), pH 4.8, incubated with 0.5 ml crude enzyme extract at 50 °C for 30 minutes. Laccase (EC 1.10.3.2) activity was measured spectrophotometrically using syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehydeazine, Sigma, Germany) as substrate at 525 nm (extinction coefficient$^{TM}_{525}$ of 5,000 M^{-1} cm^{-1}) for about 10 min. Reaction was carried out in 3-ml cuvettes containing 0.2 ml of crude enzyme extract, 1.7 ml of 0.1 M sodium phosphate buffer at pH 6.8 and 0.1 ml of 1mM syringaldazine dissolved in absolute ethanol. Absorbance measurements were conducted by the Jasco V-530 UV-VIS spectophotometer and enzyme activities, representing the mean of 6 replicates (3 sample replicates x 2 assay replicates), were expressed as U g^{-1} (units g^{-1} of dry substrate).

Fructification in tube and bag cultivation. To induce sporophore production in tubes, sets of three replicate tubes from each one of the substrates tested (fully colonized) were subjected to a low temperature shock at 5 °C for three days, they were later placed into a 24h/day illuminated fruiting chamber (200-300 lux) at 17.5 ± 1.0 °C and the number of basidiomata was scored.

For bag-log cultivation, polypropylene-autoclavable bags were filled with 2 kg of substrate, closed with a cotton plug and sterilized twice for 1 h at 121 °C. After cooling, inoculation was carried out by adding about 60 g of grain spawn along the central vertical axis of the bag. Substrate colonisation took place in growth chambers, at 25 ± 1 °C.

For fructification and productivity evaluation, bags with fully colonized substrate were transferred to the fruiting room (used for all stages of fruiting) and subjected to a cold shock for 3 days to induce primordia formation at 15 °C, 90% relative humidity and 24 h/day illumination. During harvesting, the light intensity was set at 300 lux (24 h/day, fluorescent lamps), air exchange rates were controlled to maintain low CO_2 level (<1200 ppm), relative air humidity was adjusted between 75 and 90% and temperature was set at 17.5±1.0 °C. Mature basidiomata were harvested, counted and weighted. For all replicates, the time needed for complete substrate colonization and appearance of primordia was measured, in addition to earliness (i.e. time elapsed between the day of inoculation and the day of the first harvest). Yield (total and in individual flushes) and biological efficiency (BE: percentage ratio of fresh mushrooms harvested per dry substrate weight) were recorded.

Experimental design and statistical analysis. In all experiments, a completely randomized design was applied, using fifteen replicates per strain and substrate. Variance analysis was performed by Statgraphics Plus version 5.1 statistical package, using the Least Significant Difference (LSD) test at 5% level of probability to compare mean values.

RESULTS AND DISCUSSION

Evaluation of lignocellulosic residues for mushroom cultivation by growth characters (mycelium growth rate and biomass production). During the vegetative phase of mushroom life cycle, mycelium grows through the substrate, biodegrades its components and supports the formation of fruiting bodies. Both *L. edodes* and *Pleurotus* species reveal high efficiency in degradation of a wide range of lignocellulosic residues, due to their capability to synthesize relevant hydrolytic (cellulases and hemicellulases) and unique oxidative (ligninolytic) extracellular enzymes, responsible for the degradation of residue components into low-molecular-weight compounds that can be further assimilated to ensure fungal growth. [19].

Mycelial growth measurements of *L. edodes* and *Pleurotus* species conducted in 'race tubes' [9, 22] revealed that the type of wastes, as well as the strain used, exercised a considerable influence on the colonization rates and therefore on the time needed to complete incubation and cropping. As there is a high contamination risk at the beginning of the cultivation process, reduction of the duration of the colonization phase is of prime importance. Our experimental data suggest that both lag-phase time and mycelium extension rates play a decisive role on the efficacy of substrate colonization, with the 'race tube' method proving to be helpful for a quick evaluation of the suitability of lignocellulosic residues to serve as cultivation substrates.

Regarding *Pleurotus* spp., in our previous studies, CW (including stem-leaf residues and gin trash), WS and PS were evaluated as substrates of *P. ostreatus* and *P. pulmonarius* through

measurements of their linear growth rates in tubes. Initially, extension rates assessed on non-composted WS, CW and PS indicated that the type of substrate used had a pronounced effect on mycelium growth, with WS supporting high extension rates (8.1 – 9.6 mm/day) for all *Pleurotus* strains examined, while PS furnished the poorest results (Fig. 1). In general, WS and CW supported fast colonization and produced the best earliness values for all strains tested. Comparison of growth rates on composted and non-composted WS and CW substrates (data not shown) revealed that in the latter case faster colonization was achieved. However, a strain and substrate dependent behaviour was detected as two wild strains, one *P. ostreatus* and one *P. pulmonarius*, presented 30% reduction of colonization speed on composted WS while the growth rate of commercial strains of both species were marginally affected on composted CW as compared to non-composted CW, but it was always significantly suppressed (about 40%) in comparison to non-composted WS. However, non-composted CW promoted earlier basidiomata induction, presenting the shortest pre-harvest period of *P. pulmonarius* and *P. ostreatus* commercial strains (26 and 27 days respectively).

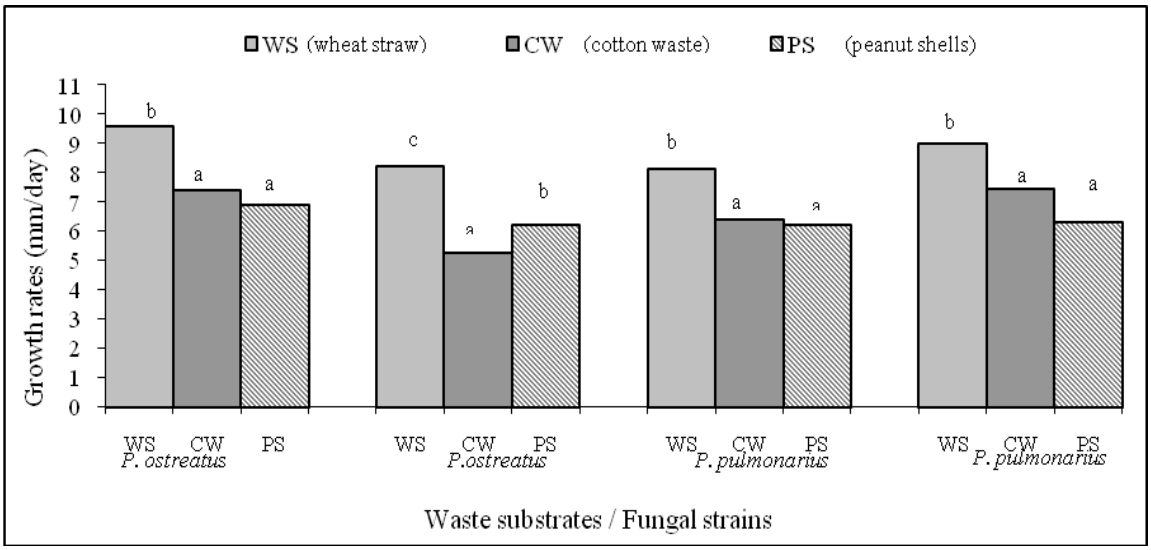

Figure 1: Effect of WS, CW and PS substrates (non-composted) on mycelial growth rates of *Pleurotus* species. (Columns within the same strain not sharing the same letters are significant different at P=0.05).

Our previous studies, evaluating several commercial and wild *L. edodes* strains as regards their efficacy of mycelium growth on WS, OS and CC, have demonstrated that OS and WS supported faster growth than CC [22]. In addition, a strain-dependent behaviour was detected since three strains performed much better on OS and WS, while one commercial strain performed satisfactorily on CC. In general, significantly lower linear growth rates were recorded for CC (Table 1). Results were verified by the fruiting technique conducted in glass tubes that furnishes a remarkable reduction in the time necessary for the first fructification (≥2 months), conducing to a quick evaluation of the production potential of tested substrates.

Recently [21], mycelium growth rates, biomass concentration (estimated as glucosamine content) and laccase and endoglucanase secretion were monitored during solid state fermentation (SSF) of wheat straw (WS), reed grass (RG) and bean stalk (BS) residues by *Lentinula edodes*

strains 119, 121 and 122. In a first experiment, these strains were subjected to screening regarding their growth rates and biomass yield, where strain 121 proved to be the fastest colonizer. However, the greatest biomass yield at the end of colonization was demonstrated by strain 122 on BS (465.93 mg g^{-1} d.w.).

Table 1: Lag time t (r=30mm), full colonization period and linear extension rates *Kr* during growth of two *L. edodes* strains on OS, WS and CC lignocellulosic substrates in 'race tubes'.

Strains	Substrates	Lag time t (r=30mm) (days)	Full colonization period (days)	Extension rate *Kr* (mm/ day)
S4080	OS	9	29	4.8 b*
	WS	12	36	4.3 b
	CC	17	45	3.4 a
SIEF 0231	OS	14	34	4.4 b
	WS	22	51	3.2 a
	CC	20	56	2.6 a

*Values within the same column followed by the same letter are not significant different (P=0.05)

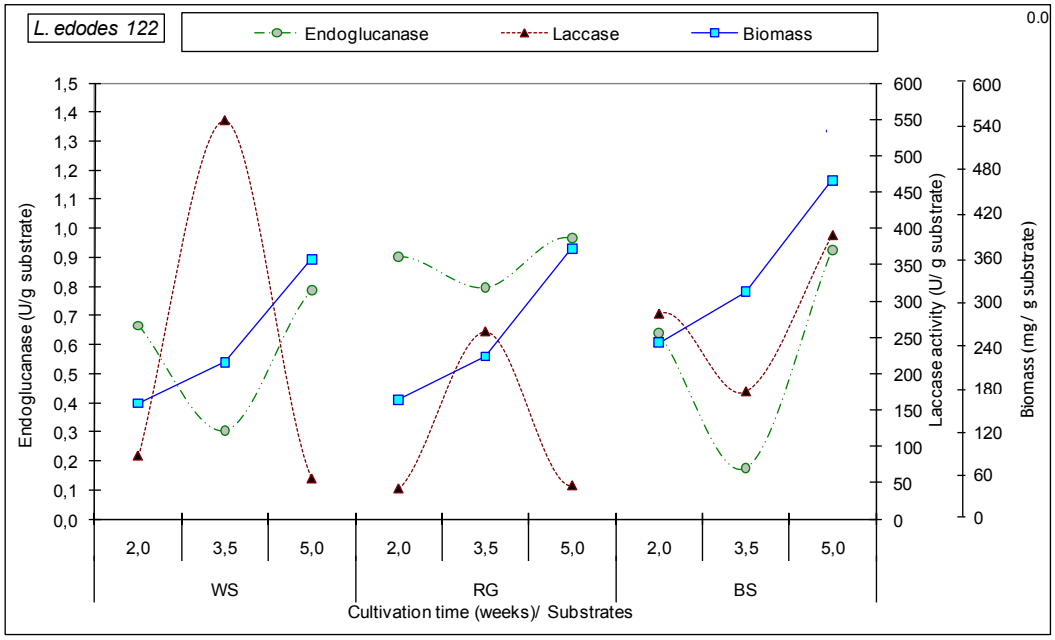

Figure 2. Patterns of biomass, endoglucanase and laccase production, monitored at 33%, 66% and 100% of WS, RG and BS colonization, during SSF by *L. edodes* strain AMRL 122.

In a second experiment, biomass, as well as endoglucanase and laccase production patterns of the selected strains 121 and 122 were monitored at three intervals i.e. at 33%, 66% and 100% of substrate colonization. BS furnished the highest endoglucanase production for strain 121, while RG for strain 122. A strain and substrate-dependent behaviour of the enzyme secretion was detected, with strain 122 presenting maximal endoglucanase activity in all substrates at the initial (33%) and final (100%) stages of colonization (0.64-0.90 and 0.79-0.97 U g^{-1} respectively)

(Fig 2). However, in strain 121 the peak of endoglucanase production was detected in the early stages of colonization (at 33% on WS and at 66% on RG and BS). Laccase activity showed increased values (maxima on WS, 353.68 and 548.67 U g^{-1} by strains 121 and 122 respectively) at 66% of colonization. Correlation analysis of growth data demonstrated negative relations between growth rate and biomass yield and between laccase and endoglucanase activities on WS and RG substrates fermented by strain 122. Correlation analysis between growth parameters and nutritional constituents of the substrates showed that biomass production rate is significantly ($p<0.05$) positively correlated with nitrogen content of the substrates ($R^2=0.99$), and with their hemicellulose content ($R^2=0.78$).

Evaluation of residues by mushroom crop yield and bioconversion efficiency (BE). The efficacy of residues bioconversion process and the productivity of the mushroom crop are assessed by the biological efficiency (BE). Apart from the type of substrate and stain used, yield response is determined by the duration of the cropping period and cultivation practice applied e.g. high spawn levels enhance mushroom yields [23].

A significant number of agro-industrial lignocellulosic materials are used as substrates for the production of *Pleurotus* species [11]. The overall BE values obtained on these residue-substrates for *P. ostreatus* and *P. pulmonarius* strains indicated that among all residues cardboard, coffee pulp, paper wastes and softwood residues, presented the highest (≥100%) biological efficiencies [24, 25, 26] BEs between 75% and 100% were recorded on cotton wastes and wheat straw [21, 27] while satisfactory productivity (BEs 50-75%) is demonstrated by most of agro-industrial residues, namely corncobs, various grasses and reed stems, vine shoots, cottonseed hulls and sugarcane bagasse [11].

In our experiments *P. ostreatus* and *P. pulmonarius* strains presented significantly higher yields and size of basidiomata on WS and CW in comparison to PS (Fig. 3). *P. pulmonarius* furnished significantly better yields than *P. ostreatus* with BE values on WS and CW, varing respectively for *P. ostreatus* between 87.37-94.39 and 70.61-116.70 and for *P. pulmonarius* between 81.39-123.07 and 92.87-97.87. Regarding straw pre-treatment, data demonstrated an approximate 20% reduction of overall BE when *P. ostreatus* is cultivated on non fermented wheat straw, as compared to fermented substrate (mean values 70.5% and 85.5% respectively). On pretreated wheat straw, supplementation with cotton seed cake and soybean cake proved to enhance productivity of *P. ostreatus* [27, 28].

Regarding *L. edodes*, studies were conducted for evaluating WS, CC and OS bio-transformation efficiency in respect of substrate colonization, earliness of sporophore production, biological efficiency and mushroom nitrogen content as well as basidiocarps number and size. WS and CC substrates furnished significantly higher crop yields (BEs) than OS, with the highest BE (80.64%), and the heavier fruit bodies (21.40 g) being recorded on CC substrate. WS appeared to promote earliness and furnished good crop productivity and quality results. However, the higher mushroom protein content was detected from OS substrate. Apart from higher N content of CC and WS which contributed to higher productivity [29], their high yields may also be attributed to their high water soluble sugar contents, particularly hemicelluloses [30], which could have exercised a positive effect during the active growth phase, prior to the breakdown of lignin and cellulose. Also, Moyson and Verachtert [31] have demonstrated that substrate decomposition by *L. edodes* is initially associated to its hemicellulose content.

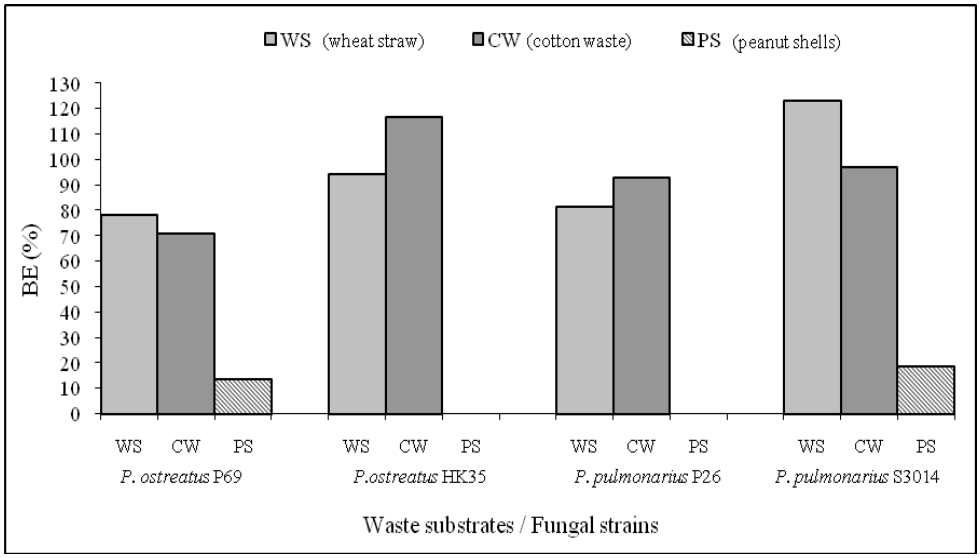

Figure 3: Productivity evaluation (BEs) of *P. ostreatus* and *P. pulmonarius* strains on WS, CW and PS waste-substrates.

Impact of lignocellulose and nitrogen composition of agro-residues on *Pleurotus* and *Lentinula* species cultivation. Solid agro-industrial residues are heterogeneous water insoluble materials having as common feature their basic macromolecular structure being cellulose, hemicellulose and lignin. Cellulose and hemicellulose (the main sources of carbohydrates) are often incrusted with lignin, which forms a physical seal around these two components. The proportions of the three structural components along with nitrogen content of residues affect mycelium growth, mushroom quality and crop yield [9, 21, 22, 38]. In fact, the general strategy of white rot mushroom-forming fungi, the most efficient degraders comprising popular cultivated species like *L. edodes* and *Pleurotus* species, is to decompose the lignin in wood, so that they can gain access to the cellulose and hemicelluloses embedded in the lignin matrix.

Understanding the impact of substrate on mushroom productivity and quality is valuable to determine the combination of suitable substrate composition and fungal strain that bioconvert effectively the agricultural residues into nutritional and medicinal food. Since the carbon sources utilized by basidiomycetes are usually of a lignocellulosic character, fungi during vegetative growth produce a wide range of enzymes to degrade the lignocellulosic substrates. Data obtained in various studies demonstrate that the type and composition of lignocellulosic substrate appear to determine the type and amount of enzyme produced by basidiomycetous fungi during vegetative growth [19, 20, 32, 38]. Finally, the nature and the nutrient composition of the substrate affect mushroom quality and crop yield of this value-added biotransformation process [9, 21, 22]. Investigations of the impact of substrate constituents on mycelium growth and mushroom production demonstrated that both are affected by cellulose, hemicellulose and lignin proportions along with nitrogen content of the cultivation substrate [22, 33].

The edible mushrooms belonging to the genus *Pleurotus* are commercially grown on pasteurized straw-based substrates or hardwood sawdust, with added supplements. However, as these fast-growing mushrooms display a complete lignocellulolytic enzyme system [34], they can use a wide spectrum of agricultural and industrial wastes that contain lignin and cellulose for growth and fruiting [35]. In our previous studies [9], CW, WS and PS were evaluated as

substrates of *P. ostreatus* and *P. pulmonarius* cultivation. *Pleurotus species* demonstrated high colonization rates and BE's on WS and CW, while PS furnished the poorest results. Chemical analysis of the substrates demonstrated that nitrogen level in WS was approximately half of that determined in CW and C/N ratio almost double of the values measured for the PS. CW presented the higher cellulose content while the cellulose: lignin ratio was found to be 5-fold more in the WS and CW than in the PS. The high lignin content of the latter was probably the cause of the slow mycelium growth, as cellulose may not be readily available as carbon source. Cellulose/lignin ratios of WS and CW substrates were positively correlated to mycelial growth rates and mushroom yields of *P. ostreatus* and *P. pulmonarius*. Regarding the influence of nitrogen availability, our studies revealed a positive correlation between the C/N ratio and mycelium growth rates of *Pleurotus* species [8]. In fact, the highly positive relation of mycelium growth with C/N and cellulose: lignin ratios were more significant for *P. pulmonarius* than for *P. ostreatus*.

Additional data furnished by the 'race-tube'method experiments provided an estimate of the potential of WS, CW, PS, poplar sawdust and CC to serve as alternative mushroom cultivation substrates [36]. Furthermore, in recent studies conducted to evaluate different grass and reed stalks as cultivation substrates of *Pleurotus* species [10, 37] bean plant residues (BS) and a mixture of reed-grass maces (RG) supported fast colonization rates for both genera strains, while BS enhanced laccase and endoglucanase activities [37].

As far as *L. edodes* cultivation is concerned, this efficient wood degrader can be grown on a variety of agro-industrial residues such as oak, ash, poplar, alder, eucalypt, beech, pine, maple and birch sawdust, cereal straws (mainly barley and wheat), corn cobs, sugarcane bagasse, sunflower seed hulls, peanut shells, cotton straw and seed hulls, vine shoots, coffee husk and pulp etc [11]. Substrate bioconversion efficacy and successful cultivation of shiitake mainly depends on the development of mycelia and complete colonization of the substrate in the first cultivation stage. As there is a high risk of contamination at the beginning of the cultivation process, reduction of the duration of the colonization phase is of prime importance.

Table 2: Correlation (R^2 values) between substrate chemical properties and mushroom production characteristics of *L. edodes* AMRL 121 grown with the 'bag-log'method.

	Chemical constituents		
Parameters	C%	N%	C/N
Earliness [a]	NS	(-) 0.8138*	(+) 0.8179*
Mushroom number	NS	(+) 0.9735**	(-) 0.9800**
BE% [b]	NS	(+) 0.9663**	(-) 0.9652**
Mushroom average weight	NS	NS	NS

[a] Period (days) from inoculation to the first sporophore harvest

[b] Biological efficiency: percentage ratio of fresh mushrooms harvested per dry substrate weight

[c]NS: Not significant. * Significant at P=0.05, ** Significant at P=0.01

Correlation analysis of carbon and nitrogen content of WS, CC and OS substrates influence on shiitake mushroom yield and quality (Table 2) revealed that nitrogen content of the substrates affected earliness of fructification and productivity [10]. Earliness and crop cycle duration were found to be related to the nitrogen content and consequently to C/N ratio values of substrates. Actually, the positive relation found between pre-harvest period and C/N ratio, which explains earliness of fructification on the substrate mixtures with lower C/N ratio, is consistent with

previous reports indicating the positive effect of nitrogen in ligninolytic activity during *L. edodes* cultivation [29, 37]. Also, Philippoussis et al. [22] have demonstrated that mycelium extension rate is related to bioavailability of nitrogen and that substrate formulation affects nutritional and porosity levels (O_2 availability). Moreover, data obtained in this investigation showed that there is a strong negative correlation between mushroom yield (mushroom number and BE%) and C/N ratio of the substrates.

In our recent work [21], the rate of lignocellulosic residues colonization, through monitoring and comparing *L. edodes* mycelium growth, biomass yield and enzyme activities in SSF of WS, RG and BS substrates was investigated, as well as possible relations of the tested growth parameters with composition of substrates regarding cellulose, hemicellulose and lignin percentages, as well as carbon and nitrogen contents (Table 4). Correlation studies revealed that biomass production rate is significantly positively correlated with nitrogen content of the substrates (R^2=0.99) and with their hemicellulose content (R^2=0.78). However, substrates as RG, with higher lignin and lower cellulose content appeared to support higher levels of endoglucanase and lower values of laccase. Correlation analyses demonstrated significant positive relation of endoglucanase production with initial hemicellulose content of the substrates (R^2=0.76) and negative relation with cellulose per hemicellulose ratio (R^2=0.90) and of laccase activity with lignin per hemicellulose ratio (R^2=0.86).

Table 4: Analysis of the main constituents of WS, RG and BS substrates at the beginning of SSF

	Carbon %	Nitrogen %	C N^{-1}	Cellulose %	Hemicellulose %	Lignin %	Cellulose $lignin^{-1}$	Cellulose $hemi\text{-}cellulose^{-1}$
Residues[1]								
WS	37.70	0.64	58.81	80.03	6.32	8.51	9.40	12.66
RG	45.30	0.63	72.02	74.68	6.78	13.13	5.69	11.01
BS	45.60	0.80	57.07	80.39	5.73	8.41	9.56	14.03
Substrates[2]								
WS	40.0	1.26	31.82	68.93	11.16	7.58	9.10	6.18
RG	46.1	1.25	36.94	64.65	11.53	11.27	5.73	5.61
BS	46.34	1.38	33.48	69.22	10.69	7.50	9.23	6.48

[1]Raw residues (before mixing with supplements)
[2]Cultivation substrates (after mixing with 12% wheat bran, 7% soybean flour and 1% $CaCO_3$)

CONCLUSION

This paper addresses evaluation of several lignocellulosic wastes for the cultivation of two important mushroom genera, *Pleurotus* and *Lentinula* that have nutritional or pharmacological value. The ability of the different mushroom species to utilize various substrates depends on both mushroom- and substrate-associated factors. Understanding the impact of residue-substrates on bioconversion process and the use of their nutritional elements by the mycelium for growth and fruiting is a prerequisite in order to determine the combination of suitable substrate composition

and fungal strain towards developing an efficient method of fermentation or cultivation to obtain palatable nutritional and medicinal food and other high value compounds.

Data concerning analysis of residue-substrate components, their colonization and bioconversion efficiencies to fruiting bodies demonstrated CW as a promising alternative substrate for *P. ostreatus* and *P. pulmonatius* cultivation, commercially grown on pasteurized straw-based substrates or hardwood sawdust. Cellulose/lignin ratios of WS and CW substrates were found to be positively correlated to mycelial growth rates and mushroom yields of *P. ostreatus* and *P. pulmonarius*, while a positive correlation between the C/N ratio and mycelium growth rates of *Pleurotus* species was detected.

Regarding *L. edodes*, the presented results support its efficient production on a mixture of WS (supports earliness and quality) and CC (supports high yield) in the framework of utilizing cereal residues as an alternative to wood. Moreover, results demonstrated the importance of simultaneous evaluation of mycelium growth rate, biomass yield and activities of hydrolytic and oxidative enzymes during residues bioconversion, along with analysis of their constituents. The results obtained showed that high mycelium growth rate and biomass yield, as well as high endoglucanase and laccase production during vegetative growth of *L. edodes* are usually negatively related variables. Their desirable coincidence depends on the fungal strain and is strongly influenced by the nature and composition of the lignocellulosic substrate.

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RECYCLING OF SPENT MUSHROOM SUBSTRATE (SMS) IN AVOCADO ORCHARDS

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ABSTRACT

Spent mushroom substrates (SMS), are usually treated as wastes. One of the main beneficial uses of SMS is as soil amendment, after further composting for horticulture. Avocado orchards in northern Israel, are grown mainly on heavy clay soils, suffering from poor drainage and limited aeration. This situation can cause yield decrease and lead to tree's degeneration. Two soil cover (mulch) treatments were compared**,** in an avocado orchard**:** fresh spent mushroom substrate (SMS) and cattle manure compost (CMC). The yield of the two avocado varieties of this orchard was higher on SMS than CMC mulch. The yield of one of the varieties was highest on control (uncovered) soils. Thick growth of avocado roots was found in and under both mulch types. Enhancement of avocado root growth into and under the mulch, will lead to improvement of avocado trees growth, especially on heavy un-drained soils. . Soil's EC values were higher under both mulch types, although they decreased due to annual rainfall. There are treatments (leaching etc.) that can be used, for minimizing the increase of soil's salinity after SMS application. There are advantages of the use of SMS, especially since it's price is only 20% of cattle manure compost due to less transportation costs and no need for composting. Therefore its use is advantageous for both avocado and mushroom growers. The commercial utilization of SMS in avocado orchards should be done carefully, with monitoring its impact on soil's EC (salinity) and taking the needed measures to avoid unnecessary damages.

Keywords: SMS, Avocado, Heavy clay Soils

INTRODUCTION

Production of "Champignon" mushrooms (*Agaricus bisporus*) is done on composts covered with casing soil. At the end of the cultivation cycle, these substrates, known as spent mushroom substrates (SMS), are moved from the growing rooms and treated as wastes. They can pollute the environment (soil, air and water) on one hand, but can be beneficial if they are treated and used properly [1,2,3,4,5]. One of the main uses of SMS is organic fertilizer for horticulture. It serves as supplier of both, organic matter that improves soils' structure and as source of macro and micro elements for plants nutrition [5,6]. The use of SMS as retardant of soil born plant diseases and notorious fungi was also demonstrated [5,7,8].

The substrates used for mushroom production in Israel are compost, made mainly of poultry manure, wheat straw and gypsum and casing soil, composed of black peat amended with lime. Most of the growers are located in northern Israel, producing, 8000 tons of mushrooms and 100,000 cubic meters of SMS, annually. The SMS are moved from the farms to remote locations for further composting treatment. Avocado orchards located close to the mushroom farms, are grown mainly on heavy clay soils, suffering from poor drainage and limited

aeration, since for good development of Avocado roots the soil must be well aerated, The present situation can cause yield decrease and lead to tree's degeneration.

MATERIALS AND METHODS

In a three years experiment in an avocado orchard two soil cover (mulch) treatments were compared: fresh spent mushroom substrate (SMS) and "conventional" agricultural cattle manure compost (CMC).

In both treatments the soil was covered with 40 m^3 mulch for hectare placed on one side of the tree line (150 liter for a tree) The first application was done on June 2008, after SMS leaching, The second application was done on January 2009 on the other side of the trees . The third application was done on January 2010 on the same side of the trees as on 2008 The control was uncovered soil, all treatments in four replicates, with two avocado varieties : Hass and Ethinger. The plots were irrigated with reclaimed effluents and fertilized according to the recommended protocol for avocado orchards in northern Israel. Data loggers (Hobo 4 Chanel) were used to collect temperature data year around. The temperature detectors were placed in the mulch cover on both sides of the trees row, in the soil under the mulch between two trees in the row and 20 cm above the mulch. Temperatures were recorded every 30 minutes. Chemical analyses of the mulch covers were made annually for: ash and total N, C/N ratio, pH and EC. In all treatments soil's chemical analysis were made once a year for: N, P, K, Cl, Na, Ca, Mg, Bo, content, pH, SAR,EC at the depths of 0-30 and 30-60 cm. Chemical analysis of avocado leaves: for N, P, K, Cl, Ca, Mg, Bo, content were also made annually. The amount of roots in the top soil (0-30cm.) was weighed in 200 gr. soil samples. Pictures were taken of avocado roots growth into the mulch. Yields of both varieties were measured annually. Yield quality was identified according to the portion of the fruits above certain dimensions.

RESULTS AND DISCUSSION

The temperatures in the soil and mulch layers are presented in Fig 1.

During January – June 2009 CMC mulch increased soil temperature more than SMS mainly with the mulch of January 2009 application. This mulch was probably un-mature manure compost and therefore more active.

Ash content of mulch layers during the first year (2008 -2009) after mulch application is presented in Fig. 2. Ash content increased faster in SMS mulch than in CMC. Increase in ash content is an indication for degradation of organic matter. The decrease of ash content after a year is explained by penetration of avocado roots into the mulch.

Other chemical analyses were done during the same period in the mulch and the results are presented in Fig. 3 -5.

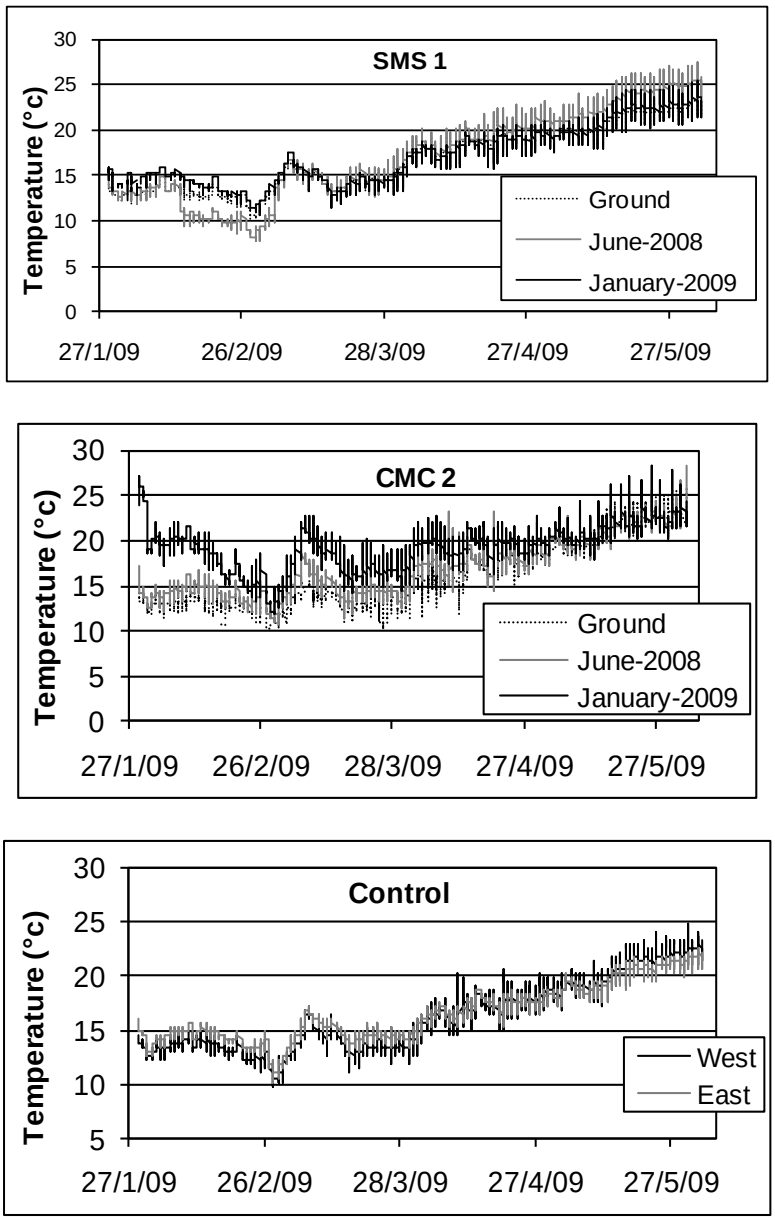

Figure 1. Temperatures in soil and mulch covers during 27.1.09 -27.5.09. Mulch was applied on June 2008 and January 2009.

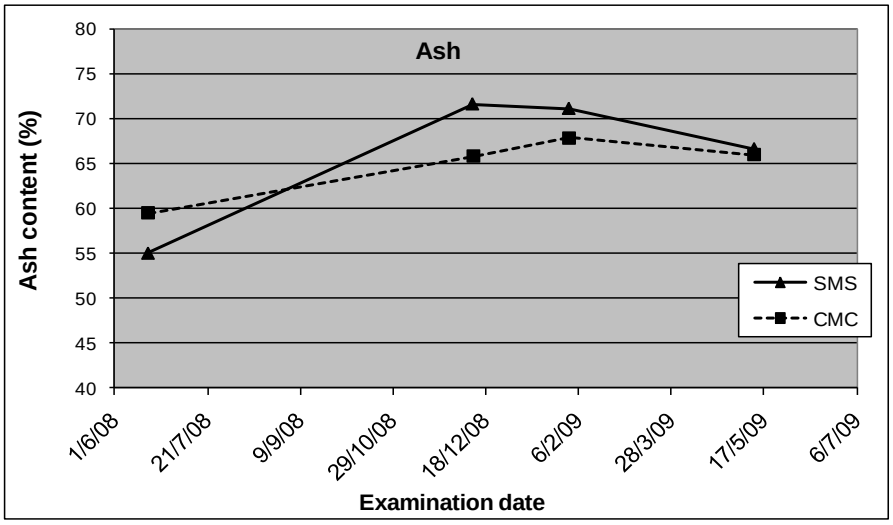

Figure 2. Ash content in mulch layers of June 2008 application.

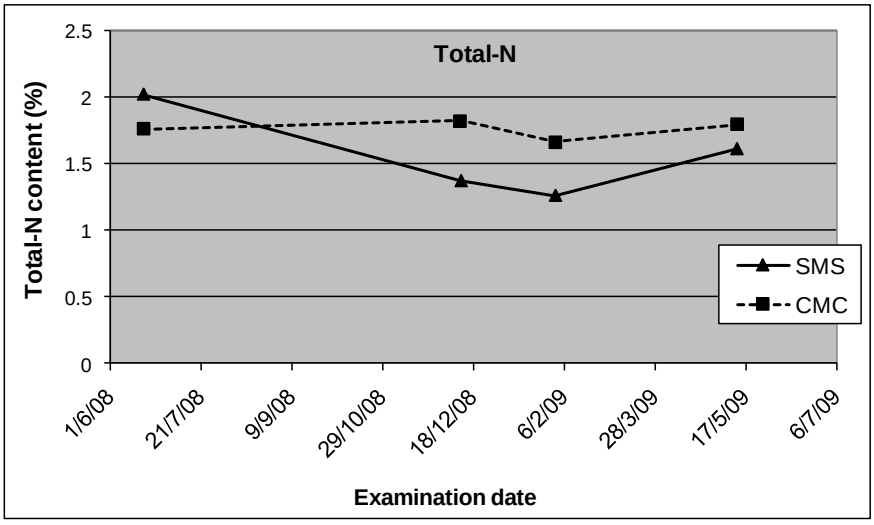

Figure 3. Total N content in mulch layers applied on June 2008.

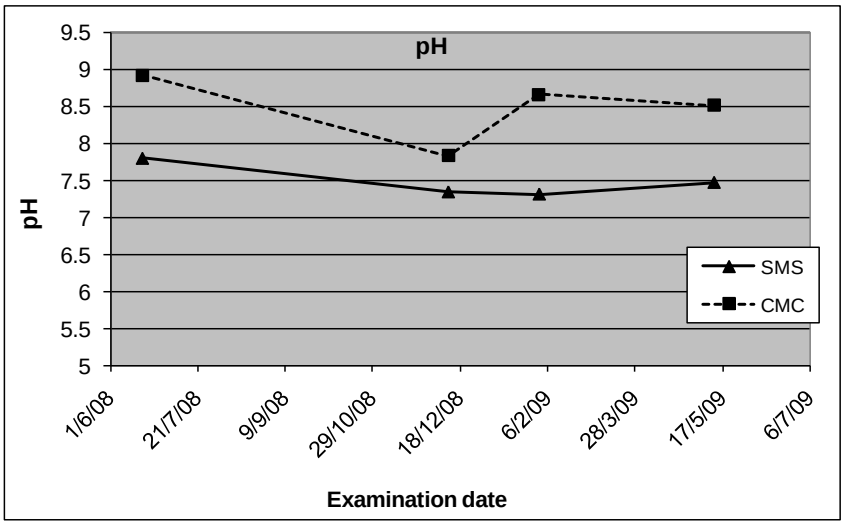

Figure 4. pH in mulch layers applied on June 2008

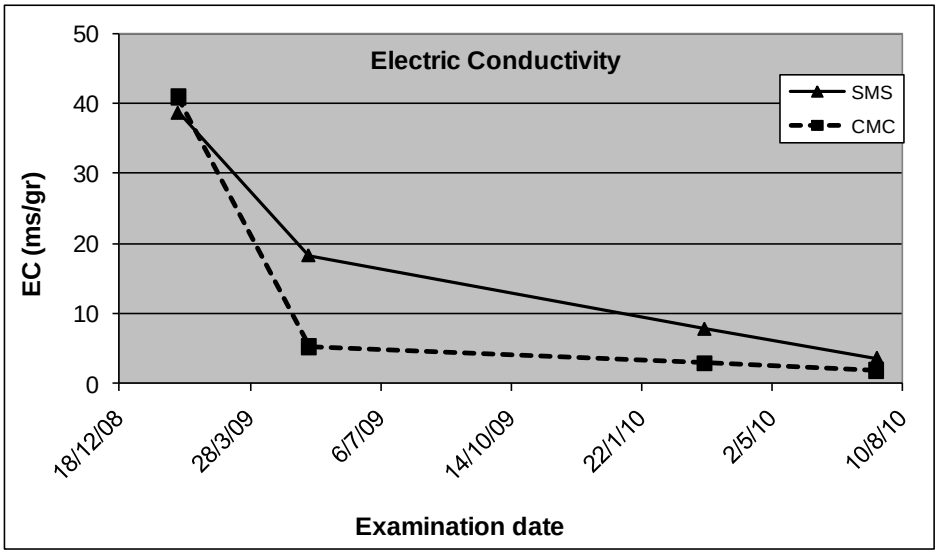

Figure 5. EC of mulch layers applied on June 2008

SMS was leached before its application to avocado orchard. CMC was un-leached compost and its EC decreased during the first winter, probably due to leaching by the rains.

Avocado yields were collected during two consecutive years. The results for the first year are presented in Figures 6 and 7.

Figure 6A and 6B : are yields of the two avocado varieties during 2009-2010 season.

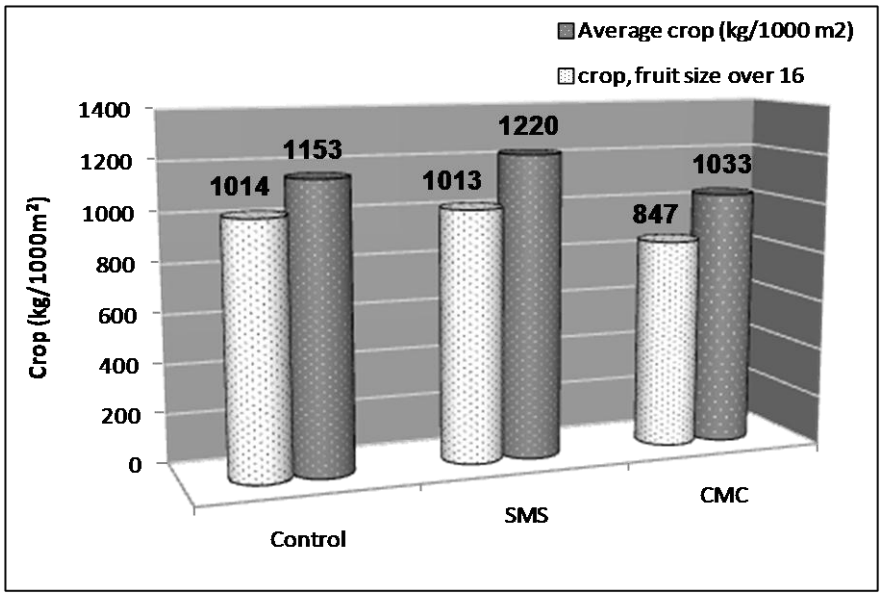

Figure 6A. Yield of Ethinger variety (first year)

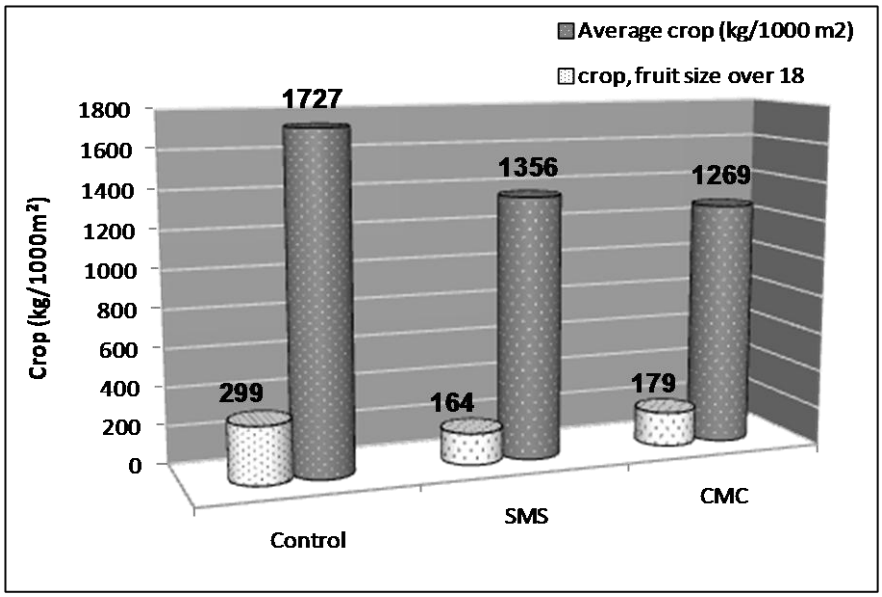

Figure 6B. Yield of Hass variety (first year)

In both avocado varieties yield of SMS plots were higher than those of CMC plots. The yield of the control was highest among the Hass variety plots.
Results for the second year (2010–2011) presented in Figures 7A and 7B.

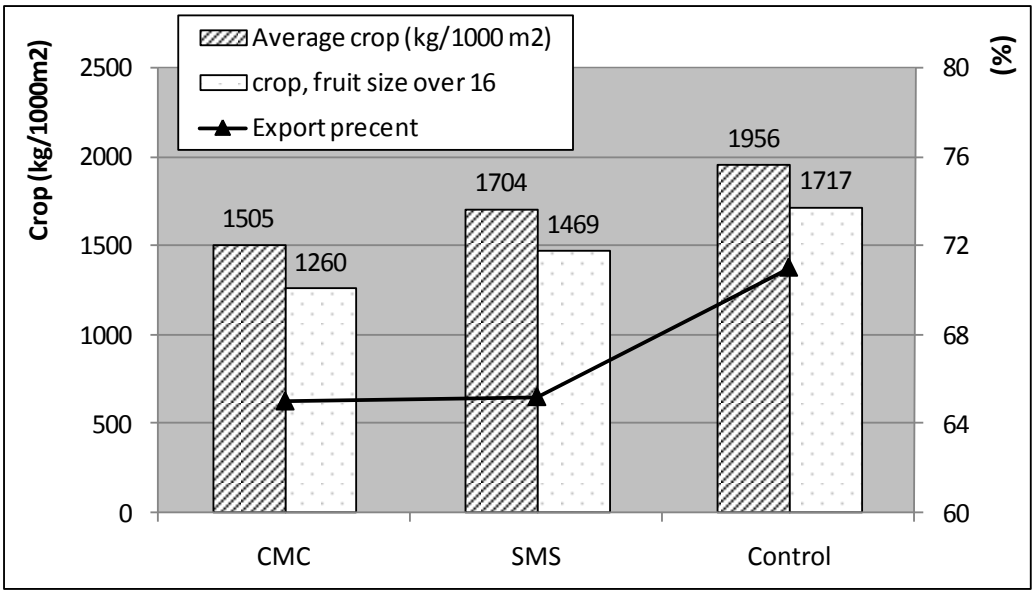

Figure 7A. Yield of Ethinger variety (second year)

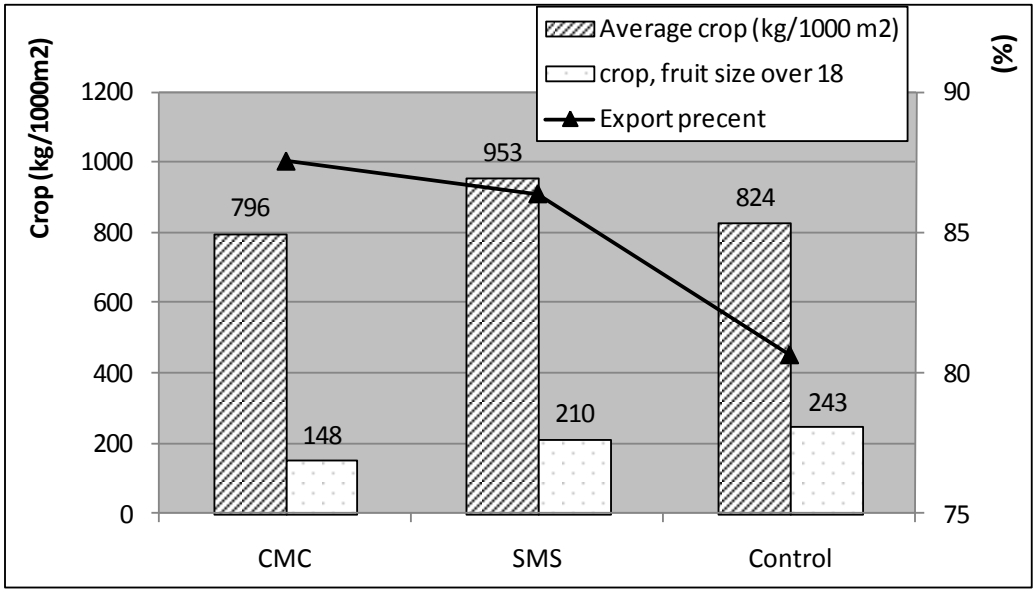

Figure 7B. Yield of Hass variety (second year)

The yield in the second year was also higher on SMS than CMC mulch in both varieties. The percentage of exported fruits is an indication for fruit quality.

No significant differences were found in avocado yields of both varieties (quantity and quality), between the above mentioned treatments, during the experiment period (the two years).

No significant differences, were also found in chemical analysis of avocado leaves, of the varieties, with the above mentioned mulch treatments.

Thick growth of avocado roots into both mulch types was documented (Fig.8).The weight of avocado roots in 200gr top soil were 4.1gr.under SMS mulch treatment and 4.8gr. under CMC mulch. In control soil only 2.0gr of roots were found

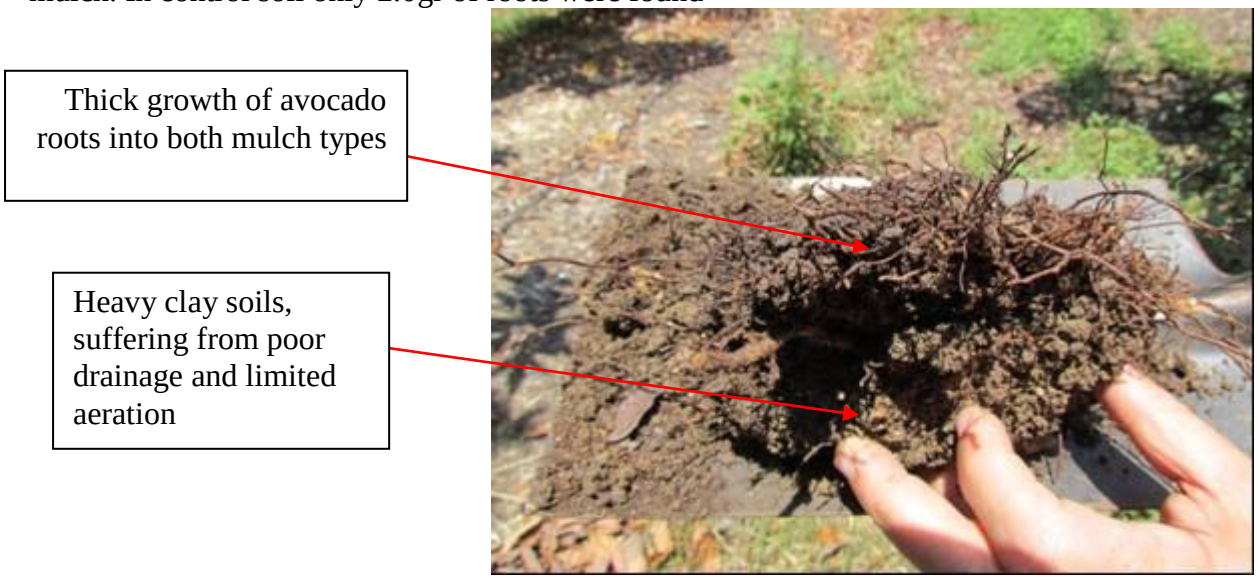

Figure 8. Growth of Avocado roots into SMS.

Soil's N and P content were higher under mulch treatments and highest under the CMC mulch. Soil's K and Ca+Mg was also higher under the mulch treatments. Soils' EC and Cl values, were higher under both mulch covers, than in the control (Table 1).

Table 1. Soil chemical analysis three years after first mulch application

CMC		SMS		Control		17.5.2011
15-30 cm	**0-15** cm	**15-30** cm	**0-15** cm	**15-30** cm	**0-15** cm	
14.3	32.9	**11.5**	25.8	6.7	14.3	N-NO3 (mg/kg)
37.9	67.2	12.4	41.6	11.3	31.8	P (mg/kg)
0.420	1.040	0.280	1.150	0.160	0.360	K in soil extract(mequ/L)
1.500	1.500	1.920	1.900	1.100	1.100	EC (dS/m)
5.00	3.60	7.50	3.40	3.20	2.30	Cl (mequ/L)
6.14	4.21	6.29	5.87	5.60	4.09	Na (mequ/L)
3.00	1.92	2.66	2.13	3.35	2.16	SAR
8.4	9.6	11.2	15.2	5.6	7.2	Ca + Mg (mequ/L)
2.60	3.50	1.30	1.80	0.70	1.00	Zn
50.7	47.3	63.6	36.0	36.2	43.2	Mn
5.3	4.7	13.5	5.8	5.4	4.6	Cu
19.6	20.0	15.7	12.7	18.6	14.5	Fe

The values of EC in these results, reveal the known negative impact of SMS and other composts on soils' salinity. There is evidence that this problem can be treated by SMS leaching or by changes in composts' composition [9]. The considerable growth of avocado roots into the mulch gives hope that this treatment will slow down orchards degeneration in the future.

Conclusions

Our results are from a three years experiment and two cropping years of an avocado orchard. This period is probably too short for evaluation of the impact of compost application, as mulch to an orchard. Nevertheless the results reveal potential advantages for recycling of SMS in this way :

a. Enhancement of avocado root growth into and under the mulch, will probably lead to improved growth, of avocado trees, especially on heavy un-drained soils. The presence of peat (from casing soil) in SMS improves soil's porosity and water holding capacity.
b. There are treatments (leaching etc.) for minimizing the increase of soil's salinity after SMS application. The annual rainfall decreases EC gradually.
c. SMS is a safe product for horticulture due to the heat treatments used during mushroom production cycle.
d. CO2 emissions of SMS utilization are lower than those of CMC, since this product is used fresh and is not produced through another composting process.
e. The yields of the SMS treated plots were higher than those of the CMC treated plots. CMC is the most widely used compost for agriculture.
f. Since the price of SMS is only 20% of cattle manure compost, its use is advantageous for both avocado and mushroom growers.
g. The commercial utilization of SMS in avocado orchards should be done carefully, with monitoring its impact on soil's EC (salinity).

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SPENT SUBSTRATE FROM MUSHROOM INDUSTRY, A POTENTAIL DYE DECOLOURIZING AGENT

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ABSTRACT

Dye decolourization using spent mushroom substrate (SMS) is influenced by the category of SMS, type of dye, initial dye concentration, form of mushroom mycelia, pH, temperature and the growing media. Among SMS of different mushrooms, the SMS from *P. sajor-caju* exhibited highest population along with variability of both fungi and bacteria. Five fungi viz., *Aspergillus fumigatus*, *Paecilomyces variotii*, *Pichia guilliermondii*, *Schizophyllum commune* and *Pezizomycotina* sp. with potential dye decolourization potential have been recorded to thrive on SMS of different mushrooms by using 5.8S rRNA gene sequencing and BLASTn techniques. Out of these *Schizophyllum commune* from *P. sajor-caju* SMS has been recorded to exhibit highest decolourization potential (95%) with in 10 days against Chicago sky blue. This fungus also exhibited dye decolourization potential of 100, 92.50, 81.60, 73.40 and 67.80% against other dyes like Starch Azure, Reactive blue, Rhodamine B, Orange II sodium salt and Methyl blue, respectively. Similarly, by using 16S rRNA gene sequencing and BLASTn, six potential bacteria viz., *Bacillus subtilis*, *B. pumilus*, *B. licheniformis*, *Pseudomonas fluorescens*, *Sphingobacterium multivorum* and *Rummelibacillus stabekissi* have also been identified, out of which *B. licheniformis* isolated from *P. sajor-caju* SMS, exhibited highest decolourization potential of 66.10% against Orange II sodium salt, followed by *Bacillus pumilus* (57.7%). Temperature and pH optima of 25ºC and 6.0 respectively, have been recorded for achieving highest level of decolourization through *B. licheniformis*. The study highlights the utility of SMS for unconventional activities like dye decolourization, which indirectly supports its use in bioremediation activities.

Key words: Fungi; Bacteria; Dye decolourization; 16S rRNA; 5.8S rRNA

INTRODUCTION

Synthetic dyes are used in a wide range of industries (textile, pharmaceutical, cosmetic and food industries) [1] and during processing, up to 40% of used dyestuffs are released in to processed water [2], producing a highly colored wastewater that affects aesthetics, transparency and gas solubility in water bodies [3]. Other main factors, which make it a problematic industrial waste, are the mutagenic, carcinogenic and toxic effect of some of these dyes [4], high volume and effluent composition [3], and their resistance towards the conventional methods of wastewater treatment [5]. Currently several physico-chemical methods are in use for decolorization of these wastewaters, but these have limitations of high cost, high salt content in the effluent and difficulties in treating concentrated waste [6]. Being eco-friendly and cost-competitive [7], white rot fungi have been recognized as most efficient, degrading dyes with the involvement of their extracellular lignin-modifying enzymes [8].

Spent Mushroom Substrate (SMS) released after mushroom cultivation, contains residual mushroom biomass along with a rich population of heterotrophic fungi and bacteria, which can act as an inexpensive source of phenoloxidases. SMS also has ability to chemically adsorb organic and inorganic pollutants, while diverse category of microbes it harbors, have capability

of biologically breaking down the organic xenobiotic compounds present in soil and water [9]. In several previous studies, role of extracellular ligninolytic enzymes and microbes from SMS of different mushrooms [10], and mushroom mycelia, especially of *Pleurotus florida*, *P. ostreatus*, *P. flabellatus* and *P. sajor-caju* have been evaluated for their use in dye decolorization activities [3, 11]. This study presents the role of different fungi and bacteria inhabiting the spent substrate of different mushrooms, and temperature and pH of medium in decolurization of different dyes.

MATERIALS AND METHODS

Isolation and identification of spent substrate microflora. Spent substrate from button (*Agaricus bisporus*), oyster (*Pleurotus sajor-caju*) and paddy straw mushroom (*Volvariella volvacea*) collected from Directorate of Mushroom Research, Solan (HP), India was used for isolation of fungi and bacteria. Fungi and bacteria were isolated using the serial dilution and plating method on Potato Dextrose Agar (potato dextrose - 24 g, agar agar - 20 g, distilled water - 1000 ml, pH - 7.2) and Nutrient Agar (beef extract - 3 g, peptone - 5 g, dextrose - 3 g, NaCl - 5 g, agar agar - 15 g, water - 1000 ml, pH - 7.2) medium, respectively and incubating at 30±2ºC for 5 days. Well isolated colonies were further purified by growing on respective media and stored thereafter at 4ºC for further use, after growing them on respective media slants. Purified fungi and bacteria were identified using 5.8S rRNA and 16S rRNA gene sequencing, followed by Nucleotide Basic Local Alignment Search Tool **(**BLASTn) techniques.

Fungal DNA extraction and 5.8S rRNA gene amplification. All fungal cultures were grown separately on Malt Extract Agar petridishes at 32 ± 2°C for 7 days. The mycelia from respective fungus plates was scrapped and placed at -85°C at least for 2 hours, followed by freeze drying for 16-18 hours. The genomic DNA was extracted from approximately 100 mg of freeze dried fungal mycelia by crushing in 1.5 ml micro-centrifuge tubes using micro-pestles. QIAGEN DNeasy Plant Mini Kit (QIAGEN GmbH, D-40724 Hilden) was used for DNA extraction as per the protocol supplied by the manufacturer.

The polymerase chain reaction (PCR) primer ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') developed by White *et al* [12] were used to amplify the ITS region of 5.8S ribosomal DNA. PCR amplification was performed in a reaction mixture of 50 µl, containing 0.2 µl *Taq* DNA polymerase (5 U μl^{-1}), 5 µl 10X PCR buffer (100 mM Tris-HCl, pH-8.3, 15 mM $MgCl_2$, 250 mM KCl), 5 µl dNTP mix (2.0 mM each), 1 µl each of ITS-1 and ITS-4 primers (0.01 mM), 1 µl glycerol (5%), 2 µl $MgCl_2$ (25 mM) and 2 µl of genomic DNA (50 ng). PCR reaction was performed in PCR Master Cycler Gradient in 36 cycles each of 95°C for 1 min, 50°C for 30 sec, 72°C for 1 min 20 sec and final elongation at 72°C for 10 min with lid heating option at 104°C. The presence and yield of PCR amplified amplicons was ascertained on 2.0% agarose gel (w/v) prepared in 1% TBE. Gel electrophoresis was carried out at 90 V for 1.30 h in 0.5X TBE buffer. Staining was done with ethidium bromide and the gel was visualized and photographed using Bio Imaging System (Gene Genius, Syngene).

Bacterial DNA extraction, quantification and 16S rRNA gene amplification. All bacterial isolates were grown separately in 10 ml nutrient broth in 20 ml screw caped tubes at 30±2ºC for 2 days. For extraction of total genomic DNA, 1.5 ml log phase broth culture was used and DNA was extracted using BACTOZOL ™ Bacterial DNA Isolation Kit from Molecular Research Centre, Inc., Cincinnati, OH. Extracted DNA samples from different bacterial isolates were run on 1.2% agarose gel (w/v) in 1.0% TBE buffer at 90 V for 1 hr along with quantification marker in one lane. Gel was visualized using Bio Imaging System (Gene Genius, Syngene).

Alternatively, the DNA concentration and purity were also checked by measuring the extinction at A_{260}/A_{280} on UV Visible spectrophotometer. The concentration was calculated assuming that one A_{260} unit is equal to 50 g of double stranded DNA/ml.

Amplicon of 16S rDNA was obtained for each bacterial isolate by PCR amplification employing forward primer (5'-GGA TCC AGA CTT TGA TYM TGG CTC AG-3') and reverse primer (5'-CCG TCA ATT CMT TTG AGT TT-3'). PCR reactions were performed in a reaction mixture of 50 µl containing 0.2 µl *Taq* DNA polymerase (5 U μl^{-1}), 5 µl 10X PCR buffer (100 mM Tris-HCl, pH-8.3, 15 mM $MgCl_2$, 250 mM KCl), 5 µl dNTP mix (2 mM each), 1 µl each of Forward primer and Reverse primer (0.01 mM), 1 µl glycerol (5%), 2 µl of $MgCl_2$ (25 mM) and 2 µl of genomic DNA (50 ng). The reactions were performed in a PCR Master Cycler Gradient with initial denaturation at 95°C for 3 min, followed by 35 cycles each of 95°C for 1 min, 50°C for 1 min, 72°C for 2 min and final elongation at 72°C for 10 min with lid heating option at 104°C. The presence and yield of specific PCR product (16S rRNA gene) was visualized as per the protocol adopted for 5.8S rRNA gene.

PCR products cleaning up, sequencing, annotation and blasting. PCR amplified amplicons of 5.8S rDNA and 16S rDNA were cleaned up by using RCB kit (Banqiao City, Taipei County 220, Taiwan) for removing of, if any undesired DNA fragments. The cleaned up PCR products were got sequenced using 3730Xl (96 capillary) electrophoresis instruments from Bioserve Biotechnologies, Hyderabad, India.

The received sequences in ab1/SCF format were cleaned up to remove the misleading sequences and were improved upon using Chromas software. The improved consensus sequences were blasted using BLASTn of NCBI (http://blast.ncbi.nlm.nih.gov/Blast) and the species against which highest total score was exhibited, was considered as the species identified.

Different dyes used and decolorization potential of different fungi. Seven dyes (Table 1) were procured from Sigma-Aldrich. Seven fungi belonging to 5 different genera [*Aspergillus fumigatus* (Ab), *Aspergillus fumigatus* (Ps), *Schizophyllum commune* (Ps), *Pezizomycotina* sp. (Ps), *Aspergillus fumigatus* (Vv), *Paecilomyces variotii* (Vv) and *Pichia guilliermondii* (Vv)] and isolated from three different spent substrates (given in parenthesis) were screened for their dye decolourization potential against Chicago sky blue. The dye decolourization potential was studied against 100 ppm concentration of the dye in Potato Dextrose Broth (PDB - potato dextrose, 24 g; distilled water, 1000 ml; pH, 7.2). Five day old culture of different fungi was inoculated separately in dye mixed 100 ml PDB in 250 ml Erlnmeyer flasks and incubated at 25 ± 1°C for 12 days in BOD incubator. Three replications were kept for each treatment keeping un-inoculated flasks as the control treatment. The decolourization if any, was measured as per the protocol elaborated under heading ‘measurement of decolorization extent’.

The potential fungus (*Schizophyllum commune*) isolated from *P. sajor-caju* spent substrate was further studied for its dye decolourization potential against 6 different dyes (Methyl blue, Orange II Sodium salt, Rhodamine B, Azure B, Reactive blue and Starch Azure). The decolourization potential was evaluated against 100 ppm concentration of each dye in 100 ml PDB filled 250 ml Erlnmeyer flasks. Five day old culture of the test fungus was inoculated separately in each flask and the flasks were incubated at 25 ± 1°C for 18 days in BOD incubator. Three flasks were kept for each treatment by keeping flasks devoid of fungal inoculation as the control treatment. The decolourization was measured as per the protocol elaborated under heading ‘measurement of decolorization extent’.

Table 1: Details of different dyes used in study

Dye	Chemical formula	CAS No.	λ max (nm)	Reference
Rhodamine B	$C_{28}H_{31}N_2O_3Cl$	81-88-9	543	[13]
Methyl Blue	$C_{16}H_{18}ClN_3S$	61-73-4	655	[14]
Orange II sodium salt	$C_{16}H_{11}N_2NaO_4S$	633-96-5	550	[15]
Azure B	$C_{15}H_{16}N_3S^+$	531-55-5	648	[16]
Reactive Blue	$C_{22}H_{16}N_2Na_2O_{11}S_3$	2580-78-1	550	[17]
Starch Azure	$C_{36}H_{55}NO_{28}$	66068-38-0	650	[18]
Chicago Sky Blue 6B	$C_{34}H_{24}N_6Na_4O_{16}S_4$	2610-05-1	650	[19]

Dye decolorization potential of different bacteria. Eight bacterial isolates belonging to 5 different genera [*Sphingobacterium multivorum*-1 (Ab), *Sphingobacterium multivorum*-2 (Ab), *Bacillus pumilus* (Ab), *Rummelibacillus stabekisii* (Ps), *B. licheniformis* (Ps), *B. subtilis* (Ps), *B. pumilus*-1 (Vv) and *B. pumilus*-2 (Vv)] and isolated from three different spent substrates (given in parenthesis) were screened for their dye decolourization potential against 100 ppm concentration of Orange II Sodium salt in PDB. Two day old culture ($\times 10^{-6}$/ml) of different bacteria was inoculated @ 0.1 ml separately in dye mixed 100 ml PDB filled in 250 ml Erlnmeyer flasks and the flasks were incubated at 25 ± 1°C for 8 days in BOD incubator. Three replications were kept for each treatment by keeping un-inoculated flasks as the control treatment. Decolourization was recorded like in case of earlier steps.

The potential bacteria (*B. licheniformis* and *B. subtilis*) isolated from *P. sajor-caju* spent substrate were evaluated for their dye decolourization potential against 2 additional dyes (Azure B and Methyl blue). The decolourization potential was studied against 100 ppm concentration of each dye in 100 ml PDB filled in 250 ml Erlnmeyer flasks. Two day old broth culture of each bacterium ($\times 10^{-6}$/ml) was inoculated separately in each flask and the inoculated flasks were incubated at 25 ± 1°C for 8 days in BOD incubator. Three flasks were kept for each treatment by keeping flasks devoid of bacterial culture inoculation as the control treatment. Decolourization was recorded like in case of earlier steps.

Temperature and pH optima of *B. licheniformis* for dye decolourization. pH range of 4.0 to 10.0 was used for studying the pH optima of Orange II sodium salt decolurization by the potential bacterium, *B. licheniformis*. Prepared broth was distributed in 250 ml Erlenmeyer flasks @ 100 ml each and sterilized at 15 p.s.i. pressure for 20 min. The 0.1 ml stock solution (1 g/10 ml) of the dye was added in 100 ml sterilized broth in flasks to give a final concentration of 100 ppm and 0.1 ml of two day old bacterial broth ($\times 10^{-6}$) was inoculated separately in each flask. Flasks devoid of bacterial broth but with PDB of different pH were kept as control treatments. Three replications were kept for each treatment and all flasks including control were incubated at 25 ± 1°C for next 8 days. Decolourization was recorded like in case of earlier steps.

Five different incubation temperatures (20, 25, 30, 35 and 40°C) were used for studying the temperature optima of Orange II Sodium salt decolorization with potential bacterium, *B. licheniformis*. Protocol for media preparation and setting up of experiment was same as was for pH studies, excepting medium pH, which was kept 7.2. Experiment was carried out in triplicate and all flasks including control were incubated at 5 different temperatures for next 10 days. Decolourization was recorded like in case of earlier steps.

Measurement of decolorization extent. Sample (3 ml) collected each time from each replication and centrifuged at 10000 rpm for 10 min was used for measuring decolorization

extent by measuring absorbance of supernatant at specific λ_{max} for each dye by using UV-Visible double beam Spectrophotometer (Unico-3802). Decolorization extent was calculated as:

Decolorization extent (%) = [100 x (OD_1-OD_t)]/OD_1

Where OD_1 is initial absorbance at 0 day, OD_t is absorbance after incubation for different periods under different experimental conditions, t is incubation time [20].

RESULTS AND DISCUSSION

Fungal and bacterial microflora in SMS of different mushrooms. Spent substrate from three different mushrooms varied both in their quantitative and as well as qualitative fungal population. SMS from *P. sajor-caju* harboured highest fungal population, dominated by *Aspergillus fumigatus*, followed by *Schizophyllum commune* and *Pezizomycotina* sp. (Table 2). SMS of *A. bisporus*, harboured next highest population exclusively dominated by *Aspergillus fumigatus*. SMS from *V. volvacea* although harboured three dominating fungi like *P. sajor-caju* SMS, but it exhibited two very distinct fungi (*Paecilomyces variotii* and *Pichia guilliermondii*) along with a common fungus (*A. fumigatus*). Although the information available on the subject is very scanty, however, in one report available; SMS from *A. bisporus* has been reported to be dominated by *Aspergillus* sp., *Trichoderma* sp., *Mucor* sp. and *Glycodium* sp. [9]. Alike earlier study, the present study has also reported highest population of *Aspergillus fumigatus* in SMS of *A. bisporus* and *P. sajor-caju*.

Table 2: Fungal population in spent substrate of different mushrooms

Spent substrate	Fungus (5.8S rDNA sequencing)	CFU (×104)
A. bisporus	*Aspergillus fumigatus*	5.0
P. sajor-caju	*Aspergillus fumigatus*	8.5
	Schizophyllum commune	3.0
	Pezizomycotina sp.	3.0
V. volvacea	*Aspergillus fumigatus*	1.0
	Paecilomyces variotii	2.0
	Pichia guilliermondii	1.0

SMS of *P. sajor-caju* did harbour highest population as well as diversity of bacteria comprised of four different species (*B. licheniformis, Bacillus subtilis*, *Rummelibacillus stabekisii* and *Pseudomonas fluorescens*) compared with SMS of *A. bisporus* and *V. volvacea*, which harboured three and one species, respectively (Table 3). *V. volvacea* SMS harboured second highest bacterial population, dominated by only one species (*Bacillus pumilus*). Reports specifically on bacterial population dynamics in SMS of different mushrooms are not available in great numbers. However, in one report on *A. bisporus* SMS, a total 14 different operational taxonomic units have been assigned using 16S rDNA sequencing technique, out of which 12 were of Gram-positive bacteria, while rest two have link to *Comamonas* and *Sphingobacterium*. Among Gram-positive bacteria, the important were *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Brevibacterium*, *Arthrobacter*, *Microbacterium* etc. [21].

The present findings also prove the dominance of *Bacillus* sp. in SMS of *A. bisporus* and *V. volvacea*, while *Rummelibacillus stabekisii* and *Pseudomonas fluorescens* in *P. sajor-caju* SMS. The presence of *Sphingobacterium multivorum* in *A. bisporus* SMS has also been presented.

Table 3: Bacterial population in spent substrate of different mushrooms

Spent substrate	Bacterium (16S rDNA sequencing)	CFU ($\times 10^7$)
A. bisporus	*Bacillus subtilis*	9.0
	Bacillus pumilus	2.1
	Sphingobacterium multivorum	4.0
	Sphingobacterium multivorum	1.0
P. sajor-caju	*B. licheniformis*	0.64
	Bacillus subtilis	0.20
	Rummelibacillus stabekisii	14.9
	Pseudomonas fluorescens	10.0
	Pseudomonas fluorescens	10.0
V. volvacea	*Bacillus pumilus*	0.64
	Bacillus pumilus	29.0

Dye decolourization by different fungi. Out of seven fungi from SMS of three different mushrooms, highest decolourization of Chicago sky blue (95.0%) after 12 days of incubation was recorded with *Schizophyllum commune* isolated from *P. sajor-caju* SMS, followed by *Pezizomycotina* sp. again from the same SMS (Table 4). It was followed by three isolates of *A. fumigatus* isolated from three different SMSs (33.6 to 49.0%). Decolourization was completely absent in *Pichia guilliermondii* isolated from *V. volvacea* SMS. Decolourization increased with time of incubation and become near static after 10 days of incubation. In several earlier studies, researchers have reported the dye decolourization potential of microorganisms isolated from different ecosystems like soft wood chips [22], composting environment [23], dye house effluent [24], refuse dump soil [25] and Brazilian ecosystems [26] and have reported their relative importance in dye decolourization. Although the workers have emphasized the importance of microorganism from specific ecosystems but amongst fungi the potential dye decolourizers have been identified as mesophilic white rot fungi *Schizophyllum commune* [27-28] and *Aspergillus* spp. [29-30] similar to the present study.

Table 4: Decolourization of synthetic dye Chicago sky blue with different fungal isolates isolated from spent substrate of different mushrooms

Fungus	Dye decolourization (%) at different interval of time (days)						
	0-day	2-day	4-day	6-day	8-day	10-day	12-day
Aspergillus fumigatus (Ab)	0	6.7	22.9	28.7	30.3	31.8	33.6
Aspergillus fumigatus (Vv)	0	12.3	37.0	41.8	43.7	47.1	49.0
Paecilomyces variotii (Vv)	0	0	11.2	25.7	27.3	29.4	29.7
Pichia guilliermondii (Vv)	0	0	0	0	0	0	0.0
Aspergillus fumigatus (Ps)	0	11.0	32.1	36.0	34.9	36.2	40.5
Schizophyllum commune (Ps)	0	0	60.4	79.8	90.7	95.3	95.0
Pezizomycotina sp. (Ps)	0	0	53.5	54.7	73.8	87.8	92.0
$CD_{0.05}$	-	0.096	0.064	0.075	0.1405	0.039	0.081

The potential dye decolourizer fungus, *Schizophyllum commune* was further evaluated for its decolourization potential against 6 additional dyes for 18 day of incubation. This fungus exhibited significantly higher decolourization of Starch Azure (100%), followed by Reactive blue (92.5%), Rhodamine B (81.90%), Orange II sodium salt (73.40%) and Methyl Blue (67.8%). Negligible decolourizarion was recorded in case of Azure B (Table 5). Earlier studies

carried out on *S. commune* have also proved the dye decolourization potential of this fungus against Congo red [27] and Solar Golden Yellow R [28], a direct textile dye.

Table 5: Decolourization of different dyes by a promising fungus *Schizophyllum commune* isolated from oyster mushroom spent substrate

Dye	Dye decolourization (%) at different interval of time (days)						
	0-day	3-day	6-day	9-day	12-day	15-day	18-day
Methyl blue	0	0	15.9	29.6	46.2	67.2	67.8
Orange II Sodium salt	0	0	16.9	34.4	59.7	73.1	73.4
Rhodamine	0	0	13.6	14.3	18.6	27.8	81.6
Chicago sky	0	0	2.2	2.5	2.2	2.2	2.2
Azure B	0	0	4.1	3.4	4.1	4.0	4.0
Reactive blue	0	0	20.5	22.5	44.6	81.0	92.5
Starch Azure	0	0	100	100	100	100	100
$CD_{0.05}$	-	-	0.075	0.041	0.069	0.060	0.079

Dye decolourization by different bacteria. Out of eight different bacteria studied for their dye decolourization potential against Orange II sodium salt for 8 days of incubation, highest decolourization (66.1%) after 8 days of incubation was with *B. licheniformis* isolated from *P. sajor-caju* SMS, followed by *B. pumilus* (57.0%) isolated from *V. volvacea* SMS (Table 6). Lowest decolourization was with *B. subtilis* and *Rummelibacillus stabekisii* both isolated from *P. sajor-caju* SMS. The three isolates of *B. pumilus* isolated from SMS of three different mushrooms also varied in their dye decolourization potential. In majority of the dyes, highest decolourization was achieved up to 6 days of incubation, after which it remained almost static. Like in present study, the microorganisms from different ecosystems have also been tested earlier for their decolourization potential [22-26] and one aerobic non-filamentous bacterium from composting environment [23], *Clostridium bifermentans* from contaminated soil [7], *B. cereus* from dye house effluent [24] and *Micrococcus* sp. from refuse dump soil [25] have been reported to have significant dye decolourization potential.

Table 6: Decolourization of synthetic dye Orange II sodium salt with different bacteria isolated from spent substrate of different mushrooms

Bacterium	Dye decolourization (%) at different interval of time (days)				
	0-day	2-day	4-day	6-day	8-day
Bacillus pumilus (Vv)	0	0	48.4	57.7	57.0
Sphingobacterium multivorum-1 (Ab)	0	10	30.4	32.9	30.2
Bacillus pumilus (Vv)	0	22.0	28.6	44.7	45.9
Sphingobacterium multivorum-2 (Ab)	0	23.1	40.5	41.0	42.1
Rummelibacillus stabekisii (Ps)	0	12.7	15.9	11.6	10.0
Bacillus pumilus (Ab)	0	26.8	44.3	40.2	40.0
B. licheniformis (Ps)	0	17.3	17.8	62.7	66.1
B. subtilis (Ps)	0	1.1	1.3	1.4	0.0
$CD_{0.05}$	-	0.146	0.228	0.129	0.109

The two potential bacteria, *B. licheniformis* and *B. subtilis* isolated from *P. sajor caju* SMS were restudied from their decolourization potential against two more recalcitrant dyes (Azure B and Methyl blue), where in *B. subtilis* decolourized two dyes up to 44.6 and 91.3%, respectively. Decolourization of these two dyes was comparatively lower (15.0 and 75.8%) in case of *B. licheniformis* (Table 7). In most of the earlier studies, the dye decolourization potential of specific bacterium have been studied against several dyes and bacteria belonging to *Bacillus* spp. have been reported to have good decolourization potential [23-24].

Table 7: Decolourization of Azure B and Methyl blue with two potential bacteria *B. licheniformis* and *B. subtilis* isolated from SMS of *P. sajor-caju* under nutrient deficient conditions

Bacterium	Dye	Dye decolourization (%) at different interval of time (days)				
		0-day	2-day	4-day	6-day	8-day
B. licheniformis	Azure B	0	1.7	24.2	15.1	15.0
	Methyl blue	0	9.8	43.1	52.9	75.8
B. subtilis	Azure B	0	2.5	36.4	40.3	44.6
	Methyl blue	0	43.7	58.0	66.0	91.3
$CD_{0.05}$		-	0.045	0.064	0.194	0.113

pH and temperature optima for dye decolourization by *B. licheniformis.* The test bacterium was studied for its decolourization potential against Orange II Sodium salt at seven different pH levels, where in highest decolourization (85.6 and 81.1%) was recorded against pH levels of 6.0 and 7.0, followed by 8.0. Decolourization was almost similar at pH 5.0 and 9.0, while lowest at 4.0 (Table 8). The decolurization was almost negligible up to first 4 days of inoculation, which suddenly increased on day 6 of incubation.

Table 8: Effect of pH of the growing medium on decolourization of Orange II sodium salt with potential bacterium *B. licheniformis* isolated from spent mushroom substrate

pH	Dye decolourization (%) at different interval of time (days)				
	0-day	2-day	4-day	6-day	8-day
4.0	0	0	0	56.6	74.1
5.0	0	3	5.5	3.0	75.4
6.0	0	1.6	1.9	78.0	85.6
7.0	0	0	1.4	71.8	81.1
8.0	0	0	0	62.6	79.4
9.0	0	0	3.5	74.1	75.9
10.0	0	0	0	43.2	59.9
$CD_{0.05}$	-	0.026	0.053	0.089	0.064

Like pH, temperature of incubation has direct role is sustaining enzymatic activities and growth of different microorganisms in a cultural medium, which ultimately influences the dye decolourization process. The temperature optima of test bacterium for decolourization of Orange II Sodium salt was studied at 5 different temperatures. Highest decolourization (62.2% and 61.7%) was recorded at 25°C, closely followed by 20°C. The decolourization level decreased with increasing temperature of incubation (Table 9). Prior to this, several researchers have studied the pH and temperature optima of the microorganism of their interest and have reported wide range of pH (5-11) and temperature (20-40°C) for optimum decolourization by those

microorganisms [7, 31]. However, pH near 6-7 and temperature from 25 to 35°C have been reported more suitable for optimum decolourization. In literature, a vide variation in pH requirements for decolourization of different dyes through different microorganisms has been cited and it varied from 10 in case of *Clostridium bifermentans* SL186 for Reactive dyes [7], 7.0 in *Citrobacter* sp. CK3 for Reactive Red [32] and 5.0 in *Aspergillus niger* SA1 for Acid Red 151 [1]. Again for temperature, specific temperature has been indicated for decolorization of specific dye through a specific microorganism [1, 32]. However, unlike present study, the optimum decolourization of Reactive red has been cited through *Citrobacter* sp. CK3 at 32°C, while that of Acid red 151 through *Aspergillus niger* SA1 at mesophilic temperature (25-45°C) and Remazol brilliant blue R through *Streptomyces psammoticus* at 32°C. However, in none of the cases, the temperature optima of SMS inhibiting bacteria have been studied.

Table 9: Effect of temperature of incubation on decolourization of Orange II sodium salt with potential bacterium *B. licheniformis* isolated from SMS of *P. sajor-caju* under nutrient deficient conditions

Temperature	Dye decolourization (%) at different interval of time (days)					
(°C)	0-day	2-day	4-day	6-day	8-day	10-day
20	0	33.9	45.7	57.7	58.4	61.7
25	0	35.9	51.3	60.0	58.4	62.2
30	0	43.3	53.4	56.0	53.0	51.0
35	0	48.3	43.3	54.7	50.0	45.0
40	0	49.6	49.0	44.1	39.0	35.0
$CD_{0.05}$	-	0.149	0.071	0.067	0.194	0.081

In present study, variation in temperature optima for decolorization of Orange II sodium salt through *B. licheniformis* was mainly because of variation in bacterium and the dye used in study. Decolourization was higher at 25 and 20°C, because of 20-25°C is the optimum temperature for mycelial growth and fruiting of *P. sajor-caju*. Accordingly, the SMS of *P. sajor-caju* will also supports the microorganisms with their temperature optima in this temperature range. In few earlier studies, the decolourization has been reported at par at 15, 25 and 35°C, which is attributed to 15°C as the optimum temperature for fruiting of *A. bisporus* and *L. edodes* [33].

Proper disposal of SMS has always remained a challenging task for mushroom growers' as till now mushrooms are considered as prime product of mushroom cultivation and left over residual substrate, which contains a variety of nutrients and microbes is discarded unsystematically. Present study highlights both quantitative and qualitative variation in microflora of SMS from three different mushrooms and same has been proved earlier, as spent substrate from different mushrooms vary in their physico-chemical properties; hence also in inhabiting microbial population [31]. The study has also revealed higher population and more diversity of both fungi and bacteria in SMS from *P. sajor-caju*, which has again been proved in some earlier studies. Amongst the inhibiting microflora from SMS of different mushrooms, the fungus (*Schizophyllum commune)* and bacterium (*B. licheniformis)* have been found to have higher decolourization potential than others and like wise in earlier several reports, the SMS of oyster mushroom or the *Pleurotus* mycelium have been reported to have higher decolourization than other SMS or microbes from SMS of other mushroom [34]. *P. sajor-caju* is grown on pasteurized wheat straw/paddy straw substrate. Although, this mushroom has pH and temperature optima of 7-8 and 20-28°C, respectively for vegetative growth and fruiting, but its spent substrate also harbor other microorganisms of mesophilic nature, which have significant ligninolytic enzymes activity [10, 31] and contribute towards dyes decolourization [9].

CONCLUSION

Spent substrate from *P. sajor-caju* has been found to harbour highest and diverse population of both fungi and bacteria. Out of the isolated bacteria and fungi from SMS of three different mushrooms, the fungus (*Schizophyllum commune)* and bacterium (*B. licheniformis)* isolated from *P. sajor-caju* SMS, exhibited highest decolourization against different dyes. The pH and temperature optima for decolourization was recorded to be in the same range as was for the growth and fruiting of *P. sajor-caju*, which supports the suitability of oyster mushroom SMS as such in decolourization of different dyes.

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MEDICINAL MUSHROOMS CULTIVATION THROUGH THE SOLID-STATE AND SUBMERGED FERMENTATIONS OF AGRICULTURAL WASTES

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ABSTRACT

The main aim of this work consists in screening the optimal biotechnology of medicinal mushroom cultivation from the solid-state fermentation and the submerged one by using different kinds of wastes coming from cereal crop processing as well as the agro-food industry. The both fermentation technologies were tested through the controlled cultivation of the medicinal mushrooms *Ganoderma lucidum* and *Lentinula edodes* on different growing substrates made of cereal, fruit and vegetable wastes. All the experiments were performed by using a modular system that provided the automatic operations of compost sterilization, aseptic inoculation in a completely clean chamber, incubation as well as mushroom fruit body formation in special growing chambers with controlled atmosphere and the picking up of mushroom fruit bodies. By applying these automatic procedures, all physical and chemical factors that could influence fungal biomass production as well as mushroom fruit body formation could be strictly driven and controlled in order to get high quality products through safe biotechnological models. Comparative results of the chemical investigations regarding some biological active compounds of both fungal pellets from submerged fermentation and mushroom fruit bodies from solid state cultivation are presented.

Keywords: Agricultural wastes; Biomass; Medicinal mushrooms; Solid state cultivation; Submerged fermentation.

INTRODUCTION

Solid-state fermentation of plant wastes from the agro-food industry is one of the challenging and technically demanding of all biotechnologies known to humankind so far [1, 2]. The industrial activities related to wine processing have generally been matched by a huge formation of wide range of wastes [3-5]. Many of these organic wastes cause serious environmental pollution effects if they are allowed to accumulate nearby wine making factories or much worse if they are incorporated in the soil matrix [6-9]. In this respect, the major group of fungi which is capable to degrade cellulose and lignocellulose wastes belongs to the Basidiomycetes Class [10].

MATERIALS AND METHODS

According to the main purposes of this work, two fungal species of the Basidiomycetes group, namely *Ganoderma lucidum* (folk name: Reishi) as well as *Lentinula edodes* (folk name:

Shiitake), were used as pure mushroom cultures isolated from the natural environment and preserved in the local collection of the University of Pitesti. Stock cultures were maintained on malt-extract agar (MEA) slants (20% malt extract, 2% yeast extract, 20% agar). Slants were incubated at 25 °C for 120-168 h and stored at 4 °C.

The pure mushroom cultures were expanded by growing in 250-ml flasks containing 100 ml of liquid malt-extract medium at 23 °C on rotary shaker incubators at 110 rev min^{-1} for 72-120 h. After expanding, the pure mushroom cultures were inoculated into 100 ml of liquid malt-yeast extract culture medium with 3-5% (v/v) and then maintained at 23-25°C in 250 ml rotary shake flasks [10-12].

Experiments of inoculum preparation were set up under the following conditions: constant temperature, 25 °C; agitation speed, 90-120 rev min^{-1}; initial pH, 5.5–6.5. All seed mushroom cultures were incubated for 120–168 h and were then inoculated into liquid culture media (20% malt extract, 10% wheat bran, 3% yeast extract, 1% peptone) at pH 6.5 previously distributed into rotary shake flasks of 1,000 ml.

During the incubation time period, all the spawn cultures were maintained in special culture rooms designed for optimal incubation at 25 °C. There were prepared three variants of culture compost made of marc grapes in the following ratios: 1:1, 1:2, 1:4 (w/w).

The winery wastes were mechanically pre-treated by using an electric grinding device to breakdown the lignin and cellulose structures to make them more susceptible to the enzyme actions [10-12].

All the culture compost variants made of winery wastes were transferred into 1,000 ml glass jars and disinfected by steam sterilization at 120 °C for 60 min. When the jars filled with composts were chilled, they were inoculated with the liquid spawn already prepared.

Each culture compost variant for mushroom growing was inoculated using liquid spawn having the age of 72–220 h and the volume size ranging between 3–9% (v/w).

RESULTS AND DISCUSSION

During a period of time of 18–20 d after inoculation, all the mushroom cultures had developed a significant mycelia biomass on the culture substrates made of marc of grapes [12]. The effects induced by some additional ingredients such as nitrogen and mineral sources upon the mycelial growth during the incubation were investigated. Of the tested nitrogen sources, wheat bran was the most efficient upon the mycelia growth and fungal biomass production at 35-40 g% fresh fungal biomass weight, being closely followed by malt extract at 25–30 g% [12]. Peptone, tryptone and yeast extract are well known nitrogen sources for fungal biomass synthesis but their efficiency in experiments was relatively lower than the mycelia growing and fungal biomass production induced by the wheat bran added as natural organic nitrogen source (Table 1).

Among the various mineral sources examined, $CaCO_3$ yielded the best mycelia growth as well as fungal biomass production at 28-32 g% and for this reason it was registered as the most appropriate mineral source.

The experiments were carried out for 288 h at 25 °C with the initial pH 6.5 and all data are the means of triplicate determinations carried out on the compost variants containing marc of grapes in the ratio 1:4, as it is shown in Table 2.

Table 1: The effect of nitrogen source on mycelial growth of *L. edodes* and *G. lucidum*

Nitrogen source (1%, w/v)	Fungal biomass dry weight (g/l)		Fungal biomass fresh weight (g/l)		Final pH	
	L. edodes	*G. lucidum*	*L. edodes*	*G. lucidum*	*L. edodes*	*G. lucidum*
Rice bran	6.47 ± 0.14	7.05 ± 0.10	57 ± 0.05	63 ± 0.23	5.5	5.1
Malt extract	6.41 ± 0.23	6.83 ± 0.12	55 ± 0.03	69 ± 0.20	5.3	5.7
Peptone	4.45 ± 0.15	5.43 ± 0.03	41 ± 0.12	57 ± 0.15	4.6	5.3
Tryptone	5.23 ± 0.09	6.95 ± 0.15	28 ± 0.70	55 ± 0.17	5.1	5.9
Yeast extract	5.83 ± 0.35	7.15 ± 0.21	30 ± 0.01	61 ± 0.14	4.3	5.1

All data are the means ± S.D of triplicate determinations

Table 2: The effect of mineral source on mycelial growth of *L. edodes* and *G. lucidum*

Mineral source (5 mg)	Fungal biomass dry weight (g/l)		Fungal biomass fresh weight (g/l)		Final pH	
	L. edodes	*G. lucidum*	*L. edodes*	*G. lucidum*	*L. edodes*	*G. lucidum*
KH_2PO_4	5.71 ± 0.09	6.05 ± 0.15	45 ± 0.07	53 ± 0.12	5.5	5.9
K_2HPO_4	6.98 ± 0.13	5.93 ± 0.07	57 ± 0.05	59 ± 0.07	5.1	5.7
$MgSO_4 \cdot 5H_2O$	6.18 ± 0.20	7.01 ± 0.25	55 ± 0.09	63 ± 0.28	5.6	6.1

*All data are the means ± S.D. of triplicate determinations

During the stage of mushroom fruit body formation, the culture parameters were set up and maintained at the following levels: air temperature, 15–17 ^{O}C; air flow volume, 5–6m^3/h; air flow speed, 0.2–0.3 m/s; relative moisture content, 80–85%, light intensity, 500–1,000 lux for 8–10 h/d.

The final fruit body production of the mushroom species used was registered between 1.5-2.8 kg per 10 kg of compost made from winery wastes. The whole mycelia growing during the incubation period from the moment of inoculation up to the mushroom fruit body formation lasted between 30–60 d, depending on the fungal species used in experiments.

In order to characterize the chemical composition of the collected mushroom pellets, the sugar as well the nitrogen content were investigated. Firstly, the dry weight of the fungal biomass was established for each mushroom species. Secondly, the sugar content of dried mushroom pellets collected after the biotechnological experiments was determined by using Dubois method. Mushroom extracts were prepared by suspension of dried pellets in a solution of NaOH (pH 9) in a ratio of 1:5. All these dispersed solutions containing the dried pellets were maintained 24 h at the precise temperature of 25 ^{O}C, in full darkness, with continuous homogenization to avoid oxidation reactions. After removal of solid residues by filtration, the samples were analyzed as above.

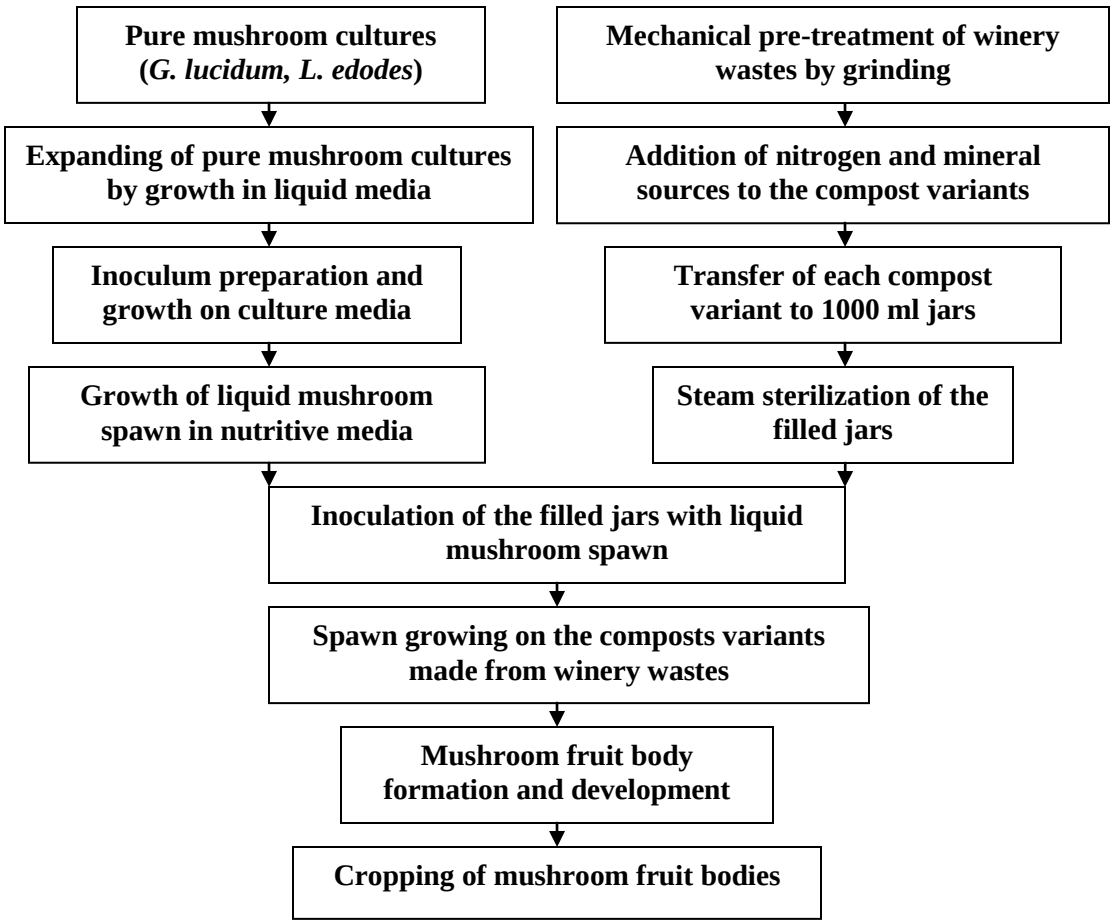

Figure 1: Scheme of enhanced cultivation of edible mushrooms on organic winery wastes

The nitrogen content of the same samples of mushroom pellets was analyzed by the Kjeldahl method. All the registered results are related to the dry weight of mushroom pellets that were collected at the end of each biotechnological culture cycle (Table 3).

Table 3: Sugar and total nitrogen contents of dried mushroom pellets

Sample number	Mushroom species	Mushroom pellets d. w. (%)	Sugar content of dried pellets (mg/ml)	Kjeldahl nitrogen of dried pellets (%)
G-1	*G. lucidum*	17.64	4.93	5.15
G-2	*G. lucidum*	14.51	3.70	5.35
G-3	*G. lucidum*	20.16	5.23	6.28
L-1	*L. edodes*	19.67	4.35	6.34
L-2	*L. edodes*	17,43	3.40	5.03
L-3	*L. edodes*	15.55	4.75	6.05

Comparing all the registered data, it can be seen that the correlation between the dry weight of mushroom pellets and their sugar and nitrogen contents is kept at a balanced ratio for

each tested mushroom species. Among all the mushroom samples that were analyzed, *G. lucidum* G-3 showed the best sugar composition values and total nitrogen content (Table 3).

In this stage, 70-80% of the former fungal pellets were separated by collecting them from the culture vessel of the bioreactor and separating from the broth by slow vacuum filtration. On the base of registered results the optimal *in vitro* culture conditions were evaluated and the best values of all physical and chemical factors which could influence the evolution of biotechnological processes for fungal biomass synthesis were established. Taking into consideration all these registered results, it was established the biotechnology of mushroom pellets producing by controlled submerged fermentation including the most important stages (Fig. 2).

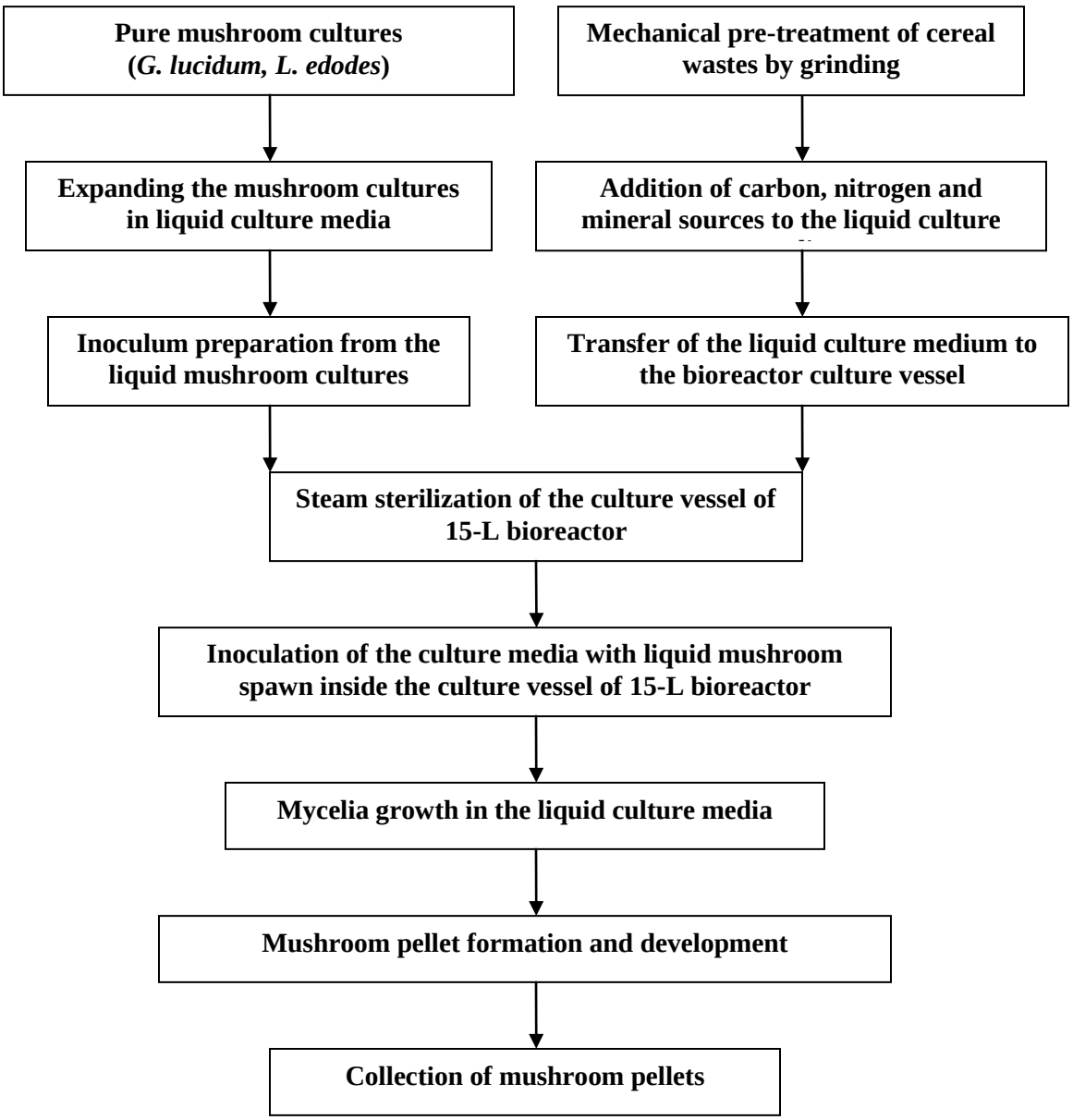

Figure 2: Flow chart showing the biotechnology ofr mushroom pellet production in submerged culture

CONCLUSIONS

1. Among the five nitrogen sources examined, wheat bran was the most efficient upon the mycelia growing and fungal biomass production of *L. edodes* and *P. ostreatus*, at 35-40 g% fresh fungal biomass weight, closely followed by malt extract at 25–30 g%.
2. The best mineral source was $CaCO_3$ that yielded the optimal mycelia growing as well as fungal biomass production at 28-32 g%.
3. The final fruit body production by the two mushroom species was registered between 1.5–2.8 kg per 10 kg of solid composts made from winery wastes.
4. Among all mushroom samples that were analyzed, only *G. lucidum* G-3 had shown the best values of their composition in sugars and total nitrogen content.
5. The collected mushroom pellets having high nutritional value were produced by applying the biotechnology of controlled submerged fermentation in order to be used in functional food producing.

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THE POTENCY OF OIL PALM PLANTATION WASTES FOR MUSHROOM PRODUCTION

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ABSTRACT

Indonesia has become the world's largest palm oil producer with total area of oil palm plantation being 7.3 million ha in 2009 wich produced a huge quantity of biomass by-product such as empty fruit bunches (EFB) estimated at 43 million tons, as well as produced liquid and solid wastes from palm oil mill effluent (POME). This research was aimed to study the potency of EFB and POME on growth of white oyster mushroom (F isolate of *Pleurotus* sp.) and GKSA isolate of *Ganoderma boninense*. F isolate was grown on EFB, *Paraserianthes falcataria* sawdust and mixture of both substrates with proportion 1:1 respectively. The results showed that EFB could be used as a substrate of *Pleurotus* fruit body production with biological efficiency that reached 152%, and even the growth went faster than the other substrates. As well as GKSA isolate could used the EFB as a substrate of fruit body production and it could decrease C/N ratio up to 84% at vegetative phase and lignin concentration up to 66% at reproductive phase. In addition the POME have induced significant increasing of the GKSA isolate growth up to 62% at 20% of liquid POME concentration in malt media and reached 64% of growth at 10% of solid POME in the same media. Based on these results the abundant wastes from the oil palm plantation were considered suitable for mushroom production and spent mushroom substrates can be used as soil fertilizers at oil palm plantation as well as for animal feed.

Keywords: Oil palm, wastes, cultivation, *Pleurotus* sp., *Ganoderma boninense*

INTRODUCTION

Oil palm tree has become a plantation icon in Indonesia and neighboring Malaysia. Both countries have now become the world's biggest producers of palm oil. Data from Indonesia Crude Palm Oil Council showed that total area of oil palm plantation in Indonesia was 7.3 million ha in 2009 which produced a huge quantity of by-product biomass such as empty fruit bunches (EFB) estimated at 43 million tons, as well as produced liquid and solid wastes from palm oil mill effluent (POME). In a palm oil mill with modern technology, every ton of fresh fruit bunch (FFB) produced 0.23 ton of EFB, 0.13 ton of mesocarp fiber and 0.55 ton of kernel shells as well as 0.2 m^3 of POME and 0.6-1.2 m^3 of waste water. Annually, 27,600 tons of EFB and 96,000 m^3 of POME are produced by a 30 tons/hour capacity mill with an input of 120,000 tons FFB. On average processing of 1 million ton FFB in palm oil mills generates 230,000 tons

of EFB and 650,000 tons of POME as residues [10]. The palm oil industry also produces fronds and trunks pruned when harvesting fruit bunches and felled during the replantation. For most mills, EFB and POME are still considered as an unwanted wastes because of their storage, distribution, and treatment costs. Concerning the environmental problem especially high air pollution, the EFB incineration is prohibited therefore palm oil mills have started to bring EFB back to the plantation and just dump them.

Millions of tons of agriculture industrial wastes are discarded, burned and neglected. They mainly content the lignocellulose compounds. These useless by-products can be recycled to produce value-added mushrooms. With the exploitation of these wastes as a raw materials for cultivation of mushrooms, the wastes can be curbed and the nutritional quality of the diet of people in plantation region improved.

The use of EFB as a substrate for the cultivation of *Pleurotus* spp. and *Volvariella* sp. is a viable alternative for the management of the solid waste and nutritional security in the oil palm plantation region. *Volvariella* sp. grows naturally on EFB. Only several reports about oyster mushrooms cultivated on EFB were published [5, 1, 10, 8]. Bioconversion of two formulations of substrates containing only 10% and 20% oil palm fiber respectively using *Pleurotus ostreatus* and *P. eous* was reported by [12]. The results showed that both species displayed a higher rate colonization and yielded on corncob-based formulations than on cocoa and rice husk-based media containing oil palm fiber. Lower biological efficiency (BE) of 50-70% was achived by *Pleurotus* spp. grown on coffee pulp. Then BE were improved with ensiled coffee pulp, reaching 82% in the cultivation of *P.sajor-caju* and 73% with *P. ostreatus* [9]. The BE of *P. ostreatus* grown on a mixture of 70% *Digitaria decumbens* grass and 30% coffee pulp varied between 59.79% and 93% [4]. High yield of *P. ostreatus* was obtained by waste paper amended with 20% husk rice [2] with BE of 140%. For *P. sajor-caju*, 200% BE has been recorded [3].

This research was aimed to study the potency of empty fruit bunches on growth of white oyster mushroom and *Ganoderma boninense* which is one of important pathogen of oil palm. We also studied the influence of liquid and solid wastes from palm oil mill effluent on growth of *G. boninense*.

MATERIALS AND METHODS

Isolates. F isolate of *Pleurotus* sp.was obtained from mating between two monocaryotic isolates of white oyster mushrooms (BNK 2 and BBR 14 isolates). Both of those dicaryotic isolates were isolated from fruit body bought from the markets in Bangkok, Thailand and Madiun, Indonesia respectively. GKSA isolate of *Ganoderma boninense* was isolated from fruiting body grown on standing oil palm in Adolina, The Fourth State-Owned Estate, North Sumatera, Indonesia.

Growth of F isolate of *Pleurotus* sp. F isolate was grown on *Paraserianthes falcataria* sawdust, empty fruit bunches (EFB) and mixture of both substrates with proportion 1:1 respectively. Initially, EFB was chopped and shredded into smaller pieces, water soaked for one night to gain 75% moisture content. All substrates were added with 15% rice bran, 1.5% gypsum, 1.5% $CaCO_3$. Afterwards all substrates were placed in 30 x 20 cm sized polyethylene bags. Each bag containing of 500 gr substrate was sterilized, spawned with grain spawns and incubated in a range of 28-30^{o}C. After completion of spawn running, the bags were unfolded at the upper parts for cropping. Five replications were used for each growing trial. Biological efficiency of each

treatment was determined as follows: BE = (fresh weight of harvested mushrooms/dry weight of substrate) x 100%.

Growth of GKSA isolate of *Ganoderma boninense* on empty fruit bunches (EFB). GKSA isolate was grown on chopped and shredded EFB supplemented with 15% rice bran, 1.5% gypsum, 1.5% $CaCO_3$ and added with water to gain 75% moisture content. Each of 70 gr of substrate was placed in a small bottle, then sterilized, spawned with grain spawns and incubated in a range of 28-30^{o}C. The substrate analysis at various stages of growth i.e before inoculation, vegetative phase (after spawn run), during reproduction phase were determined. Lignin and cellulose contents were determined by Van Soest method. Organic-C content was measured by titration method and N content measured by Kjeldahl. The analyses were achieved at Laboratory of Science and Animal Feed, Faculty of Husbandry, Bogor Agricultural University and Services Laboratory, SEAMEO BIOTROP, Bogor.

Growth of GKSA isolate of *Ganoderma boninense* on liquid and solid wastes from palm oil mill effluent (POME). One hundred milliliter of 1.5% malt extract liquid medium was poured into a 250-L Erlenmeyer flask. The flasks were added with 10% (v/v), 20% (v/v) of liquid POME, 5% (w/v) or 10% (w/v) of solid POME respectively. Both POME were obtained from sixth ponds. Water or soil from oil palm plantation were used as the controls. The surface of the medium was inoculated with one disc of *G. boninense* solid medium (7-mm diameter) punched out from the edge of a 7 day-old colony grown on malt agar. The fungus was grown at 28-30^{o}C without agitation. At the end of the incubation period the mycelium was collected, dried at 105^{o}C, and weighed.

Data analysis. Results are expressed as means ± standard deviation (S.D.). The analyses of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were performed with a program of Microsoft Excel 2007 and SPSS Statistics 17.0.

RESULTS AND DISCUSSION

Growth of F isolate of *Pleurotus* sp. Based on this results, F isolate could grow and produce fruit body on all substrates i.e. *Paraserianthes falcataria* sawdust (PFS), empty fruit bunches (EFB), mixture of both substrates (M) with fresh weight and biological efficiencies (BE) for all substrates were not significantly different (P>0.05) in a range of 190-209 gram/bag for 8 flushes and 152-167% of BE but the highest results were obtained from PFS substrate with 209 gram/bag of fruiting body fresh weigh for 8 flushes and 167% of BE (Fig. 1). PFS is the principal substrate for oyster mushroom growing in Indonesia, although adequate production can be achieved through use of sawdust with addition of supplements that substantially increase the yield per unit weight. In addition PFS contains cellulose 48.3%, lignin 27.8%, pentosan16.2% [7] but EFB contains lower cellulose of 36%, lignin 27.3%, carbon 64.7%, N 1.1% and C/N ratio 57.8 (Sudirman, unpublished).

Vegetative phases (VP) on all substrates were observed between 25-31 days and fruit body production phase (RP) between 86-101 days and total of growth and development phase (GDP) between 111-132 days with shorter GDP on EFB and PFS substrates reaching 111 and 119 days respectively (Fig. 2).

The quantity of fruit body/bag for 8 flushes and pileus diameter of those three substrates were not significantly different (P>0.05) in a range of 31-34 pieces and 4.1-4.7 cm (Fig. 3). Number of fruit body and pileus diameter were related to the bag removal method [3] or were dependent on opened surface area for cropping. In case of larger opened surface area, the quantity of mushrooms could be larger but the diameter of pileus will be smaller.

Growth of GKSA isolate of *Ganoderma boninense* and bioconversion on empty fruit bunches (EFB). Based on these results, GKSA isolate of *G. boninense* could grow and produce fruiting body, only one flush was obtained. The vegetative phase took 17 days, longer than other substrates such as oil palm leaf and root which were only 8-10 days (unpublished). Nutrition contents of substrates after vegetative and during reproduction phase are presented at figure 4. C/N ratio decreased as much as 84% with initial C/N ratio 49.1 and 7.8 after vegetative phase, but increased thereafter during reproduction phase, but not as high as before inoculation. Similar patterns were shown with cellulose and carbon contents. Cellulose content decreased as much as 41% with initial content 50.6% and 29.8% after vegetative phase, but increased thereafter during reproduction phase. This pattern was followed by decreased carbon content up to 81% with initial content 58% and 10.9% after vegetative phase, but increased during reproduction phase. Differently with lignin degradation, its content decreased up to 66% during reproduction phase with initial content 31.4%, thereafter 10.6%. The degradation pattern indicated that the rate of lignin breakdown was slow during spawn run. The most extensive degradation of lignin occured during reproduction phase, implying the release of cellulose increased during reproduction phase as well. The lignin moiety can act as a barrier to cellulose degradation [6]. Therefore, the degradation of lignin serves to increase the availability of cellulose for development of fruit body. Nitrogen content in the substrate slightly gradually increased during decomposition reaching 1.5% during reproduction phase.

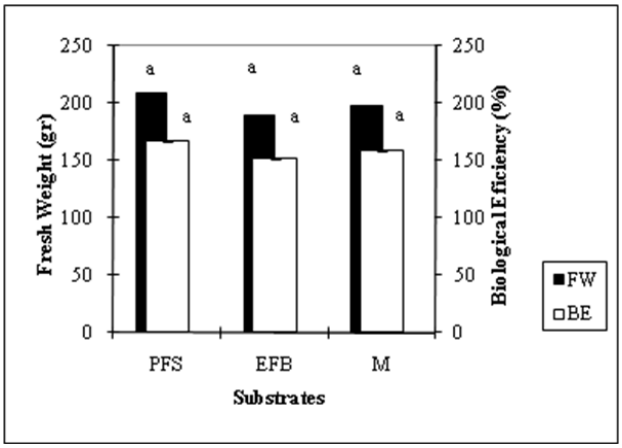

Figure 1: Fresh weight of fruiting body (FW) and biological efficiency (BE) of F isolate of *Pleurotus* sp. grown on three kinds of substrate. PFS: *Paraserianthes falcataria* sawdust, EFB: empty fruit bunches, M: mixture of PFS and EFB substrates (1:1)

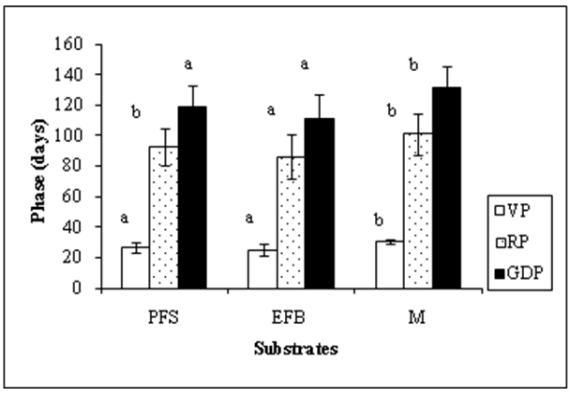

Figure 2: Growth phases of F isolate of *Pleurotus* sp on three kinds of substrate. PFS: *Paraserianthes falcataria* sawdust, EFB: empty fruit bunches, M: mixture of PFS and EFB substrates (1:1), VP: vegetative phase, RP: reproduction phase, GDP: growth and development phase.

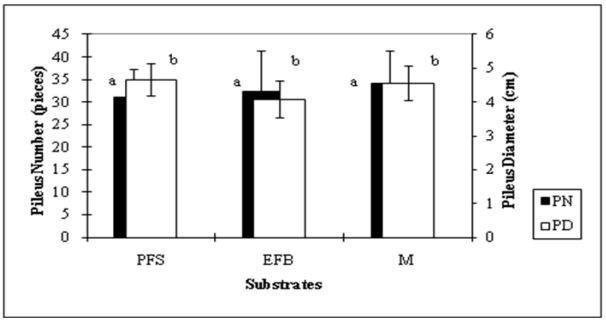

Figure 3: Pileus number (PN) and diameter (PD) of F isolate of *Pleurotus* sp on three kinds of substrate. PFS: *Paraserianthes falcataria* sawdust, EFB: empty fruit bunches, M: mixture of PFS and EFB substrates (1:1).

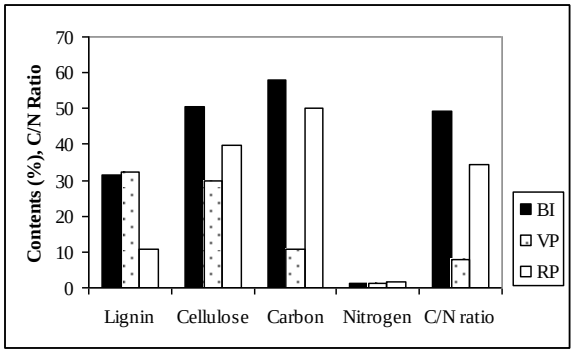

Figure 4: Bioconversion of empty fruit bunches by GKSA isolate of *Ganoderma boninense*. BI: before inoculation, VP: vegetative phase, RP: reproduction phase.

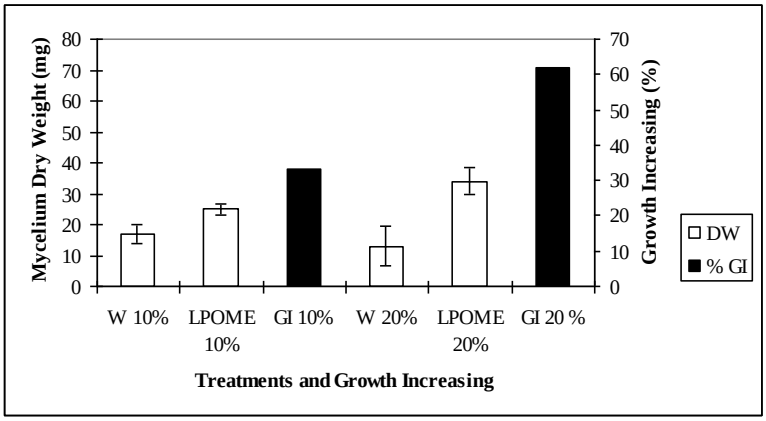

Figure 5: The growth of GKSA isolate of *Ganoderma boninense* on malt extract media containing liquid palm oil mill effluent (LPOME). W 10% or 20%: concentration of water at 10% or 20%, LPOME 10% or 20%: concentration of LPOME at 10% or 20%, DW: dry weight, GI: growth increasing.

Growth of GKSA isolate of *Ganoderma boninense* on liquid and solid wastes from palm oil mill effluent (POME). GKSA isolate could grow on malt extract media containing liquid and solid POME respectively, even stimulated the growth of GKSA isolate with the growth increase of 33 and 62% at 10 (v/v) and 20% (v/v) of liquid POME concentration in malt media respectively (Fig. 5). Similar results were achieved for solid POME, the growth increasing reached 23 and 64% at 5 (w/v) and 10% (w/v) of solid POME concentration in malt media respectively (Fig. 6). POME as a nutrient source can speed up the process of decomposition by reducing the wide C/N ratio of the EFB [7]. POME are started to be reused in oil palm plantation (land irrigation).

Composting EFB through mushroom production could be a possible way to transform the bulky bunches into a valuable, manageable product as market product or for use in plantation. The disadvantage using EFB in mushroom production is in its preparation. Initially, EFB must be chopped and shredded into smaller pieces that need much labor but it is not needed for ready use of sawdust.

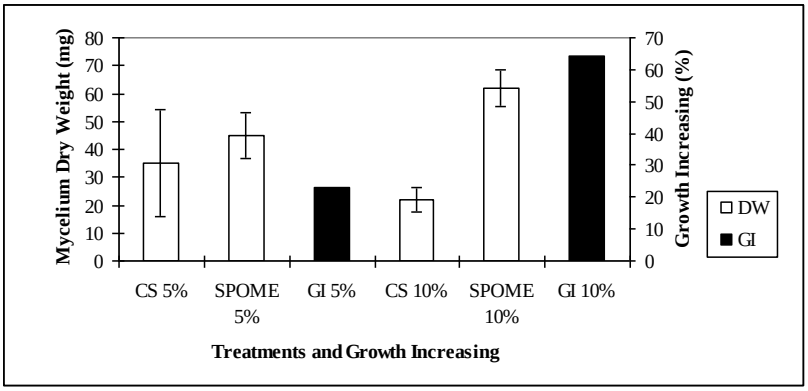

Figure 6: The growth of GKSA isolate of *Ganoderma boninense* on malt extract media containing solid palm oil mill effluent (SPOME). CS 5% or 10%: concentration of control soil at 10% or 20%, SPOME 5% or10%: concentration of SPOME at 5% or10%, DW: dry weight, GI: growth increasing.

CONCLUSION

The empty fruit bunches (EFB) could be used as alternative substrate for mushroom production. Based on this research it was suggested to build mushroom industry located near the palm oil factory where the main substrates are abundantly available. The liquid as well as solid wastes from palm oil mill effluent (POME) stimulated the growth of mushrooms. In addition spent mushroom substrates can be then used as soil fertilizers in oil palm plantation.

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YIELD PERFORMANCE AND NUTRITIONAL ANALYSIS OF *PLEUROTUS CITRINOPILEATUS* ON DIFFERENT AGROWASTES AND VEGETABLE WASTES

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ABSTRACT

Pleurotus citrinopileatus was cultivated on paddy straw, brassica straw, pea pod shell, cauliflower leaves and radish leaves separately and on various combinations of paddy straw and aforementioned wastes. The mushroom failed to grow on pea pod shell, cauliflower leaves and radish leaves when it was cultivated separately on these vegetable wastes. However, it grew very well on paddy straw in combination with other substrates. Total yield and biological efficiency of the mushroom was found to be lower on paddy straw than paddy straw and other wastes combinations. 70% paddy straw and 30% other wastes combination supported maximum biological efficiency of mushroom followed by 80% paddy straw and 20% other wastes combination. The mushroom cultivated on paddy straw mixed with other lignocellulosic wastes i.e. brassica straw, pea pod shell, cauliflower leaves and radish leaves contained better nutrient content than the mushroom cultivated on paddy straw alone. The protein content, total sugar and nonreducing sugar content was found to be higher in the mushrooms grown on paddy straw and other agrowastes combination than on paddy straw alone. Similarly, six essential amino acids i.e. leucine, isoleucine, valine, threonine, methionine and phenylalanine content was higher in the mushrooms cultivated on paddy straw and other agrowastes combination than on paddy straw alone.

Keywords: Biological efficiency; Nutritional analysis; *Pleurotus citrinopileatus*

INTRODUCTION

The problem of malnutrition with ever-increasing ‘protein gap’ is quite obvious in Asian, African and many developing countries, since, the traditional source of protein has not kept pace with population growth. It is desired, therefore, to explore and exploit the possible source of protein production to help the country to overcome the malnutrition. Malnutrition posses a continuing constrain to India’s development. Mushroom, one of the highest protein producers per unit area and time from agrowastes can be very effective weapon to fight malnutrition.

Of more than 2000 recorded species of the edible mushrooms, India accounts for nearly 300 species belongs to 70 genera [1]. Out of 2000 species 100 are widely picked, 15-30 species are commonly eaten, 80 species are experimentally cultivated and 5 – 6 are produced on large scale [2]. Among these *Agaricus* and *Pleurotus* contribute maximum to total world production of cultivated edible mushroom. The oyster mushrooms are botanically species of *Pleurotus* called as ‘Dhingri’ or ‘Abalone’. They grow naturally in temperate or tropical forest on dead and decaying wooden logs or sometimes on outer bark of living trees. The fruit bodies of this mushroom are distinctly shelly or oyster shaped with different shades of white, cream, grey, yellow, pink or light brown depending upon the species. The oyster mushroom confers many advantages over other

mushroom in terms of its ease for cultivation, role in biodegradation and bioremediation, extracellular enzymes production and nutriceuticals production [3-9].

Nutritional attributes of the oyster mushroom is being increasingly realized in recent times. Low in calories and high in protein as compare to rice, wheat, cabbage and milk, they are good sources of several vitamins including thiamine, riboflavin, niacin, biotin and ascorbic acids. The oyster mushrooms are good source of minerals and rich in carbohydrate and fibres.

Various workers have reported nutritional and medicinal attributes of mushroom. But there is wide variation in the values reported for the same species by different workers [10]. The difference may be due to the variation in the genetic make-up, substrates, cultivation technology and conditions at the stage of harvests as well as post harvest, which affect the composition.

MATERIALS AND METHODS

In the present research work the oyster mushroom *Pleurotus citrinopileatus* was cultivated on different vegetable and agricultural wastes viz. brassica straw, cauliflower leaves, radish leaves and pea pod shell alone and in combination of different proportion with paddy straw. The pure cultures of *P. citrinopileatus* were procured from NRCM, Solan (HP), India and maintained on malt extract agar (MEA) medium at temperature 25 ± 2°C and pH 6 - 6.5 and subcultured at periodic interval of three weeks.

Collection of agricultural and vegetable wastes. Five different agrowastes were collected from the different agricultural fields and Sabjimandi of district Jaunpur (UP), India. Radish leaves and cauliflower leaves were collected from old Sabjimandi, Kotawali, Jaunpur and New Sabjimandi, Chaukiya, Jaunpur. Brassica straw and paddy straw were collected from agricultural field of village Dewkali and Kukuripur just behind the V.B.S. Purvanchal University and pea pod shell from different house holds. Old Sabjimandi and New Sabjimandi are 10 km away from the University Campus.

Preparation of spawn. Spawn is referred to as the vegetative mycelium of the fungus, which is grown on cereal grains. Wheat grain spawn was prepared by the following method. Wheat grains were well washed in tap water and then half boiled in water. After that water from wheat grains was drained out. To remove excess water, wheat grains were spread over a tilted platform. This was followed by mixing of buffers $CaCO_3$ and $CaSO_4$ in 3:1 ratio (30 gm $CaCO_3$ and 10 gm $CaSO_4$ per kg of half boiled wheat grains). The wheat grains were now half filled in bottles and plugged by cotton. The half filled bottles were autoclaved at the temperature 121°C and pressure 15 psi for 40 minutes then left for overnight followed by inoculation of bottles by transferring inoculums *P. citrinopileatus* from cultured plate. Then bottles were incubated in BOD incubator at temperature 25 ± 2°C.

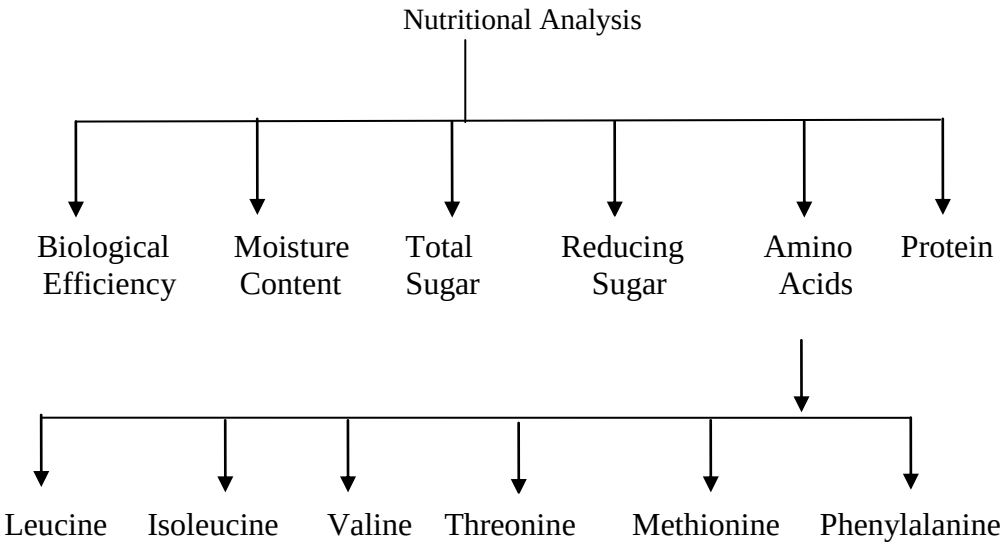
After 3-4 days of inoculation fungal mycelium started spreading on the grains. The mycelium is white net web like in appearance. The bottles were nearly half filled in 10-12 days and in 18-21 days these were completely filled with white mycelial growth.

Preparation of substrate, spawning and cultivation. The collected vegetable wastes i.e. cauliflower leaves, radish leaves and pea pod shell were spread in open area to sun dry for 30 to 40 days. These dried substrates were autoclaved at the temperature 121°C and pressure 15 psi for 40 minutes. Vegetative substrates separately and in various combinations with paddy straw were used for cultivation experiment. The paddy straw before mixing of vegetable wastes was completely dipped in water (50 litres for every 10 kg dry chopped paddy straw) in a drum or big bucket and was allowed to stay in water for 12 hours. After that excessive water was drained out. After draining, the paddy straw was again completely dipped in hot water (temperature 70-80°C)

for an hour. Then water was drained out and paddy straw was evenly spread on platform and mixed with dried autoclaved vegetable wastes (radish leaves, cauliflower leaves, brassica straw and pea pod shell) in two combinations i.e. 70% paddy straw and 30% other wastes and 80% paddy straw and 20% other wastes.

Spawning is the process of mixing spawn in the sterilized substrates. 3% wet weight basis spawn grain was mixed with the substrate and filled into polythene bags. The mouth of the bag was tied with rubber band and 12 holes of about 1cm diameter were made two at each corner at the base, four each on the broader area and one each on the narrow, rectangular side to drain out extra water and for proper aeration. Five bags of each combination of substrates (equiv. 300 g of dry substrate) spawned with *Pleurotus citrinopileatus* were filled and kept in mushroom house on the iron racks on the bricks.

Nutritional analysis. The nutritional analysis of mushroom fruit bodies were done after drying the mushroom samples taken separately from each bag in hot oven at a constant temperature of 40°C. The parameters selected for nutritional analysis is depicted in the flow diagram given below.



Biological efficiency

The four bags for each substrate and *P. citrinopileatus* were kept for evaluation of yield performance and biological efficiency in mushroom house under *in vivo* condition. The yield was expressed as of fresh fruit bodies produced per bag. Biological efficiency (B.E.) was calculated as the percentage conversion of dry substrates to fresh fruit bodies [11] i.e.

$$\text{Biological Efficiency} = \frac{\text{Fresh weight of mushrooms per bag (x)}}{\text{Dry weight of substrate per bag (y)}} \text{ X } 100$$

*Moisture content***.** It was done by picking fresh fruit body of the *P. citrinopileatus* and dried in hot air oven at 60°C for 15 hours.

$$\text{Moisture content} = \frac{\{\text{Fresh weight of mushroom (A)} - \text{Dry weight of mushroom (B)}\}}{\text{Fresh weight of mushroom (A)}} \text{X } 100$$

Sugar, amino acids, protein estimation. Total sugar estimation was done by using sulphuric acid phenol method [12] and the reducing sugar estimation was done by Dinitrosalicylic acid method. Amino acids were estimated by following the method of Moore and Stein [13]. Protein estimation was done by Lowry et al. method [14].

Data analysis. All the experiments were carried out in quadruplicates and the results are expressed as mean with standard deviation. Statistical significance was analyzed by ANOVA following Duncan's multiple comparison test ($P<0.05$) and student t-test. Each bar represents mean ± standard deviation (n=4). Asterisks show significant difference from control statistically at $P<0.05$.

RESULTS AND DISCUSSION

When the *Pleurotus citrinopileatus* was cultivated separately on radish leaves, pea pod shell and cauliflower leaves, the mushroom failed to grow on these three vegetable wastes. On the other hand when the mushroom was cultivated on paddy straw alone and paddy straw in combination with vegetable wastes, the fructification took place. Brassica straw alone as well as in combination with paddy straw supported better growth of the mushroom. The mean yield of *Pleurotus citrinopileatus* on different agrowastes in various combinations and their biological efficiency are given in Table 1 and Figure 1.

Table1: Yield and Biological Efficiency of *Pleurotus citrinopileatus* on different combinations of agrowastes

Substrates	Flush I (g/ bag)	Flush II (g/ bag)	Flush III (g/ bag)	Flush IV (g/ bag)	Total ((g/ bag)	B.E. (%)
100% PS	150.00	76.67	45.00	xx	271.67	90.55
30%BS+70%PS	148.75	65.50	55.00	13.75	283.00	94.33
30%PP+70%PS	148.75	65.50	55.00	13.75	283.00	94.33
30%CF+70%PS	138.75	70.00	56.25	12.5	277.5	92.50
30%RL+70%PS	136.25	16.75	57.50	12.50	273.00	91.00
20%BS+80%PS	136.25	62.50	58.75	12.50	270.00	90.00
20%PP+80%PS	145.00	66.25	57.50	18.33	282.50	94.16
20%CF+80%PS	160.00	63.33	48.33	xx	271.67	90.56
20%RL+80%PS	150.00	78.30	45.00	xx	275.00	92.78

PS = Paddy Straw, BS = Brassica Straw, PP = Pea Pod, CF = Cauliflower Leaves, RL=Radish Leaves. BE = Biological Efficiency.

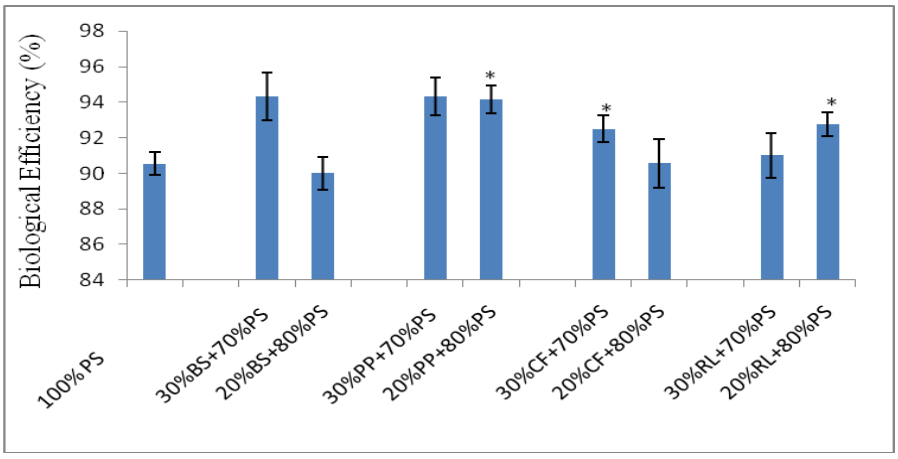


Figure1: Biological efficiency of *Pleurotus citrinopileatus* on different combinations of agrowastes.

PS = Paddy Straw, BS = Brassica Straw, PP= Pea Pod, CF = Cauliflower Leaves, RL=Radish Leaves.

70% paddy straw and 30% other agrowastes supported significant mushroom yield and bioefficiency than 80% paddy straw and 20% other agrowastes combination. Paddy straw and vegetable wastes combination gave better result in terms of total yield and bioefficiency than paddy straw alone. In all the cases first flush fruit bodies gave much more yield than second and subsequent flushes. There was decrease in the mushroom yield in the subsequent flushes.

The result of moisture content, protein and sugar content is given in Table 2 and Figure 2. The moisture content of fresh mushroom fruit bodies grown on various substrates ranged from 87.84 to 90%. Brassica Straw and Pea Pod with paddy straw in all combination in this experiment showed similar moisture retention capacity i.e. 89%.

Table 2: Nutritional content of *Pleurotus citrinopileatus* on different combinations of agrowastes

Substrates	Moisture (%)	Protein (mg/100g)	TS (mg/100g)	RS (mg/100g)	NRS (mg/100g)
100% PS	90.00	42.00	45.55	18.35	27.20
30%BS+70%PS	89.00	50.00	40.00	15.35	24.65
30%PP+70%PS	89.00	52.35	32.00	17.35	14.65
30%CF+70%PS	87.84	46.00	35.00	18.35	16.65
30%RL+70%PS	87.84	42.00	35.00	18.00	17.00
20%BS+80%PS	89.00	45.00	40.00	15.35	24.65
20%PP+80%PS	89.04	52.35	32.00	17.35	14.65
20%CF+80%PS	87.84	46.00	35.00	18.35	16.65
20%RL+80%PS	87.84	42.00	43.00	18.00	25.00

PS = Paddy Straw, BS = Brassica Straw, PP = Pea Pod, CF = Cauliflower Leaves, RL=Radish Leaves. TS = Total Sugar, RS = Reducing Sugar, NRS = Non-reducing Sugar.
Values of protein and sugar are expressed in mg/100g wet weight of dry mushroom

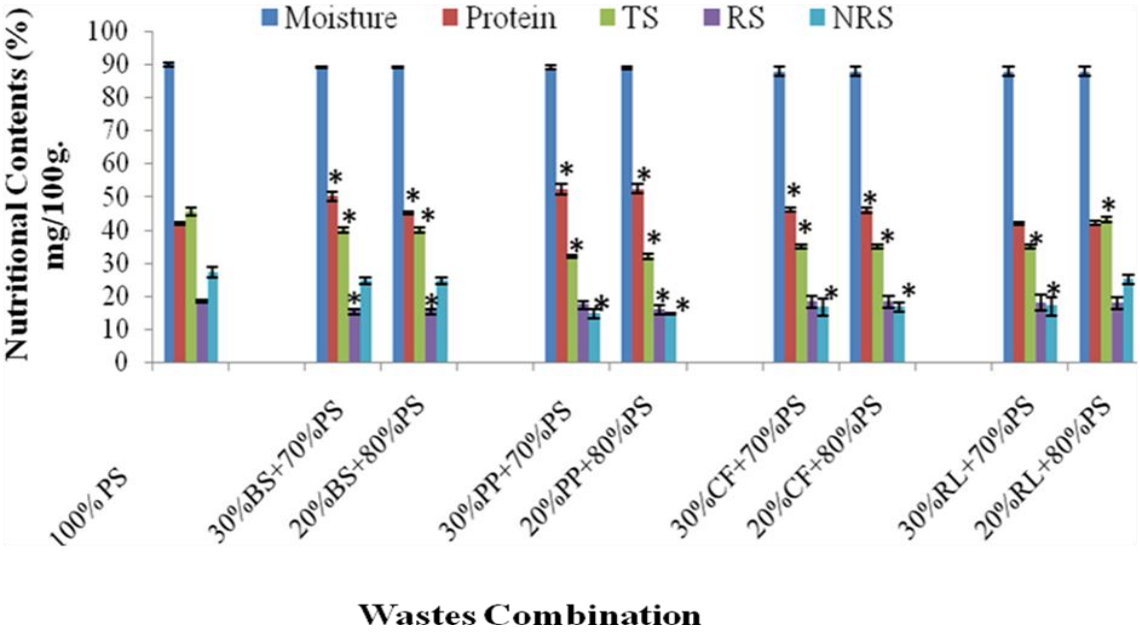


Figure2: Nutritional content of *Pleurotus citrinopileatus* on different combinations of agrowastes.

PS = Paddy Straw, BS = Brassica Straw, PP = Pea Pod, CF = Cauliflower Leaves, RL=Radish Leaves. TS = Total Sugar, RS = Reducing Sugar, NRS = Non-reducing Sugar.
Values of protein and sugar are expressed in mg/100g wet weight of dry mushroom

The protein content of mushroom fruit bodies ranged from 42 mg to 52.35 mg per 100 g of dried fruit bodies. Maximum protein content was observed in the mushroom fruit bodies when it was grown on paddy straw and pea pod combination. The mushroom grown on 70% paddy straw and 30% vegetable wastes had more protein content than 80% paddy straw and 20% vegetable wastes combination. The mushroom fruit bodies produced on paddy straw and vegetable combination showed more protein content than paddy straw alone.

Total sugar content recorded in the mushroom fruit bodies varied from 32 mg to 45.55 mg per 100 g of dried mushroom. Maximum total sugar content was observed in the mushroom fruit bodies produced on paddy straw alone. Total sugar content in the fruit bodies produced on various combinations of paddy straw and vegetable wastes was found to be lower than paddy straw. This was contrary to the protein content of fruit bodies. Non-reducing sugar in *P. citrinopileatus* cultivated on various substrates ranged from 14.65 to 27.25 mg/100mg weight of dry mushroom.

The amino acid content of *P. citrinopileatus* cultivated on paddy straw alone and combination of paddy straw and vegetable wastes is given in Table 3 and Figure 3. Six amino acids i.e. leucine, isoleucine, valine, threonine, methionine and phenylalanine determined from the fruit bodies of *P. citrinopileatus* grown on paddy straw had lower amount than on paddy straw and other agrowastes combination. Among the six amino acids amount of valine was observed as the maximum followed by threonine and other amino acids.

In the present investigation *P. citrinopileatus* failed to grow when cultivated separately on radish leaves, pea pod shell and cauliflower leaves. The probable reason for this is that these three vegetable wastes when processed for cultivation and dipped into and taken out from water they hold large amount of water. Hence due to presence of excess water in the substrate and lack of proper aeration *Pleurotus* mycelia does not grow and spread adequately and spawn run and fructification fail to occur. On the other hand when these vegetable wastes are mixed with paddy straw, these shortcomings are overcome and spawn run and fructification take place. The results of yield performance (Table 1 and Figure1) indicate that the first flush of fruiting bodies gave maximum yield in comparison to second and subsequent flushes. The lowest yield was recorded in the last flush. Block *et al.* also reported higher yield of *P. ostreatus* in first flush while yield of second flush was two third of first flush and yield of third flush was two third of second flush [15]. However, Chang *et al* observed uniform distribution of fruit bodies of *P. sajor-caju* in all the four flushes on cotton wastes while they observed higher yield of first flush (46%) than second flush (29%), third flush (15%) and fourth flush (9%) on paddy straw [11]. Bisaria *et al* reported higher yield of *P. florida* in first flush than subsequent flushes on paddy and wheat straw [16].

In the present work better yield and bioefficiency of *P. citrinopileatus* was seen when they were cultivated on paddy straw mixed with other agrowastes than paddy straw alone. This may be due to presence of various macro and microelements in the brassica straw, radish leaves, pea pod shell and cauliflower leaves which could have promoted the growth of the mushroom and ultimately yield and biological efficiency.

Moisture content *per se* may not be of any nutritional significance but it considerably influences the nutritional value of mushroom fruit bodies. In the present work the moisture content of the mushroom have been found to be 87.84% to 90%. Crisan and Sands, and Bano and Rajarathnam reported moisture content of fresh cultivated mushroom between 90 to 94% [17, 18]. The oyster mushroom *P. sajor-caju* when cultivated on paddy straw mixed with vegetable wastes had better nutrient contents than paddy straw alone. This is because of the fact that vegetable wastes are rich in minerals and vitamins. The composition of these substrates affects the nutritional value of mushroom fruit bodies. The mushroom mycelia secret extracellular enzymes which play key role in the degradation of substrates and which in turn affect the growth, development and nutritional value of fruiting bodies. In the present work it was observed that mushroom cultivated on paddy straw and other agrowastes mixed substrates are rich in qualitative

and quantitative protein. This is reflected from the higher amount of six amino acids i.e. leucine, isoleucine, valine, threonine, methionine and phenylalanine and higher amount of protein contents.

Table 3: Amino Acid content of *Pleurotus citrinopileatus* on different combinations of agrowastes.

Substrates	Leu (mg/100g)	Ile (mg/100g)	Val (mg/100g)	Thr (mg/100g)	Met (mg/100g)	Phe (mg/100g)
100% PS	0.535	0.580	0.930	0.700	0.321	0.598
30%BS+70%PS	0.600	0.722	0.995	0.725	0.325	0.635
30%PP+70%PS	0.635	0.695	1.010	0.865	0.375	0.655
30%CF+70%PS	0.625	0.600	*1.033*	0.825	0.495	0.665
30%RL+70%PS	0.725	0.695	1.035	0.875	0.500	0.695
20%BS+80%PS	0.595	0.700	0.935	0.700	0.300	0.600
20%PP+80%PS	0.615	0.655	1.000	0.800	0.325	0.635
20%CF+80%PS	0.610	0.585	1.020	0.810	0.435	0.645
20%RL+80%PS	0.700	0.680	1.000	0.825	0.475	0.685

PS = Paddy Straw, BS = Brassica Straw, PP= Pea Pod, CF = Cauliflower Leaves, RL=Radish Leaves.
Leu = Leucine, Ile = Isoleucine, Val = Valine, Thr = Threonine, Met = Methionine,
Phe = Phenylalanine. Values are expressed in mg/100g weight of dry mushroom

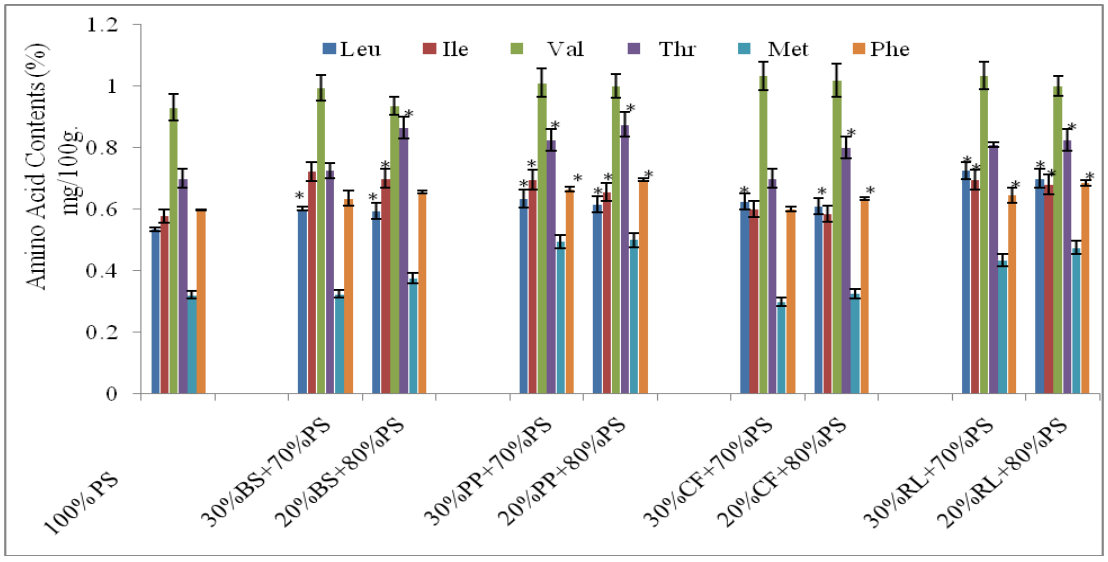


Figure3: Amino Acid content of *Pleurotus citrinopileatus* on different combinations of agrowastes.
PS = Paddy Straw, BS = Brassica Straw, PP= Pea Pod, CF = Cauliflower Leaves, RL=Radish Leaves.
Leu = Leucine, Ile = Isoleucine, Val = Valine, Thr = Threonine, Met = Methionine,
Phe = Phenylalanine. Values are expressed in mg/100g weight of dry mushroom

CONCLUSIONS

The observations of present investigation suggest that the edible mushroom *Pleurotus citrinopileatus* grown on paddy straw mixed with brassica straw, pea pod shell, cauliflower leaves and radish leaves gives fruit bodies with enhanced protein, sugar and amino acid content. Besides, these substrates also support better yield performance and biological efficiency. The three vegetable wastes i.e. pea pod shell, cauliflower leaves and radish leaves used in the present investigation are generated from every households and vegetable markets in large quantities. These wastes are generally left to rot in situ in many cities of India causing outbreak of many diseases. It pollutes the environment and causes environmental degradation. These wastes can be

utilized as resources for mushroom production with improved nutriceuticals. This can be used as an effective weapon against malnutrition particularly in those regions of the world where malnutrition related diseases and deaths are common.

ACKNOWLEDGEMENT

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THE IMPACT OF WHEAT VARIETIES AND FUNGICIDE APPLICATION, DURING WHEAT CULTIVATION, ON *PLEUROTUS* GROWTH ON STRAW

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ABSTRACT

Several wheat varieties are grown in Israel according to their different features. Fungicides for plant protection are applied when rust disease is expected.
Wheat straw was taken from an experiment plot that studied the impact of the fungicide Cyproconazole on the performance of 15 wheat varieties. The impact of fungicide application to the wheat, on *Pleurotus* growth on the straw was found to be variety dependent. Significant differences were found between fungal growth on straw of different varieties.
Fungal mycelium growth was harmed on straw from fungicide treated plots, of 7 wheat varieties. Mushroom yields were higher on straw from untreated plots, of the 2 tested varieties. Therefore wheat straw suitability for mushroom cultivation is not obvious. Quality control of wheat for mushroom production should include: The impact of wheat variety and fungicide treatments during wheat cultivation.

Keywords: Fungicides, Wheat varieties, Mushrooms, Straw.

INTRODUCTION

Fungicides are frequently applied during wheat cultivation mainly when rust disease is expected, to prevent damage the wheat crop.
Since wheat straw is widely used as *Pleurotus* substrate, it was important to study the impact of different varieties and fungicide application on the mushroom cultivation process It was demonstrated, that fungicides and growth regulators, application to wheat can have impact on straw structure [1,2] and retard mushroom mycelium growth [3].The production of *Pleurotus* mushrooms increased, in recent years, and it is now the third among mushroom species in the amounts of world-wide production. This fact is due to their taste, nutritional and medicinal values, and a wide variety of strains compatible for different climate (temperature, relative humidity etc.) conditions. Although, *Pleurorus* mushrooms are cultivated on wide range of lignocellulose substrates, selection of most suitable substrates is essential for good mushroom yields [4]. When wheat straw is used as substrate for *Pleurotus* cultivation, it is recommended to use straw that contains a lot of stems and less leaves (rich in lignin) [5]. Israel, although its small dimensions has a wide range of different climatic regions, with different amounts of annual precipitation. Therefore a wide range of wheat varieties are cultivated by the farmers. These varieties differ in their straw quality and in the use of fungicides during their cultivation. The purpose of the present study was to develop tools for selection of suitable straw source according to wheat variety and cultivation conditions, including the use of fungicides, during wheat cultivation.

MATERIALS AND METHODS

The study included 15 wheat varieties, some were commercial (names) and some were in trial process (numbers) grown in an experimental station in northern Israel. Two treatments were given to each one of them: The systemic fungicide Cyproconazole (Syngenta100mg/l) and untreated control. The fungicide was applied (400ml/hectare) two months after wheat germination. Wheat plots were harvested and grains and straw yields were measured. Straw samples were collected from each plot and were analyzed for pH and humidity, ash and total nitrogen content. For mushroom growth studies straw samples were chopped and their humidity was adjusted to 72% (w/w). For mushroom linear growth, glass Petri dishes were filled with 50 gr. chopped straw each, and pasteurized at 75^0C for 200 min. Plates were inoculated by commercial spawn of *Pleurotus ostreatus* (Mycellia 2140), by 4 grain spawn on the plate center. The plates were incubated at 25^0C and every 3 days colony diameter was measured. The thickness of the colony mycelium was visually observed and recorded on a scale of 1-5 (5=most thick). As a criterium for the strength of mycelium growth on the straw the colony diameter was multiplied by the thickness. The used substrate was analyzed for pH, humidity, ash and total nitrogen content. For fruit bodies production 410 gr. bags of the pasteurized chopped straw (of the wheat strains Bar-nir and Galil) was inoculated by 6% spawn (Mycellia 2140), and incubated. Mushroom yield was harvested and calculated as biological efficiency (BE = fresh mushroom yield to dry substrate).

RESULTS AND DISCUSSION

The effect of wheat varieties and fungicide treatment (during wheat growth) on *Plearotus* mycelium growth on wheat straw was found to be: Wheat variety dependent.

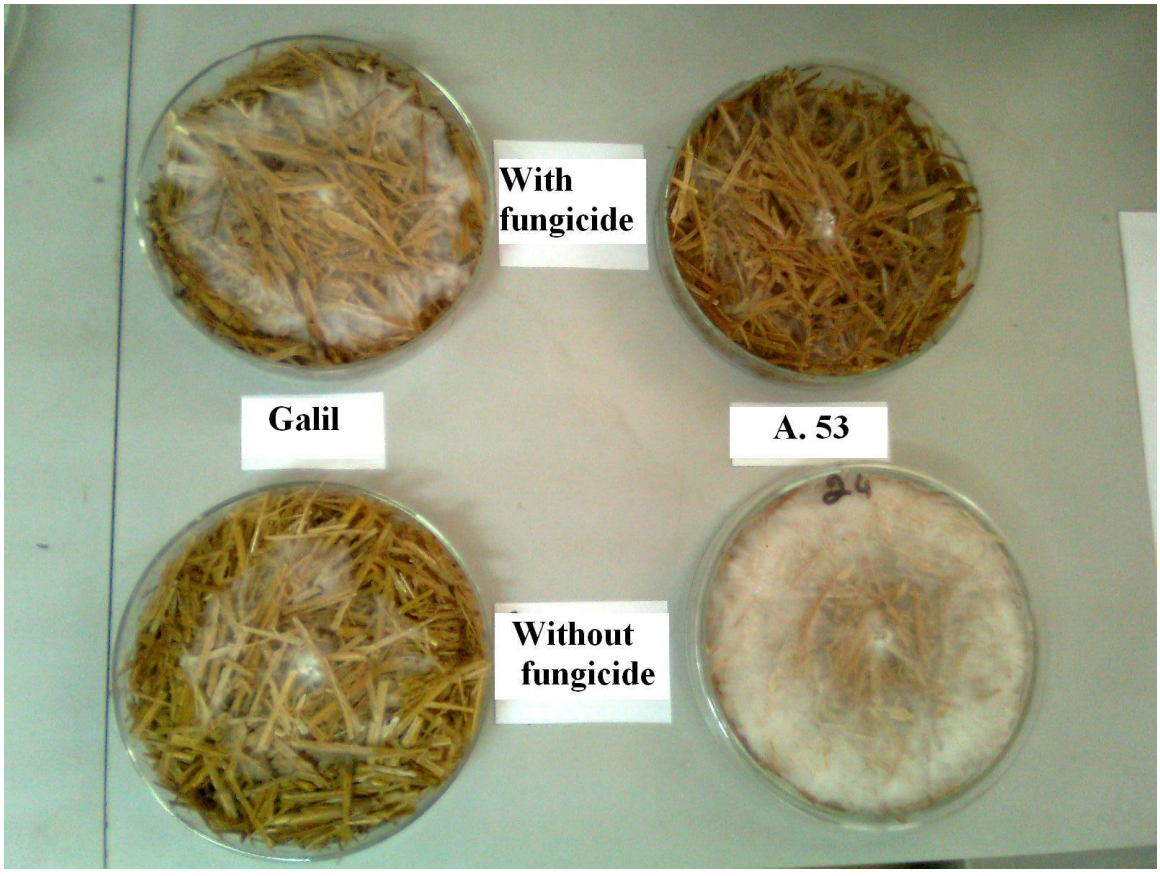

Figure 1: The impact of fungicide treatment on mycelium growth on straw of two wheat varieties

In fig.1 it is shown that fungicide application to wheat, had opposite effect on fungal mycelium growth on straw of two different wheat varieties.

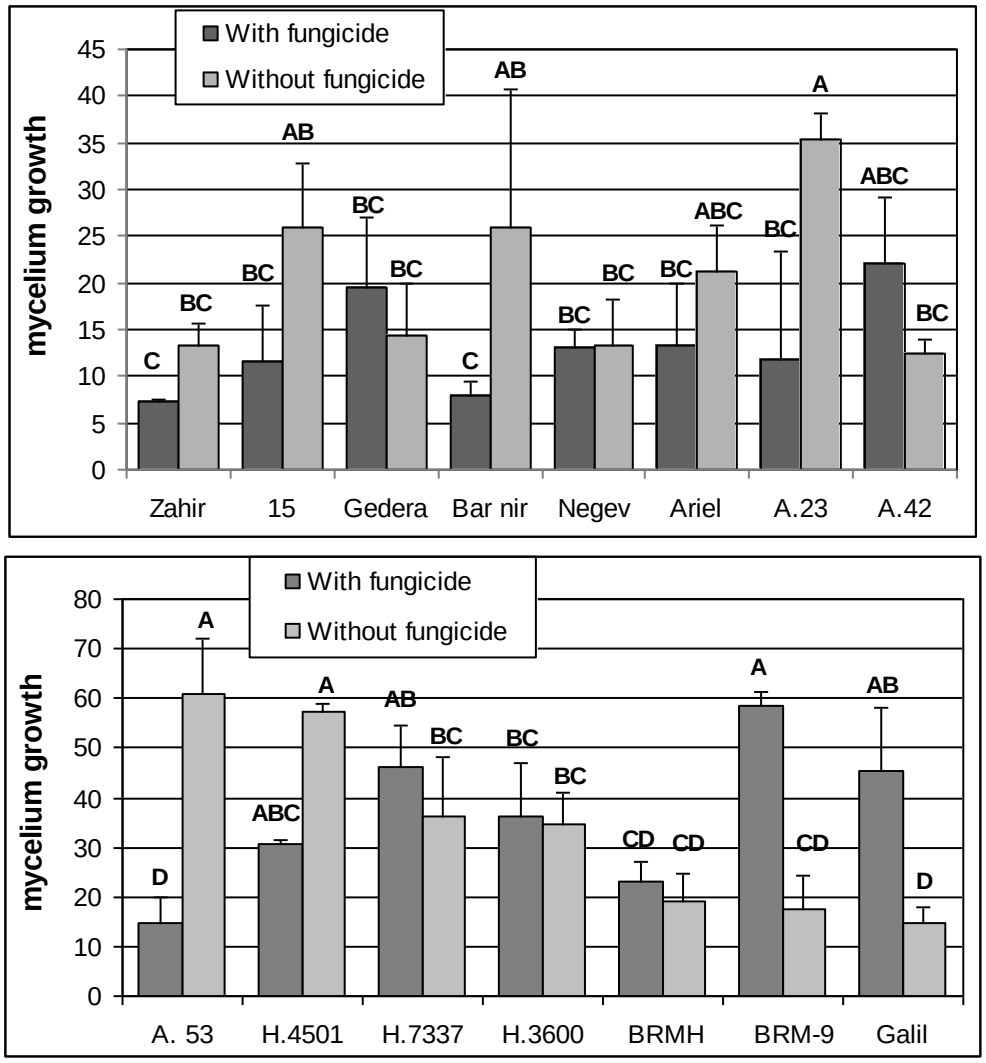

Figure 2: Growth of *Pleurotus* mycelium on straw of 15 wheat varieties

The best mycelium growth was found on straw of the varieties A53 and H4501, untreated with fungicide. For 7 wheat varieties stronger mycelium growth was found, on straw from the untreated (with fungicide) plots.

For 5 varieties stronger mycelium growth was found on wheat from fungicide treated plots. On straw of the other 3 wheat varieties no significant differences were found between the two treatments. Significant differences were found between fungal growths on straw of different varieties.

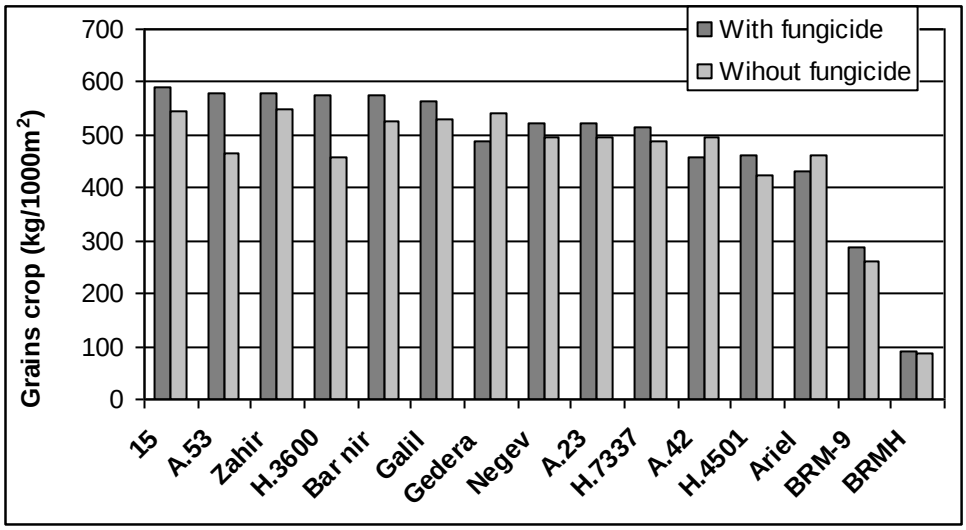

Figure 3: Grain yields of wheat varieties (2010 season)

Wheat grain yields were higher for 9 varieties with the fungicide treatment, while for 6 wheat varieties higher yields were recorded without the fungicide treatment

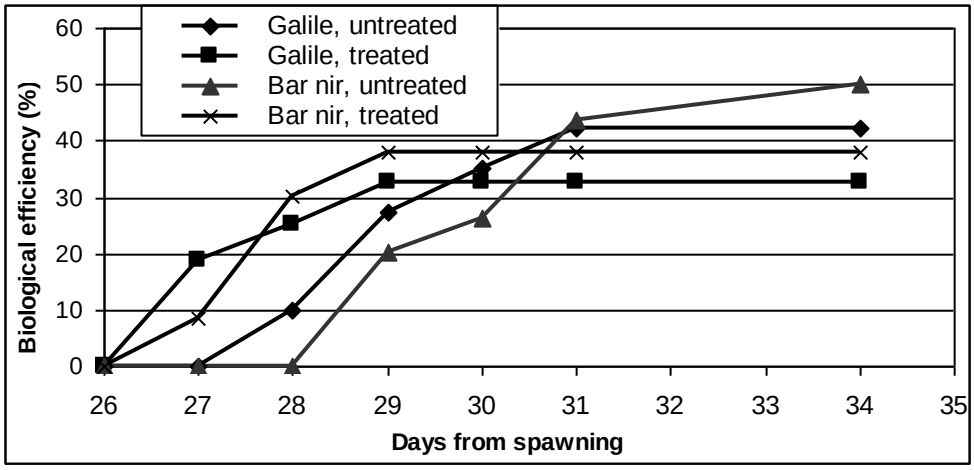

Figure 4: Mushroom yield calculated as Biological Efficiency (BE), on straw of two wheat varieties (Galile and Bar-Nir, treated and untreated with fungicide.

With these two varieties (Galile and Bar Nir), mushroom yield BE, was higher on non sprayed (with fungicide) straw, although the differences were not significant.

Table 1: The effect of *Pleurotus* growth on the composition of wheat straw of Galile and Bar Nir varieties treated and untreated with fungicide.

Treatment	Beginning of experiment			End of experiment		
	pH	Total N (%)	Ash (%)	pH	Total N (%)	Ash (%)
Galile, untreated	5.8	0.81	11.68	4.69±0.05	0.95±0.06	18.80±0.99
Galile, treated	5.66	0.93	15.93	4.97±0.35	0.90±0.03	17.40±0.54
Bar nir, untreated	5.9	1.04	19.61	4.82±0.37	1.15±0.06	21.25±1.34
Bar nir, treated	6.04	0.94	13.64	4.69±0.01	1.01±0.05	24.46±0.75

The differences in chemical composition of the straws, before and after fungal growth, between the fungicide treated and untreated straw of two wheat varieties are presented in table 1. Ash content in the straws increased during fungal growth. The effect of fungicide treatment on ash content was different between the two wheat varieties. Galile straw ash content increased more in the untreated straw. Bar Nir straw ash content increased more in the fungicide treated straw. These results showed that the effect of fungicide treatment on straw quality for mushroom cultivation was variety dependent.

CONCLUSIONS

The interactions between wheat variety and fungicide treatment, during wheat growth can impact straw suitability for mushroom cultivation. Mushroom growth on straw varies according to straw quality. The wheat variety and cultivation conditions are important factors in straw quality. For some wheat varieties, straw suitably as a source for *Pleurotus* substrate could be harmed by fungicide treatments, during wheat growth.

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PASTEURIZATION OF SUBSTRATE FOR GROWING *PLEUROTUS OSTREATUS* BY SELFHEATING

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ABSTRACT

Substrate to grow *Pleurotus ostreatus* must be pasteurized in order to be used for commercial production of Oyster mushrooms. The pasteurizing treatment normally involves steam application during a variable time/temperature procedure. In this research we studied an alternative treatment by forming a substrate compost pile with sufficient size and moisture to generate the necessary heat for pasteurization. A 1 m^3 wooden box with 80 kg dry Pangola grass *Digitaria decumbens* + 2% lime ($Ca(OH)_2$) was adjusted to 55, 60, 65 and 70% moisture. The composting treatment lasted 48 h; subsequently the substrate was cooled to ambient temperature and spawned. The treatment was efficient in preventing other organisms like flies, bacteria and competitor fungi. After two flushes of mushrooms, the biological efficiency was between 70.5 and 88.1% when compared to the control (same substrate steam pasteurized 90ºC, 1 h).

Keywords: Oyster mushroom cultivation; Substrate treatment; *Digitaria decumbens*; Composting

INTRODUCTION

Several treatments are used to prepare substrate for growing Oyster Mushrooms *Pleurotus* spp, principally pasteurization (steam, hot water), alkaline soaking and composting, among others [1, 2]. As part of a strategy to support small mushroom growers, the pasteurization process has been studied in order to replace traditional steaming pasteurization with a simpler and less costly treatment by avoiding energy expenses. For this purpose, we used the self-heating (reduced compost) method already tested by Villa et al. [3] and improved by Hernández *et al.* [4] and Barrios Espinoza *et al.* [5]. These authors reported that inducing a short composting process in a substrate pile rendered possible the use of self-generated heat to inhibit the development of non-desired organisms detrimental to *P. ostreatus* cultivation. In this study, we researched the effect of substrate moisture on substrate temperature profile during the pasteurization process. Subsequently, by using the best treatment we made supplementation tests to improve Oyster Mushroom production.

MATERIALS AND METHODS

Strain. *Pleurotus ostreatus* ECS-1123 from the fungal culture collection of El Colegio de la Frontera Sur was used since it is a strain with already tested commercial quality [5].

Substrate treatment. Self-heating: dry and coarsely ground Pangola grass *Digitaria decumbens* (80 kg, 3-5 cm long particle size) plus 2% lime ($Ca(OH)_2$ was subjected to an incomplete composting process (48 h long) as recommended by Barrios Espinosa *et al.* [5] in a $1m^3$ wooden crate. Substrate moisture was adjusted to 55, 60, 65 and 70% in order to determine the effect of moisture on the temperature profile of the pile. At 24 h incubation, the pile was tumbled and mixed once to provide aeration. At 48 h, mixing and aerating the substrate to a lower temperature (25ºC) stopped the composting process. The best treatment selected was the one that provided enough heat to the substrate during the process to reach at least 50ºC during no less than 10 h in any part of the pile. According to Overtjins [6] this time/temperature treatment should be sufficient to kill most bacteria and mushroom competitor microorganisms.

Steam pasteurization was done by providing the necessary steam to reach 90ºC during an hour inside a mass of 75 kg substrate (Pangola grass, 65% moisture and 2% lime).

Supplementation treatments. Soybean flour, ground cowpeas and wheat bran were used as supplements, according to Table 1. In all cases 6% supplement was added to the self-heating pasteurized substrate (65% moisture) at the time of spawning.

Table 1. Mixtures used to supplement the substrate (Pangola grass) after self-heating pasteurization, at the time of spawning *Pleurotus ostreatus* ECS-1123. Supplementation rate 6%.

Treatment number	Supplementation		
	Soybean*	Cowpeas	Wheat bran
T1	1	0	0
T2	0	1	0
T3	0	0	1
T4	0.67	0.17	0.17
T5	0.17	0.67	0.17
T6	0.17	0.17	0.67
T7	0	0.5	0.5
T8	0.5	0	0.5
T9	0.5	0.5	0
T10	0.33	0.33	0.33

* Numbers indicate fraction of 6% supplementation rate per treatment.

Cultivation. After pasteurization, the substrate was spawned at a rate of 3% dry weight basis with sorghum spawn [7]. The supplement was added by mixing simultaneously while spawning and then 3.0 kg of substrate was placed in polyethylene bags (30X40 cm) with 6 repetitions per treatment. Incubation lasted 17 days at 25ºC initially, although incubation temperature was changed as explained later. After full colonization, in order to induce fruit body formation the polyethylene bags were discarded and fresh and moist air was introduced to maintain 85-90% relative humidity and CO_2 content in the air below 800 ppm. The mushrooms were harvested at maturity, when the caps were fully extended and, just before the pileus border rolled up. Two flushes of mushrooms were obtained. The comparison steam/self-heating pasteurization was made between substrates pasteurized by one of those methods and without supplementing. Twenty bags per treatment were used in this case.

Sampling. From the center of the compost pile, 100g of substrate were taken at the beginning and at the end of the composting process to determine pH, moisture content and main contaminants.

Parameters. For pH, ten grams of substrate taken from the center of the compost pile was added to 90 ml distilled water and then agitated. pH value was measured with a pH/ISE Orion meter model 710A. For moisture determination in substrate**,** five grams of substrate were taken from the center of the pile and placed in a thermobalance Moisture analyzer A&D M.F. 50.

For temperature measurements, the readings were taken directly from the compost pile every three hours: three holes on the wooden box front side, situated at 15 cm below the upper substrate surface, at the center of the pile and 15 cm above the bottom level were used to insert 30 cm long bimetallic lab thermometers.

The Biological Efficiency (BE) was determined according to the formula:

$$BE = (X/S)*100$$

where X is the weight of harvested fruiting bodies and S the dry weight substrate

Microbial contamination was evaluated by two methods: 1) from the compost pile used for the evaluation of supplementation mixtures, 150 g of substrate was taken from the center of the pile at the end of the pasteurization process. Approximately 15 g sample was placed aseptically in each of 10 sterile Petri dishes. The plates containing the sample were incubated at 25ºC for 15 days to search for microbial contaminants. 2) all synthetic logs spawned (60 bags) were observed at the end of the incubation period (15 days after spawning) for contamination spots. The contaminated area for each bag was estimated (%), and then an average was calculated for each treatment (6 repetitions per treatment).

Experimental design and statistical analysis. Three compost piles were made for each moisture level reported and the mean temperature was used to monitor the temperature increase in the substrate (Fig.1). For the supplementation experiments, a randomized design with 10 treatments and 6 repetitions was used. An analysis of variance (ANOVA) and a mean separation by Tukey´s test were performed ($\propto$=0.05). The statistical package JMP 4.0 from SAS (SAS Institute Inc. Cary, NC, USA, 1998) was used.

RESULTS AND DISCUSSION

Compost crate temperature profile. Figure 1 shows the temperature profile of substrate with various moisture contents. The pile with 55% moisture (1a) presented the lowest temperature profile of all four treatments tested. It was observed there that the substrate upper layer did not reach 50ºC, the medium layer stabilized between 40-45ºC after 16 hours incubation and the lower layer stabilized around 35ºC and had presented a maximum of 40ºC after 60 h incubation. The pile with 60% moisture (1b) reached 55ºC in its substrate upper layer after 24 h incubation and maintained around that temperature until the end of the 48 h process; the medium layer showed very close temperatures to the upper layer and the lowest layer in this pile reached 50ºC

after 36 h incubation and maintained this temperature for 3-4 h, then decreased slowly. In the pile with 65% moisture (1c) the upper substrate layer reached 50ºC after 26 hours incubation, same as the medium layer, and the lower layer reached 50ºC after 34 h incubation and remained there up to the end of the composting process. The three layers of pile "d" (70% moisture, 1d) reached at least 50ºC in 40 h incubation but the lower layer stabilized around 45-48ºC after 40 h incubation.

Overtjins [6] indicated that a treatment at 46ºC during 1 h was enough to kill cecids; 50ºC during 10 minutes and 4 h prevented the development of bacterial blotch and cobweb respectively. He also pointed out that a few hours at 55ºC or 60ºC were necessary to kill other bacteria and fungi frequently encountered in white button mushroom compost. It should be mentioned that *Agaricus bisporus* is a mushroom needing a very selective substrate in order to grow optimally. However, *Pleurotus ostreatus* grows well on a less selective substrate with low simple carbohydrates, low nitrogen content, high lignin content and being slightly alkaline. That is why using a grass like Pangola + lime and a temperature of 50ºC during 10 hours may be enough to produce Oyster mushrooms.

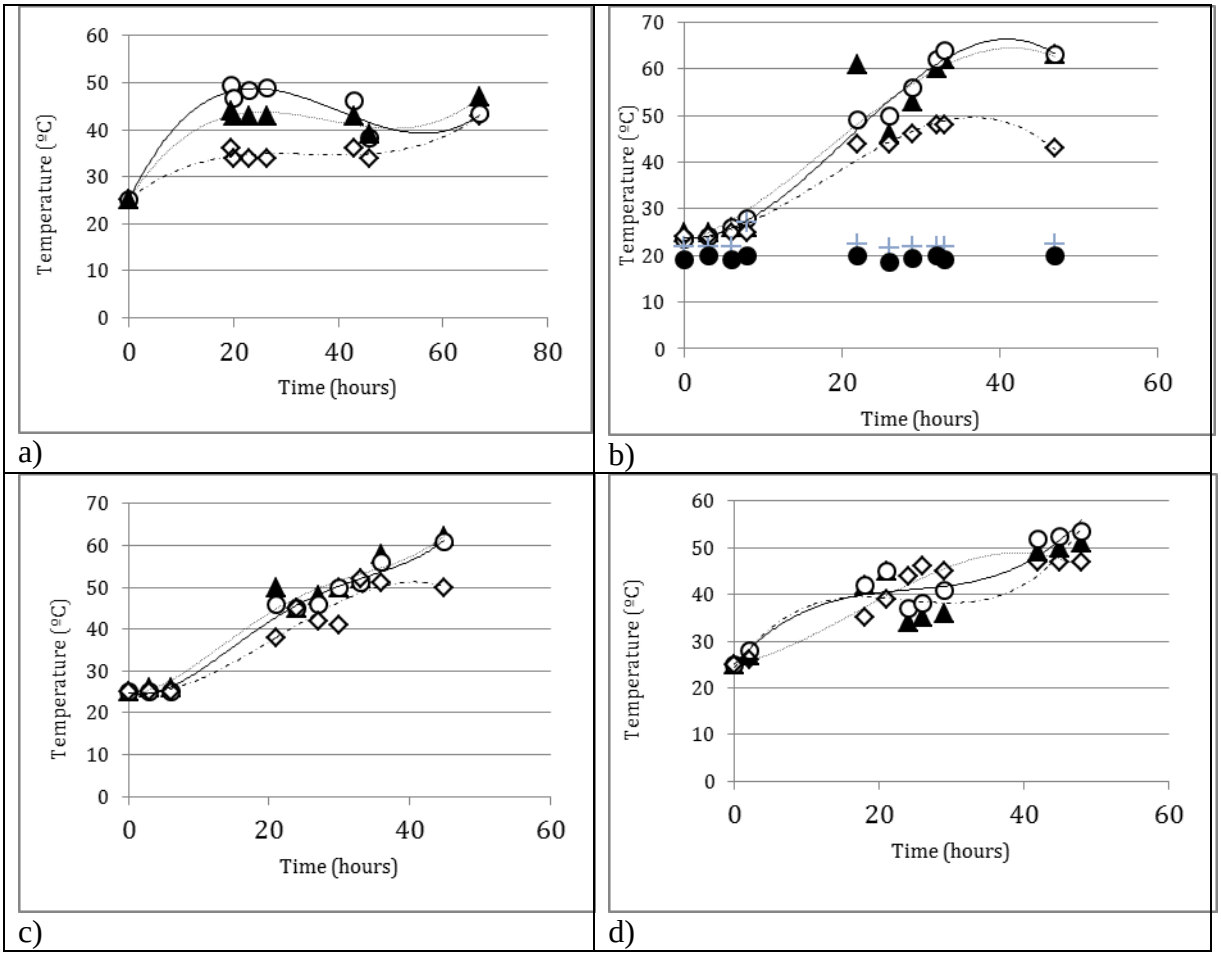

Figure 1. Temperature profile of 80 kg (d.m.) Pangola grass + 2% lime composted during 48 h in a 1m^3 wooden box, at four different moisture contents: a) 55%, b) 60%, c) 65%, d) 70%. Δ = upper layer; circle= medium layer; rhomb= lower layer; + = wet air temperature; black circle= dry air temperature.

According to the profile observed, and the need to avoid the growth of different wild organisms taken from the field in substrate (larvae and fly eggs, mites, other insects, nematodes, various fungi and bacteria), substrate piles with 60 and 65% moisture performed better since medium and upper layers reached temperatures above 50ºC in 24 h; however, only the 65% moisture pile allowed its lower layer to maintain 50ºC for 10 h. Thus the 65% moisture pile allowed a time/temperature treatment providing enough heat to kill or inhibit contaminant microorganisms. Certainly, the combined action of temperature and alkaline pH (initial pH was 9.5, data not shown) in the substrate after composting helped to inhibit most competitor organisms. The pH at the end of composting was slightly alkaline (7.5-8), allowing *P. ostreatus* spawn to develop in its selective substrate, while impeding the growth of other possible surviving fungi like *Trichoderma* spp. The absence of contaminant organisms was observed in all samples taken from the substrate and incubated in Petri plates: nor larvae nor adult flies nor pathogenic mitosporic fungi were found. Although these organisms were not added to the raw substrate, they normally come within the substrate from the field [2, 8]. On the other hand, looking at the temperature profile of each substrate layer it remains clear that substrate tumbling is important and necessary in order to aerate and provide a homogenous heat treatment to the substrate.

Self-heating against steam pasteurization. Substrates prepared by self-heating or steam pasteurization were compared by spawning strain ECS-1123 after pasteurization. The biological efficiency obtained after two harvests was 69.8±17.5% for the self-heating process and 75.6 ± 10.2% for the steam pasteurization. Statistical analysis did not find significant differences between these two treatments. These results confirm those obtained for the same strain by Barrios Espinoza et al. [5] whereby no difference between steam and self-heating pasteurization were obtained. However, these results differ from those obtained by Contreras et al. [2], as they found that steam pasteurization and alkaline disinfection were statistically better than self-heated compost, when using strain ECS-0152 of *Pleurotus ostreatus*. Although different strains were used in both cases, the contrast in findings may be due to the fact that Contreras at al., *op cit*. used a substrate with 70% moisture and a smaller crate (50x50x50 cm^3) for composting. Certainly, the temperatures attained inside the substrate were lower than those in the crate we used (1 m^3 crate, 65% moisture). Probably due to these two differences (moisture and mass), their pasteurization treatment became less effective (less self- generated heat).

Temperature profile in supplemented substrate during spawn running. A second trial was carried out to test the use of different supplementation mixtures (Table 2) in a self-heated substrate. After supplementing and spawning, all 3.0 kg substrate bags were incubated at 25ºC. The temperature at the center of each bag (Fig. 2) increased to reach after two days incubation, an average of 34ºC, and then decreased to 26ºC on the third day. This was followed by temperature increasing again and reaching an average of 32ºC. Because of this substrate temperature rise, the air temperature in the incubation room was set to 21ºC in order to avoid a further increase in substrate temperature resulting in the death of fungal mycelium. This change in air temperature allowed controlling substrate temperature, which was stabilized at approximately 25ºC by the end of the spawn run period. From these results it remains clear that temperature control is of utmost importance if substrate supplementation is required. This

conclusion confirms previous results [9, 10] indicating that supplementation increases yield but a temperature rise may occur during spawn run.

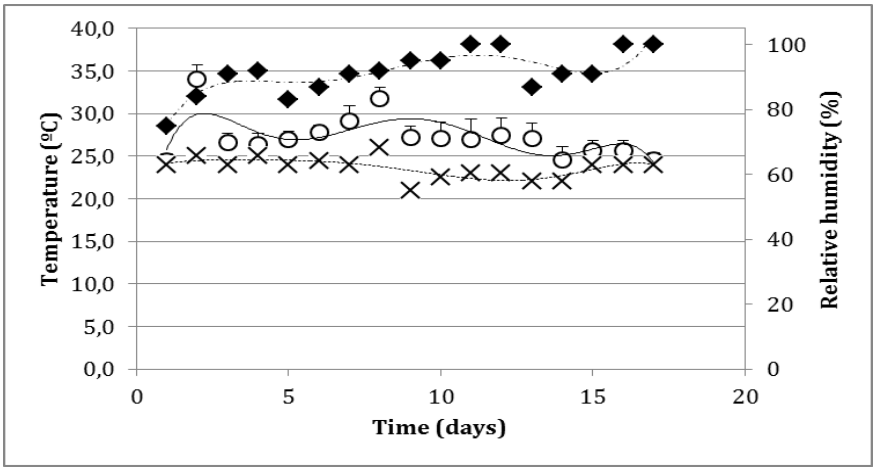

Figure 2. Average temperature profile of Pangola grass substrate supplemented with several mixtures of cowpeas, soybean meal and wheat bran (Table 1), during incubation at 25ºC (initially) then 21±1ºC. Mean of 10 treatments. Relative humidity (Rhomb), mean temperature of substrate at the center of bags (circle), air temperature (X).

Table 2. Presence of contaminated spots after colonization of substrate by *P. ostreatus* at 25ºC in each out of 6 repetitions spawned.

Treatment	Contamination (% of exposed area)						
	r_1	r_2	R_3	r_4	r_5	r_6	Mean (%)
1	0	0	0	0	25	0	4.1
2	0	5	0	0	0	0	0.8
3	0	0	0	0	0	0	0
4	0	0	0	0	0	2	0.3
5	0	0	5	0	0	0	0.8
6	1	0	0	0	0	2	0.5
7	0	1	0	0	0	0	0.2
8	2	25	0	0	0	0	4.5
9	2	0	0	0	0	0	0.3
10	0	2	0	0	0	0	0.3

Yield. Figure 3 shows the biological efficiency of *P. ostreatus* ECS-1123 grown on Pangola grass substrate pasteurized in a self-heated compost crate and supplemented with 10 different mixtures of soybean, wheat bran and ground cowpeas. Values varied between 70.5% (T_9) and 88.1% (T_4). The statistical analysis did not demonstrate any differences between treatments.

This result is lower than those reported [5] when cultivating the same strain on non-supplemented Pangola grass (110%). However, the authors of this report harvested three flushes of mushrooms while our results took only two flushes into account.

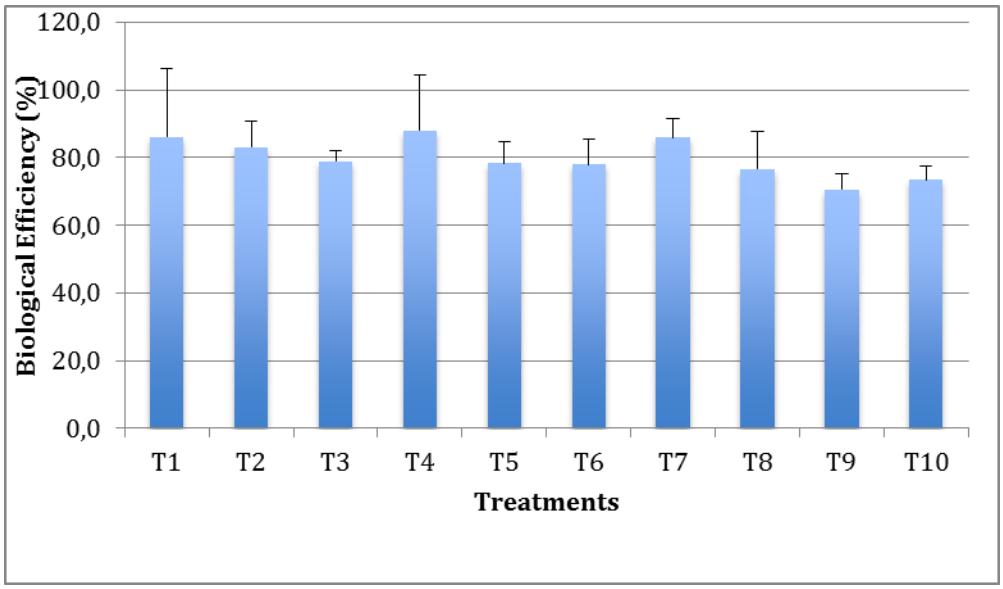

Figure 3. Biological efficiency after two flushes of *P. ostreatus* ECS-1123 on Pangola grass pasteurized by self-heating and supplemented with different mixtures of soybean, wheat bran and ground cowpeas. Incubation temperature 25ºC.

Contamination. After self-heating pasteurization, just prior to spawning, a substrate sample was aseptically taken and placed in ten Petri plates. The plates were incubated for 15 days at 25ºC to observe the appearance of microbial contaminants in the substrate. After the incubation period, it was observed that none of the plates showed presence of neither unsafe organisms nor microorganisms. The absence of contaminants or competitor fungi on the pasteurized Pangola grass suggested that at the place where the substrate sample was taken (the center of the substrate pile), the pasteurizing process was successful. It must be pointed out that temperature at the core of the compost pile (50 cm deep, in the middle of the crate) should be higher than the one reported by our thermometers (30 cm long). For future studies it would be helpful to take samples from other sectors of the compost pile, mainly near the bottom of the crate to evaluate the pasteurization treatment in the entire substrate. Table 2 shows the incidence of contaminants in substrate bags after substrate colonization by the mushroom, just prior to harvesting. Forty nine out of 60 bags spawned were totally free of contamination (82%), nine bags (15%) showed small spots with no mycelium growth in a range of 1-5% exposed surface and two bags (3.3%) were severely contaminated showing contamination spots of about 25% of exposed area. Regarding the exposed area, the total non-colonized area for all bags represented 11.8%. All but one bag showed bacterial contamination due to moisture excess in the substrate and only one bag (Treatment 1, r_5; 25% contamination) was contaminated by *Trichoderma* sp.
Based on the spreading pattern we could suggest that contamination should be decreased by a better substrate mixing to better homogenize moisture and by improving prophylactic measures after spawning.

The pasteurization method by self-heating discussed here has been successful in producing mushrooms. The use of Pangola grass as raw material with 65% moisture and 2% lime

[$Ca(OH)_2$] is suitable for self-pasteurization. The method did not decrease mushroom production and may well be considered as an ecological pasteurization treatment because it uses lime (an input allowed for organic growers) and its self-generated heat from the compost pile to provide a substrate thermo-treatment. It does not require an external energy source for pasteurization. However, the method is not yet ready to be recommended to mushroom growers: whether it is later commercially used or not remains unclear; nevertheless, it opens up new alternatives for future research. It must be further studied to confirm that after repeatedly use, certain thermophilic fungi and/or actinomycetes resistant to high temperature may affect production. On the other side, the use of more thermo-resistant materials to provide better insulation thus reducing heat losses during the process is another research alternative.

ACKNOWLEDGMENTS

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EFFECT OF BACTERIAL AND CYANOBACTERIAL CULTURE ON GROWTH, QUALITY AND YIELD OF *Agaricus bisporus*

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ABSTRACT

In the mushroom growing process, *Agaricus bisporus* function of casing soil is to provide an environment for fruit formation. Presence of *Pseudomonas* species in casing soil is important for mushrooms formation and development. They can represent up to 50 percent of the total bacteria. The result of this study showed that total number of bacteria and population of pseudomonas species increased dramatically after casing. The maximum number of bacteria was recorded at the primordia formation stage. Population of pseudomonas in casing soil was increased with addition of bacterial inoculum to the casing soil. There was a significant difference of mushroom yield as compared to the control.
Until now most research and applications of cyanobacteria have been conducted with green plants growing. Findings of this research strongly supported that the production of promoting substances such as auxins, sugars and vitamins by the algae may be partly responsible for the greater mushroom growth and yield. Inoculation of cyanobacteria into the casing soil significantly increased mushroom yield and quality.

Keywords: *Agaricus*; *Pseudomonas*; Primordial; Cyanobacteria; Phytohormones

INTRODUCTION

Following colonization of mushroom mycelia in pasteurized compost, a 1.5 inch layer called casing soil is applied on top of the compost surface. The casing soil enhances the retention of irrigation water on production beds and promotes mushroom fruit body formation. An important process during mushroom growing is pinning. Addition of casing is necessary for shifting the vegetative phase to the reproductive phase by pin formation. The casing soil supports an active, aerobic bacterial flora [2] among them fluorescent *Pseudomonas* spp. play an important role in initiation of pinning and mushroom fruit body development. There is a controversy regarding populations of pseudomonads in casing soil. Samson [12] demonstrated that fluorescent pseudomonads may represent up to 50 percent of the total bacteria in casing samples, whereas Doores et al. [1] indicated that they represented only 2 percent of the total casing bacteria. Miller et al. [7] showed that populations of casing bacteria changed over the *A. bisporus* growth cycle. *Pseudomonas putida* has been identified as an important species involved in fruiting body initiation [3, 10]. Inoculation of bacterial culture on mushroom growing media, besides the effect on pin formation, caused an increase of mycelial growth rate up to 1.6 fold, promoted the rate of radial hyphal extension, and suppressed the frequency of branching [6].

Soil is a habitat of some terrestrial blue-green algal species that are beneficial organisms for soil fertility by fixing atmospheric nitrogen, binding soil particles, helping to maintain moisture and preventing erosion. These bacteria supply substances that promote the growth of plants. There are many reports that cyanobacteria produce phytohormones such as cytokinin, auxin and auxin-like substances in soil [5, 13, 14, 15, 16]. Other plant growth regulator (PGR) substances such as amino acids, sugars, vitamins that may have a positive influence on growth of vascular plant are produced by cyanobacteria [5, 8, 9]. Until now most research and applications of cyanobacteria have been conducted with green plants. This is a first attempt to study the effect of cyanobacteria on mushroom. Moreover, cyanobacteria may enhance production of secondary metabolites. These phenomena may be controlled with or mediated by hormones [11, 13]. Cyanobacteria are another group of microorganisms that might have a positive effect on mushroom yield.

MATERIALS AND METHODS

In this work two experiments were conducted. The first study concerned the bacterial population in casing soil and effects of *Pseudomonas patuda* used as bacterial inoculum, on yield and quality of mushroom. A similar objective was followed in the second trial but algal culture was used for inoculation.

Experiment 1. Isolation and cultivation of *Pseudomonas* species from casing soil. After colonization of compost with mushroom mycelium, the substrate was covered with casing soil. Sampling was carried out in Malard mushroom research farm. To determine *Pseudomonas* population, casing soil was sampled periodically until primordia formation. Specified amount of the dilutions transferred on to sterile plates of nutrient agar (NA) and Chromagar Pseudomonas (PS822) media. Inoculated plates were incubated at 30^{o}C for 24h. Colonies that appeared at the end of incubation were counted, the unit expressed in terms of colony-forming units per gram (CFU/g) of original sample. The isolates were further subjected to standard biochemical tests. Bacterial identification of isolates was carried out by comparing the results obtained with *Bergey's Manual of Determinative Systematic Bacteriology*. The predominant *Pseudomonas* species isolated from casing soil was tested to study the growth of mycelium and pin formation.

Inocula were prepared by growing the selective strains in nutrient broth (NB) medium. After incubation at 30^{o}C for 18h, the densities of culture were determined at OD600. Then cultures were diluted further in serum until final bacterial cell numbers were 10^8 cells/ml. Bacterial suspensions (5 L) were sprayed on 42 m^2 of casing soil at time of casing.

Protein contents of fruit bodies were calculated from the nitrogen content (N×6.25) as determined by micro-Kjeldahl method [4].

Experiment 2. Cyanobacteria cultivation. Among the cyanobacteria isolated from different paddy fields one heterocyestous cyanobacteria (Nostoc) was selected because it showed a high growth rate. Isolates were grown in BG11 nitrogen free medium in a 2 L container at 24°C and a 12/12 h light/dark cycle with artificial illumination (2000-2500 Luxes) and constant stirring and aeration. After three weeks, the culture was harvested and used as inoculum. Addition of algal culture was carried out at the primordia formation stage, before second and third flushes.

Mushroom production. The compost formulation consisted of wheat straw, chicken manure and gypsum prepared in conventional yard and pasteurization tunnels. Mushrooms were grown in controlled and standard growing room. After colonization of compost with *A. bisporus* (commercial strain Sylvan A15) mycelium, the substrate was covered with 4-5 cm of casing soil. Mushroom yield was determined over a 3-weeks production period. Mushrooms were weighted after removal of stipes.

Statistical analysis. Data were subjected to analysis of variance (ANOVA) and means were compared using LSD test ($P<0.05$ and $P<0.01$). A completely randomized design was used with four replications for each treatment.

RESULT AND DISCUSSION

The results of this study showed that the total aerobic bacterial populations ranged from 7.5 to 7.8 Log of colony-forming units (CFU)/g of casing soil while fluorescent *Pseudomonas* ranged from 6.8 to 7.2 Log CFU/g. Periodic sampling of casing soil indicated that number of bacteria increased gradually with growth of mushroom mycelium into the casing soil. With the initiation of pin formation the number of bacteria increased dramatically. The maximum number of bacteria was recorded at the first flush primordia formation stage between day 18 and day 20 (Fig. 1).

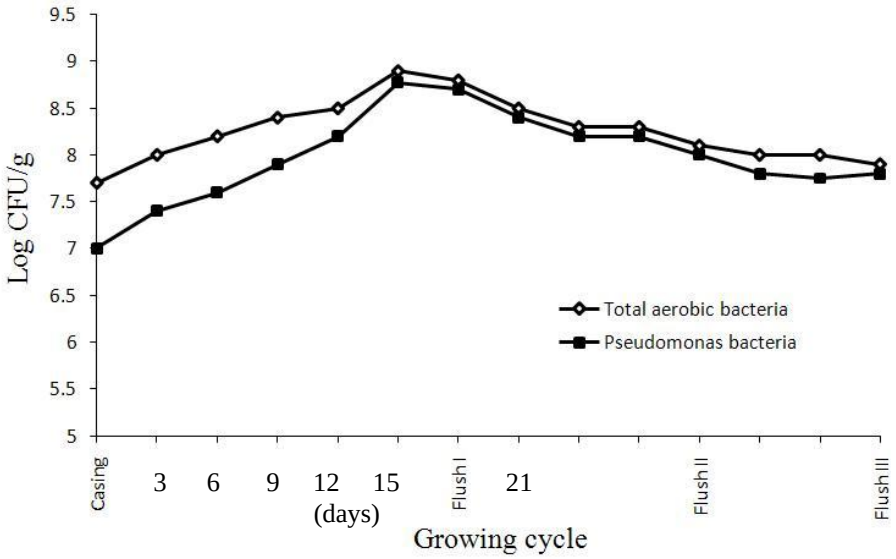

Figure 1. Total numbers of aerobic bacteria in the casing soil at different cropping stages.

Among the different strains of *Pseudomonas* isolated from soil adhering to primordia, only one was selected. Casing soil inoculated with bacterial culture (*Pseudomonas putida*) promoted faster and more uniform size of primordia. Mushroom yield increased 0.3 kg/m^2 in first break and 2.1 kg/m^2 in second break, but decreased it by 0.4 kg/m^2 in the third break. Addition of bacteria culture to the casing soil also improved quality of mushrooms. Dry matter of mushrooms increased with 0.5 percent in the first break, 0.6 percent in the second break and 0.7

percent in the third break. Protein content of treated mushrooms also increased with 3 to 5 percent in three breaks compared to untreated mushrooms (Table 1).

Table 1. Effect of bacterial culture on yield, dry matter and protein content of mushroom

	First picking			Second picking			Third picking		
Sample	Yield Kg/m^2	D.M %	Protein %	Yield Kg/m^2	D.M %	Protein %	Yield Kg/m^2	D.M %	Protein %
Control	11.8	8.2	37	7.9	7.9	34	4.8	7.5	32
Test	12.1 ns	8.7 *	40*	10 * *		8.5 * *	4.4 ns		8.2 * *
				39 * *			37 * *		
Diff.	0.3	0.5	3.0	2.1	0.6	5.0	- 0.4	0.7	5.0

* * significance < 1 % level, * significance < 5 % level, ns: not significant

Casing soil was irrigated with algal culture at the primordia formation stage, before second and third breaks. There was a significant difference in mushroom yield in three flushes treated with cyanobacterial culture as compared to the control. Mushroom yield was increased 0.661 kg/m^2 in first, 2.2 kg/m^2 in second flush and 0.053 kg/m^2 in the third flush as compared to the control. The result also showed that inoculation of casing soil with cyanobacteria had a positive effect on quality of mushroom. Dry matter content of mushrooms increased in the first flush. Dry matter was measured at 9.75 % in treated and 8.35 % in untreated mushroom. However, the amount of dry matter and protein declined in second and third flush as compared to the first flush. There was a slight difference in dry matter in treated and untreated mushroom in second and third flushes. Addition of algal culture had a positive effect on protein content of mushroom. The amount protein was increased significantly in first flush, but there was a slight difference between treated and control in second flush. Whereas, in the third flush protein content of treated mushroom was less than control (Table 2).

Table 2. Effect of algal culture on yield, dry matter and protein content of mushroom

Sample	First Flush			Second Flush			Third Flush		
	Yield Kg/m^2	D.M %	Protein %	Yield Kg/m^2	D.M %	Protein %	Yield Kg/m^2	D.M %	Protein %
Control	12.59	8.35	48.1	7.58	8.3	45.1	2.75	7.40	41.1
Test	13.2 *		9.75 * *	9.78 * *		8.6 *	2.80 ns		7.50 ns
	51.9 * *			45.5 ns			39.1 ns		
Diff.	0.61	1.4	3.8	2.2	0.3	0.4	0.05	0.10	-2.0

* * significance < 1 % level, * significance < 5 % level, ns: not significant

CONCLUSION

In cultivation of *A. bisporus*, compost and casing soil are two major elements. Studies have demonstrated that populations of pseudomonas in the casing layer on which the mushroom fruit body develops is very important. In most of the studies, total bacterial populations ranged from 8.0 to 8.5 log CFU/g casing material. With a slight difference, this result is same as other workers. The majority of bacterial population in casing was attributed to pseudomonas species. In this study they present more than 80 present of the bacterial population in the casing layer. The result showed that there is a close relation between growth of mycelium and number of bacteria in casing soil. Sampling of casing soil at different periods of mushroom growing cycle revealed that number of bacteria increased simultaneously with increase growth of mycelium into casing soil. At the pining stage, populations of pseudomonas species especially *P. putida* were important since they are playing a key role in fruiting body formation. We conclude that inoculation of native *P. putida* isolated from casing soil at the primordia formation stage will be very efficient for increasing mushroom yield and quality.

In the second experiment the application of cyanobacterial culture on casing soil and its effect on mushroom yield and quality was investigated. Irrigation of casing soil with cyanobacterial culture increased yield, dry matter and protein content of mushrooms. Production of promoting substances such as auxins, sugars and vitamins by the algae are the main factors for this issue.

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INFLUENCE OF THERMOPHILIC FUNGI *HUMICOLA INSOLENS* ON THE GROWTH OF *AGARICUS BRASILIENSIS (A. BLAZEI)*

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ABSTRACT

Cultivated medicinal mushrooms are a good source of bioactive substances that may be useful for human medicine. Some of these mushrooms are edible and contain valuable nutrients that are good for human health.

During our previous investigation of influence of thermophilic fungi *Humicola insolens* on the growth of *Agaricus bisporus*, *Agaricus bitorquis* and some other edible mushrooms we have found out that it stimulated the mycelium growth of some these tasted mushrooms. Our experiments on the interaction of *H. insolens*on with *Agaricus brasiliensis* showed that investigated thermophilic fungi stimulated the growth of this medicinal mushroom on straw agar media (SA). On this media the growth of *A. brasiliensis* on the surface of the *H. insolens* colony was twice or more faster than on the media without thermophilic fungi. Preliminary experiments on straw which was previously inoculated by *H. insolens* (grew during 3-5 days, temperature 42ºC), showed that fruit bodies of *A. brasiliensis* were produced the on obtained substrate.

Keywords: *Humicola insolens, Agaricus brasiliensis (A.blazei),* nutrition media, interaction, substrate, cultivation.

INTRODUCTION

During preparation (fermentation) of mushroom compost more than 20 species of thermophilic fungi were isolated from it [1-5]. Some of them play the main role in bioconversion of initial ingredients to selective substrate for development of *Agaricus bisporus* and some other mushrooms. Thermophilic fungi at optimum for them conditions (temperature, pH, humidity etc.) grow on different cellulose-lignin substrates [6-7].

During Phase I and II of compost fermentation and beginning of spawn run some thermophilic and mesophilic fungi are able to produce antibiotics which can protect mushrooms against negative effects of some fungi and bacteria [8-10]. They also produce some important substances for mushrooms development, like growth regulators - auxins, cytokines, gibberellins etc.; vitamins, amino acids; volatile compounds, such as carbon dioxide and are efficient in humus formation [11-13].

Our experiments showed different interactions on agar nutrition media of thermophilic fungi from mushroom compost [14] and it was found out that among them only *Humicola grisea var. thermoidea*, *H. insolens* and *Scytalidium thermophilium* (syn. *Torula thermophila),* so-called *Scytalidium* (*Torula) Humicola* complex, have been mentioned as dominant species at the end of

fermentation and beginning of *A. bisporus* spawn run [1, 4, 9]. The taxonomy and nomenclature of this thermophilic species are specified [15, 16]. The role of thermophilic fungi in mushroom compost preparation and formation of selective substrate for *A. bisporus* mycelium growth have been studied extensively [17-22]. It was found that in the nutrition media, sterilized mushroom compost and grain spawn, pre-incubated by *S. thermophilium*, *H.insolens* and some other thermophilic fungi *(Chaetomium* sp., *Myriococcum thermophilium)* promoted mycelium growth of *A. bisporus* but also of such mushrooms as *A. bitorquis, Coprinus cinereus, C. comatus, Lentinus edodes, Pleurotus ostreatus* [5, 9, 21-23].

Mushrooms are widely recognized as good sources of bioactive substances that may be useful for human medicine. Some of these mushrooms are edible and contain valuable nutrients that are good for human health. *Agaricus brasiliensis* is known by his medicinal properties (anticarcinogenic, antimutagenic, antitumour effects etc.) [24-28].

The cultivation of *A. brasiliensis* was started first in Brazil in the field (outdoors), but this type of production of this mushroom was risky because of uncontrolled factors in the environment. On the other hand, fruit bodies of *A. brasiliensis*, which where obtained in this way, now are in demand of Japanese importers, who use this mushroom for production of different medical additives etc. At present most of Brazilian growers of *A. brasiliensis* use the technology similar to growing of *A. bisporus* (the substrate formulation, its composting, pasteurization, and conditioning, inoculation, and incubation at controlled conditions [25, 29-30].

The aim of the present work was to study the interaction of thermophilic fungi *H. insolens* with medicinal mushroom *A. brasiliensis* on SA medium and growth of its mycelium and fruit body formation on straw pre-inoculated by this thermophilic fungi.

MATERIALS AND METHODS

Strain. *Humicola insolens* Cooney et Emerson, IBKF-519, (IMI-354859, ATTC-201434), *Agaricus brasiliensis* S. Wasser et al. (*A. blazei* Murrill) commercial strain.

Media. Two solid agar media were used. Yeast glucose agar (YGA) comprising yeast extract («Serva»), 4.0 g; peptone («Sigma»), 2.0 g; glucose monohydrate («Merck»), 10.0 g; agar, 15.0 g; 1000 ml distilled water. Autoclaved for 1h at the temperature of 120°C. Straw agar (SA) comprising milled air-dried wheat straw (mesh size 0.3-0.5 cm), 200.0 g; agar, 20.0 g; 1,000 ml distilled water. Autoclaved for 1h at the temperature of 120°C.

The pure culture of thermophilic fungi *H. insolens* and cultivated mushrooms with medicinal properties *A. brasiliensis* were grown on YGA medium in tubes (for subculture and storage) and YGA, SA (on which *H. insolens* formed more conidia) media in Petri dishes (for inoculation) in the dark at 42±1°C and 28±1°C, accordingly.

For the observation in the scanning electron microscope to study the growth and interaction of *H. insolens* and *A. brasiliensis* the sterile pieces (0.9 x 0.9cm) of the cover glass or straw were put on SA medium on different distance from the inoculum of thermophilic fungi and mushroom. After overgrowth and interaction of their colonies on glass or straw they were collected at different times (days) and fixed [32, 33]. For the research we use scanning electron microscope (SEM) Jeol JSM-35 and Jeol JSM-6060.

Grain for obtaining grain spawn in the flasks was prepared by standard technology [29]. After sterilization and cooling grain in flask was inoculated by pure culture of *A. brasiliensis* from Petri dishes with YGA medium and grown in the dark at 28±1°C.

For studying the interaction of *H. insolens* with *A. brasiliensis* in agar nutrition medium one part of Petri dishes with SA medium was inoculated by thermophilic fungi (diameter of inoculum 1.0 cm) from the YGA. The Petri dishes with *H. insolens* were cultivated for 3-5 days

at 42±1°C °C in the dark. After that another part of Petri dishes (opposite of *H. insolens* colony) was inoculated by pure culture of *A. brasiliensis*. These Petri dishes with thermophilic fungi and mushrooms were incubated in the dark at temperature 28±1°C. As control we used Petri dishes with pure culture of *A. brasiliensis* or *H. insolens* on SA medium that also were kept at 28±1°C.

For the study of the growth of mycelium and fruit body formation of *A. brasiliensis* on the experimental substrate (straw pre-inoculated by *H. insolens*) straw was meshed to the size 1.0-3.0 cm, and watered up to the 60-75%, and then put into polypropylene boxes (approx.1.0-1.2 kg of watered straw per box), covered by folia and autoclaved for 1h at 120°C. Boxes with sterile straw were inoculated by conidia of *H. insolens* (water suspension from the surface of SA medium from one Petri dishes per one box). After that inoculated boxes again covered by folia and shook for the better mixture of thermophilic fungi conidia in the straw. The boxes with straw which was inoculated by conidia of *H. insolens* (treatment) and control substrate - without inoculation with them (not treatment-sterile straw) were put into the thermostat cabinet at temperature 42±1° C for 3-5 days in the dark. There were six replications of the each experiment with Petri dishes and boxes. At the end of the growth of *H. insolens* on the straw (treatment substrate) and straw without treatment by thermophilic fungi (control) were inoculated with grain spawn of *A. brasiliensis* (10-12 g/kg of substrate), covered and arranged in growing room. Depending on the used substrate spawning run lasted 10-15 days, without light, at temperature of 28±1°C and relative humidity between 85 and 90%.

The cover paper was removed and the two types of substrates were covered by 4-5 cm of casing soil (mixture of 80% black and 20% brown peat) which was used in mushroom complex "Valentina Ltd.". Then these boxes were re-covered and placed on the shelves in growing room. The incubation lasted 10 days at 28±1°C, in the dark and a relative humidity of 90%.

In our experiments any type of supplements, like soybean meal, ChampFood etc., were not used and a traditional mushroom compost was used as control.

Under environmental control in growing room (which uses in "Valentina" mushroom complex), the first fruit body of *A. brasiliensis* started to appear at 18-25 days after casing.

RESULTS AND DISCUSSIONS

After the partially growth (aprox. 3.0 cm from the edge of inoculum) of colony of *H. isolens* on SA medium at temperature 42±1° C these Petri dishes were inoculated by pure culture of *A. braziliensis* and incubated at temperature 28±1° C (Fig. 1, 2). At this temperature, growth of the colony of *H. isolens* decelerated and continued to grow, but much more slowly. During this period, on the surface of the tested colony of thermophilic fungi a great number of conidia were formed. It is a very important moment, because our previous examinations have shown that mycelium of some species of genus *Agaricus* grow faster on the colony of *H. isolens* which formed a lot of conidia [21, 22]. During the contact of the colony of *H. isolens* with *A. braziliensis* the latter inhibited the growth of thermophilic fungi (Fig. 1). After said contact the colony of tested mushrooms started to grow on the surface of the colony of *H. isolens*. During first days of growth of the colony of *H. isolens*, *A. brasiliensis* grew rapidly, but it formed rare, thin mycelium (Fig. 1). On the twelfth – fifteenth day of the interaction of tested thermophilic fungi and mushrooms, the colony of *A. braziliensis* almost completely overgrew on the colony of thermophilic fungi with formation of a typical colony for these mushrooms with mycelium which formed the strands (Fig. 2). At the same time, mycelium of *H. isolens* continued to grow on those areas of the medium which had no contact with *A. brasiliensis* (Fig. 1 a-black line with bulbs). In the sequel, after the contact of thermophilic fungi with mushrooms, the latter overgrew on *H. isolens* (Fig. 2b).

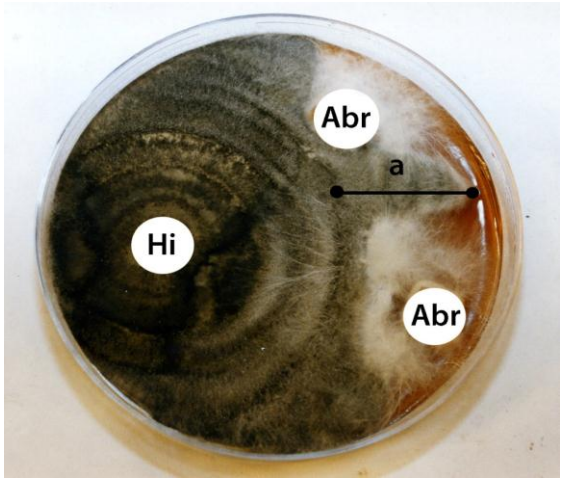

Figure 1: 5 days growth of *A.brasiliensis* (Abr) Colony on the surface of colony of *H. insolens* (Hi)

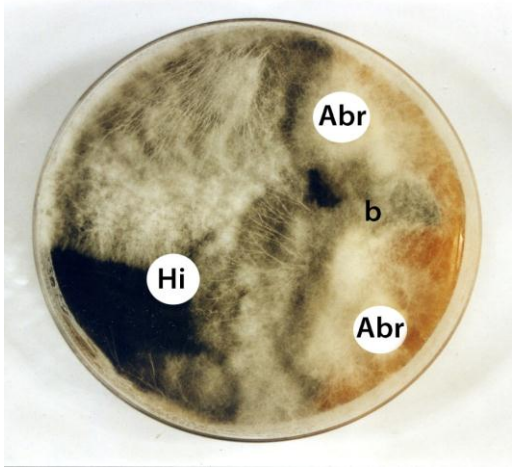

Figure 2: 14 days growth of *A.brasiliensis* (Abr) colony on the surface of colony of *H. insolens* (Hi)

Examination of interaction of *H. isolens* with *A. braziliensis* using the SEM is shown on Fig. 3, 4. During first days after the contact of the colony of thermophilic fungi with mushrooms, mycelium of *A. brasiliensis* started to grow on the surface of conidia *H. isolens* (Fig. 3). During subsequent interaction (12-15 days) of the thermophilic fungi with the tested mushroom, on the surface of the colony of *H. isolens* growth of mycelium and strands formation is observed (Fig. 4).

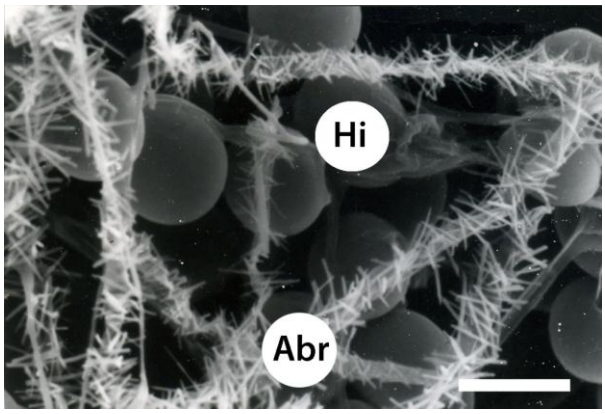

Figure 3: Growth of mycelium of *A.brasiliensis* (Abr.) on the conidia of *H. insolens* (Hi). SEM. Bar = 10 µm

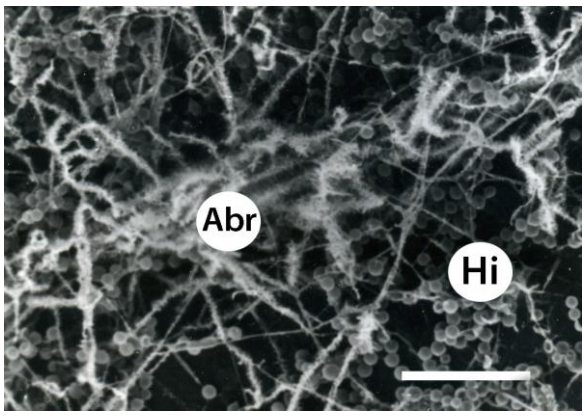

Figure. 4: Growth of mycelium and strands formation of *A.brasiliensis* (Abr.) on the conidia of *H. insolens*(Hi). SEM. Bar = 100 µm

Our preliminary experiments on the influence of *H. insolens* on the growth of *A.brasiliensis* showed that these thermophilic fungi stimulated the growth of mycelium of this mushroom on SA medium.

Next stage of our work was to study the possibility of mycelium growth and fruit body formation of *A. brasiliensis* on the straw pre-inoculated by *H. insolens* (experimental substrate) and development of this mushroom without pre-inoculated straw (control). Our study has shown that the mycelium growth of *A .brasiliensis* on the experimental substrate was 1.5-2 times more rapid (Fig. 5) than on the control one. After covering of the experimental substrate with casing

soil, the mycelium growth of *A. brasiliensis* was typical for this mushroom (Fig. 6). On the control substrate, the growth of mycelium of tested mushrooms was very slow or there was no growth at all through the casing soil. And only on the experimental substrate, fruit bodies of *A. brasiliensis* were formed (Fig. 7 a,b).

The task of our preliminary experiments was to study the influence of the thermophilic fungi *H. insolens*, strain IBKF-519, on the growth of mycelium and fruit body formation of the medicinal mushrooms *A. brasiliensis* on cellulose-lignine containing substrates, in our case it was the straw, and we obtained a positive result. On the other hand, we did not have a task to examine the impact of these thermophilic fungi on productivity of *A. brasiliensis.* We understand that it is necessary to conduct additional studies using different mixtures for the selective substrate preparation, various supplements (soybean meal, ChampFood, etc.), increasing density of the experimental substrate and its C/N relation, etc.

Figure 5: Spawn run of *A.brasiliensis* on the straw pre-inoculated with *H. insolens*

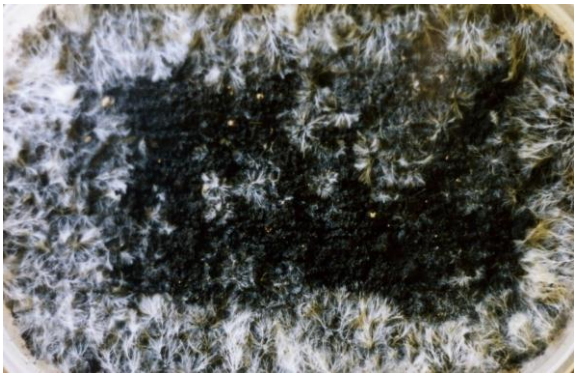

Figure 6: Growh of *A.brasiliensis* on the casing the substrate pre-inoculated by *H. insolens*

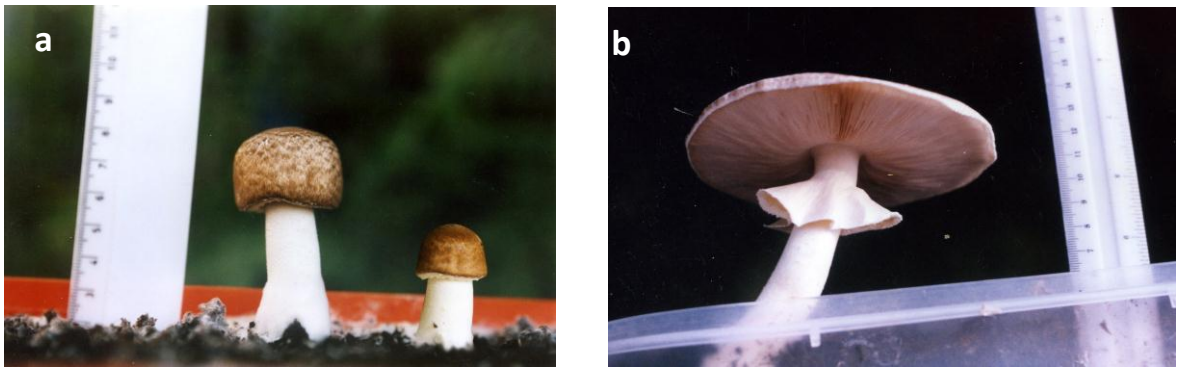

Figure 7: Different stages of fruit body formation of f *A.brasiliensis* on the substrate with *H. insolens*

CONCLUSIONS

In our previous investigation of influence of strain *H. insolens* IBKF-519, selected by us, on the growth of *A. bisporus* and *A. bitorquis* we have found out that this thermophilic fungi stimulated the growth of these two mushrooms and some others [21, 22]. We obtained the same results during the present studies. They showed that on SA medium *A. brasiliensis* inhibited the growth of *H. insolens* colony. After that these thermophilic fungi stimulated the growth of mycelium of tested mushrooms on its sporulated colony. The growth of *A. brasiliensis* on surface of the colony of *H. insolens* on SA medium was twice faster than on this media without thermophilic fungi. On the straw pre-inoculated with *H. insolens* (experimental substrate) this thermophilic fungi positive influence on the growth of mycelium and fruit body of *A. brasiliensis* formed on

it. That impact was not observed on straw without pre-inoculation of thermophilic fungi (control).

Further studies are needed to look for new initial components, waste of plants, food, etc., as well as supplements and formulation of different substrate (compost) for development of *A. brasiliensis*, including the use of thermophilic fungi *H. insolens*, strain IBKF-519 or another ones.

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"INDOOR" METHOD OF COMPOSTING AND GENETIC BREEDING OF THE STRAINS TO IMPROVE YIELD AND QUALITY OF THE ALMOND MUSHROOM *AGARICUS SUBRUFESCENS.*

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ABSTRACT

The aim of the present work was to evaluate the potential efficiency of an *indoor* composting method and the genetic breeding of strains on the agronomic performance (yield, number and weight of basidiocarps, precociousness and earliness) and quality of *A. subrufescens* mushrooms. The experiment followed a factorial combination (3 composts types x 4 strains) with five replicates per treatment. One strain was a hybrid between French and Brazilian isolates. Strains and composts affected all variables analyzed (yield, number of basidiocarps, precociousness and earliness), except the weight of basidiocarps harvested. According to agronomic performance, yield was positively correlated with the number of basidiocarps and precociousness but was negatively correlated with earliness. According to chemical characteristics of basidiocarps, moisture was positively correlated with the amount of fat; protein was negatively correlated with the amount of hemicellulose and finally, hemicellulose was negatively correlated with the amount of cellulose present in the mushrooms. Despite the observed differences between composts, the best composting process for the cultivation of *A. subrufescens* is still unknown, requiring further research with management approaches, methods and formulations to be used for the commercial production of a selective substrate. The intercontinental hybrid possessed improved quality characteristics while yielding similar to its better parent. Breeding programs for improving mushroom quality and yield of *A. subrufescens* would be warranted.

Keywords: *Agaricus subrufescens*; compost; genetic breeding; chemical characterization; agronomic performance.

INTRODUCTION

Since the first tests performed in 1980 by Takatoshi Furumoto, agronomist, production of *Agaricus subrufescens* (formerly *A. blazei*, *A. brasiliensis*) was done on basis of cultivation practices adopted for the production of *Agaricus bisporus*. Even today, little has changed especially with

growing practices to improve yield (15-25%), earliness (70% of total yield in the first half of the crop), duration of flushes (4 days of harvest), interval between flushes (3-5 days) and crop cycle (50-60 days).

Strains used for the cultivation of *A. subrufescens* in Brazil are marketed as varieties collected indigenously, that were selected through domestication and adaptation to the cultivation conditions of the farms (type and formulation of the compost and local environmental conditions). The consequences are a great variability in yield, a long growing cycle and a lack of control over the specific growth characteristics of the strains.

Agaricus subrufescens has been characterized as a tropical mushroom with fruiting temperatures used during cultivation usually between 25 and 29°C. However mycologists have collected fruiting bodies in temperate countries such as Belgium and France [1; Guinberteau, pers. com.], showing the species has an extended geographic distribution. Because of this great geographic distribution, the important work of genetic breeding and acquisition of new hybrids can be performed, creating individuals with specific characteristics for production in different conditions worldwide.

In Brazil, the traditional process of composting has been widely practiced by growers, following the steps of: pre-wetting (4-7 days), fermentation (formation of the windrow 2 m wide x 2 m high, with intervals of turning every 2-3 days), pasteurization (58±2°C) and physical, chemical and biological conditioning (47±2°C) [2]. The raw materials commonly used as bulk compost are: sugar cane bagasse (*Saccharum officinarum*), various grasses (*Braquiaría* sp., *Cynodon dalactylon*, *Panicum maximum*, etc), cereal straw (*Triticum aestivum*, *Avena sativa*, *Oryza sativa*, etc.) and manure. Already as concentrated material (nitrogen source or not) soybean, wheat, corn and cotton meal, urea, ammonium sulfate, superphosphate, calcium carbonate and gypsum are used [3].

In 1986, the first method of "indoor" composting used for the production of *A. bisporus* was proposed [4], later called "environmental control" [5] and "accelerated" [6] composting, in order to accelerate the composting process to limit anaerobiosis and bad smells, to decrease the loss of material during the composting process, to reduce the physical space of the operations, and the use of machines [7]; and especially to increase process efficiency and productivity. Productivity is a direct consequence of operating quality practiced during the composting process, both with respect to the design of the theoretical formulation, as well as a civil structure existing and used [8].

As important as the agronomic performance of the species, the final quality of mushrooms (physical, chemical and biological control of harvested mushroom) should also be taken into consideration. It can be defined as physical aspects: size, degree of maturation, absence of pests and diseases, etc.; chemical aspects: the amount of β-glucan, no heavy metal, high presence of proteins and minerals, etc.; and finally biological activities: bactericidal, antitumor and antioxidant activity.

In general, it is difficult to compare the chemical results obtained and cited in the literature by several authors working with the same species, since there are many variables influencing the nutritional composition of mushrooms [9], such as differences between strains, composition of compost, type of casing layer, environmental conditions and methods of cultivation, besides the inherent inaccuracy in methods of analysis and precision of the analyst [10]. New cultivation technologies should be investigated to increase the agronomic performance without changing the physical-chemical characteristics of harvested mushrooms. Thus, the present study focused on evaluation of potential efficiency of *indoor* composting and genetic breeding of strains on yield, number and weight of basidiocarps, precociousness, earliness and quality of *A. subrufescens*.

MATERIALS AND METHODS

Spawn. Four strains were used described as follows:

- 99/30: strain stored in the mycology collection, Mushroom Research Center (FCA/UNESP), isolated in Piedade (1999) from a commercial farm of the Atushi Group, São Paulo State (Brazil).
- CA454: originated from Brazil, corresponding to ATCC 76739, deposited as the original strains of *A. blazei* Murill used for the development of the cultures.
- CA487: wild strain isolated by Jacques Guinberteau in 2006 at Saint-Léon, Gironde, France, on waste of leaves lawn mowing
- CA454 x CA487: hybrid between Brazilian (C454) and French (C487) strain obtained by crossing mycelia from single spore isolates. All the CA strains are from the CGAB collection (INRA, UR MYCSA, France)

Production of spawn followed procedures adopted by Zied et al. [11].

Compost (Phase I and II). Three composts were used, made from different plants of "Indoor" composting, that were produced by different methods.
Compost 1: wheat straw was moistened for 6 days, then the straw was mixed and transferred to the 1st Bunker with chicken manure and concentrated ingredients where they remained for 5 days; afterward the compost was mixed and transferred to the 2nd Bunker where it remained for another 5 days, finally the compost was mixed again and transferred to the 3th Bunker where it remained for an additional two days. Phase II lasted 7 days (8 hours at 60°C and 6 days at 45-50°C).
Compost 2: wheat straw and chicken manure were moistened for 8 days then held 3 days and turned; then the compost was transferred to the 1st Bunker together with the concentrated ingredients where it remained for 2 days; afterward the compost was mixed and transferred to the 2nd Bunker where it remained for 2 days. Finally the compost was mixed again and transferred to the 3th Bunker where it remained for 2 days. Phase II lasted 7 days (13 hours at 57°C and 6 days at 45-50°C).
Compost 3: wheat straw and chicken manure were moistened for 6 days with turning on the 3rd day; then the compost was transferred to the 1st Bunker together with the concentrated ingredients where it remained for 2 days; afterward the compost was mixed and transferred to the 2nd Bunker where it remained for 2 days; then the compost was mixed again and transferred to the 3th Bunker where it remained for 2 days. Finally the compost was transferred to 4th Bunker where it remained for 2 days. Phase II lasted 8 days (8 hours at 58°C and 7 days at 45-50°C).

Table 1 shows the characteristics of each compost type at the end of Phase II of the composting process.

Inoculation and spawn run. The compost was inoculated with 1% spawn in relation to the wet weight of the compost and incubated at 28±2°C with relative humidity at 50±10% for 15 days.

Casing layer. A mixture of casing with black peat + soil (4:1, v/v) added calcium carbonate and formaldehyde in the amount of 50 ml per m^3 of material was used. With fully developed mycelium, the casing was added over the compost at a depth of 3 cm (2.6 liters of material per plastic box containing 6 kg of compost). The boxes with compost and casing were taken to a chamber with air temperature of 26±1°C, compost temperature of 27±1°C, relative humidity of 90±5% and CO_2 content of 2,100 ppm, during 8 days following the methodology presented by Minhoni et al. [12].

Table 1. Physico-chemical characteristics of three compost types (at end of Phase II).

Parameter	Compost 1	Compost 2	Compost 3
pH, 1:5, v/v	7.35	7.24	7.51
Moisture, g kg^{-1}	678	675	668
Nitrogen, g kg^{-1}	23.8	21.7	24.7
Protein, g kg^{-1}	104.2	95.0	108.1
Ash, g kg^{-1}	245.5	294.5	297.1
Organic matter, g kg^{-1}	754.5	704.5	702.9
C/N	18.4	18.8	16.5

Pinning and harvest. The environmental variables were controlled in order to obtain 4 flushes of production over the crop. For this the temperature, relative humidity and aeration were conducted according to methodology presented by Zied [13]. Fig. 1 demonstrates the behavior of environmental variables and reflects the flush of production according to the strain used.

The total production time of the crop was 70 days, and the presence of primordia was observed at 17 days. The mushrooms were collected manually with the largest weight possible before pileus opening and lamella breaking. Then, mushrooms were evaluated for their agronomic performance and chemical characteristics.

Experimental design and data analysis. The experiment was conducted using 4 strains and 3 composts, totaling 12 treatments. Each treatment consisted of five repetitions of boxes with 6 kg of compost. The Sisvar 3.2 statistical program was used to separate treatment means with Tukey's test ($P \leq 0.05$). Linear correlation between agronomic performance and chemical characteristics of *A. subrufescens* was done using the statistical software Sigma Stat 3.5.

Evaluated data. The agronomic performance was evaluated by yield [14], number and weight of basidiocarps [10], precociousness [12] and earliness [15]. The chemical characteristics were evaluated by moisture [16], protein, N-free extract and ash [17], fiber [18], fat [19] and hemicellulose and cellulose [20; 21].

RESULTS AND DISCUSSION

Strain CA487 had the highest yield, followed by CA454 x CA487, 99/30 and CA454, which demonstrates the potential production of the strains from a temperate country and also of the hybrid (Table 2). Regarding the compost used, compost 2 had the highest yield, followed by compost 1 and 3, and shows that compost with a low C/N ratio and high nitrogen content may result in low yield. Another factor that may have influenced the low yield obtained for compost 3 is a lower compost moisture (66%).

Kopytowski-Filho [22] emphasizes that compost obtained with an initial mixture having a high C/N ratio, (40-33/1) tends to show higher yield than compost obtained from an initial C/N ratio around 29-26/1.

The behavior of production flushes of strains according to management of environmental variables controlled is shown in Fig. 1. The strains 99/30, CA454 and CA454 x CA487 showed flushes well distributed during cultivation at 27°C. This distribution responded to the phase of induction by decreasing temperature to 20°C and increasing to 27°C. Strain CA487 was harvested at ± 27°C as the others, but it also produced fruiting bodies during the decrease in temperature to 20°C, as observed on days 43 and 58. More studies are required on the management of environmental variables to obtain the flushes of production for this strain of temperate origin.

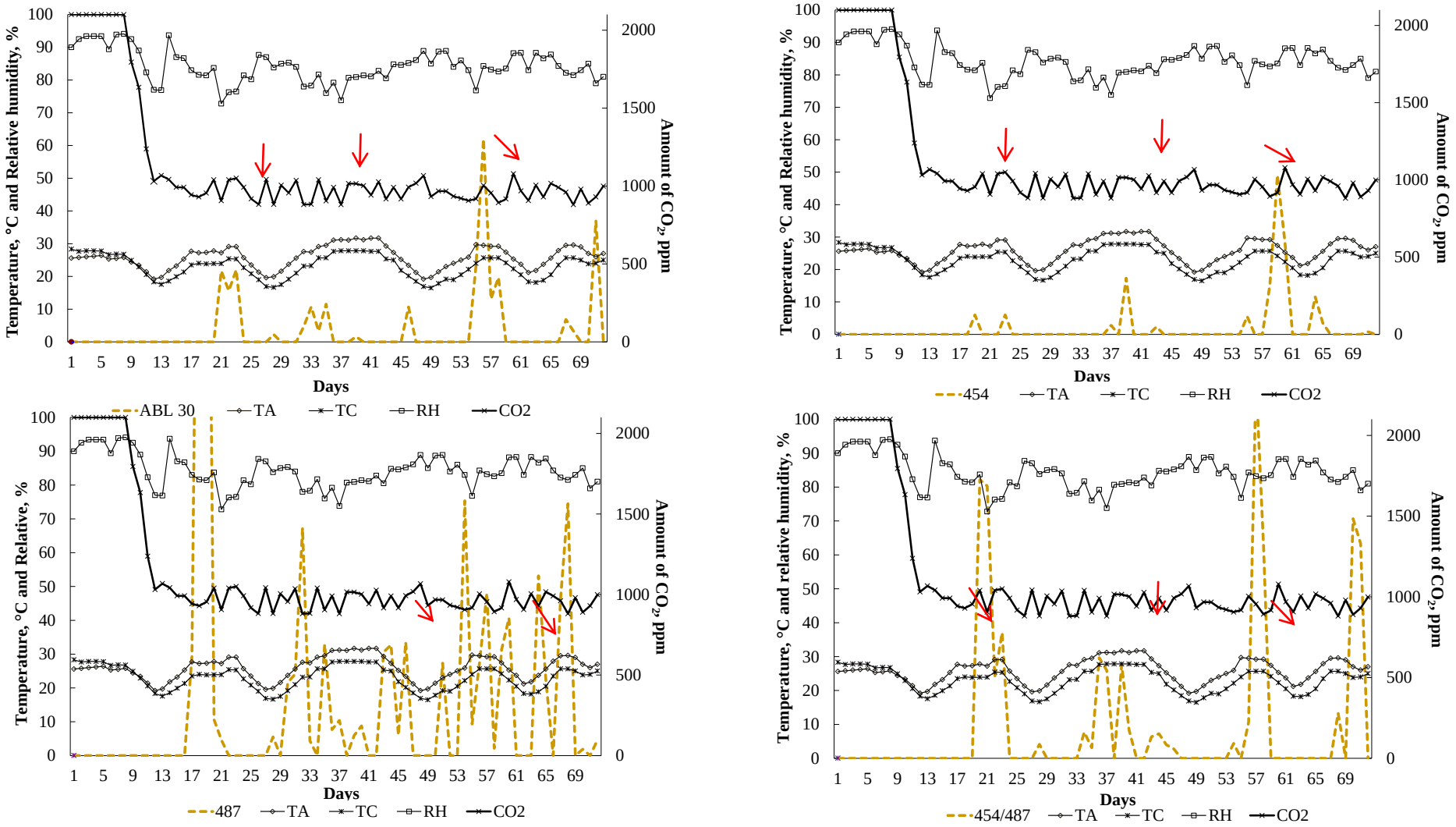

Figure 1. Environmental variables and crop flushes during the 70 days of cultivation, where: ABL 30, 454, 487 and 454/487 indicates the production of the strains (grams) of harvested mushrooms during the growing period; TA, air temperature; TC, compost temperature; RH, relative humidity and CO_2, the amount in environment.

Table 2. Agronomic performance of four strains of *Agaricus subrufescens* produced on three different composts.

Strain	Compost		
	1	2	3
Yield (%)			
99/30	4.7 a CB	3.2 ab C	1.2 b C
454	1.9 a C	2.3 a C	1.1 a C
487	14.4 a A	18.6 a A	13.5 b A
454 x 487	6.1 b B	11.2 a B	8.0 b B
Number of basidiocarps, u			
99/30	21 a B	13,8 abC	5,8 b C
454	3.8 a C	6,8 a C	3,6 a C
487	72.4 a A	74,8 a A	65,6 a A
454 x 487	25.6 b B	41,4 a B	30,8 ab B
Weight of basidiocarps, g			
99/30	27.2 a A	19.4 a A	10.9 a A
454	17.9 a A	15.2 a A	24.5 a A
487	17.0 a A	12.6 a A	15.5 a A
454 x 487	18.7 a A	15.2 a A	17.4 a A
Precociousness, %			
99/30	30.5 a AB	37.8 a A	27.7 a AB
454	12.6 a B	25.2 a A	20.0 a B
487	64.4 a A	59.4 a A	65.1 a A
454 x 487	51.4 a A	52.6 a A	28.1 a AB
Earliness, days			
99/30	28.7 a A	26.8 a AB	38.0 a AB
454	50.0 a B	44.0 a B	52.8 a B
487	18.8 a A	18.2 a A	18.5 a A
454 x 487	29.0 a A	25.4 a AB	25.5 a A

Lowercase letters compare the results on the same line and capital letters compare the results in the same column in the Tukey's test ($P \leq 0.05$).

Another positive factor that should be highlighted is the convenience and ease in working with the CA487 strain that in just 70 days of crop had a yield between 13.5-18.6%, high number of mushrooms (mean of 71 u), precociousness (mean of 63%) and earliness of production (18.5 days). Similar yields were observed with commercial strains by Siqueira et al. [23] and Zied et al. [3] with values of 16.3% and 18.3%, respectively, but with crop time above 110 days of production.

The numbers of mushrooms followed the patterns of yield, that had a positive correlation with precociousness ($r = 0.868$, $P = 0.001$), and a negative correlation with earliness ($r = -0.829$, $P = 0.001$). Thus high yield in the crop is associated with a large number of mushrooms harvested, concentrated in the first half of the crop; but with low yield in the first flush (this trend was clear for the Brazilian strains).

The present work illustrates the interest of intercontinental breeding programs for the development of efficient new varieties, since the first hybrid used responded exactly the goals that has been developed; maintained a good agronomic performance (close to its better parent) and increased the weight of harvested mushroom (although no significant difference at Tukey's test, was observed that the hybrid increased approximately 12.3% of the weight of mushrooms when compared with the CA487 strain).

The compost used did not affect the weight of mushrooms, precociousness and earliness, but the strain affected the earliness and precociousness. The best composting process for the cultivation of *A. subrufescens* is still unknown, requiring further research with management approaches (performance of "traditional" composting or composting in Bunkers with Phase II and

III together), methods (production of substrate composted or sterilized "axenic") and formulations (range in C/N ratio, content N, organic matter and ash) to be used for the commercial cultivation.

According to Table 3, little variation in chemical characteristics of mushroom were observed according to strains and composts used, but some features need to be mentioned. 99/30 and CA454 strains had higher amount of protein when grown in compost 3, on the other hand the CA487 and CA454 x CA487 strains has higher amount of protein when grown in compost 1. The higher levels of fiber were observed in 99/30 and CA454, and the highest levels of fat were observed in CA 487 and CA 454 x 487 strains.

Table 3. Physico-chemical characteristics of mushrooms according to strain and compost type

Strain	99/30			454			487			454 x 487		
Compost	1	2	3	1	2	3	1	2	3	1	2	3
Moisture, %	85.3	85.4	84.1	84.7	85.8	85.0	83.0	87.6	88.3	86.1	86.5	86.0
Protein, %	30.3	32.8	33.3	28.9	30.1	30.5	30.7	26.2	29.0	34.9	33.7	28.6
Ash, %	5.8	7.0	6.1	6.2	7.1	6.8	6.3	7.1	7.2	6.6	6.3	5.9
Fiber, %	6.8	6.8	5.6	8.2	7.8	8.1	5.1	6.7	5.1	5.6	6.7	5.1
Fat, %	0.97	0.86	1.13	0.94	1.04	1.00	0.96	1.71	1.66	1.26	1.18	1.52
N-free extracts, %	56.0	52.3	53.7	55.5	53.8	54.0	56.8	58.1	56.8	51.5	51.8	58.7
Hemicellulose, %	19.1	19.6	17.5	21.5	17.2	19.0	21.0	22.7	21.4	18.8	18.1	20.6
Cellulose, %	6.6	6.8	6.9	6.2	8.9	7.0	5.7	3.8	4.5	7.1	7.7	6.9

Comparing the results of protein, ash, fiber and fat of *A. subrufescens* mushrooms with those obtained by Hernández [9], Andrade et al. [24] and Pardo et al. [10] for *Agaricus bisporus* and *Lentinula edodes* we have, protein: *A. subrufescens* (32.17%), *L. edodes* (20.33%) and *A. bisporus* (23.22%); ash: *A. subrufescens* (6.35%), *L. edodes* (3.10%) and *A. bisporus* (12.62%); fiber: *A. subrufescens* (6.4%), *L. edodes* (8.04%) and *A. bisporus* (20.41%) and finally fat: *A. subrufescens* (0.98%), *L. edodes* (2.00%) and *Agaricus bisporus* (5.2%).

It should be noted that the moisture content had a positive correlation with the amount of fat ($r = 0.780$, $P = 0.002$); the protein had negative correlation with the amount of cellulose ($r = -0.712$, $P = 0.009$) and finally cellulose had a negative correlation with the amount of hemicellulose ($r = 0.623$, $P = 0.030$) present in the mushrooms.

Polysaccharides are the main chemical compounds found in fungal cell walls. Glucose – usually as glucans, β(1→4) cellulose, α(1→4) and α(1→6) glycogen, β(1→3) and β(1→6) yeast glucan – constitutes from 80 to 90% of the cell wall material of many species, and glucosamine (in chitin) constitutes from 1 to 58% (range of values), usually 5 to 20% [25].

Park et al. [26] compared the amount of β-glucan in mushrooms produced in Brazil (greenhouse and field) and in Japan (greenhouse), concluded that *A. blazei* cultivated in greenhouses have a lower amount of β-glucan (7.6 ± 2.8g $100g^{-1}$ mushroom produced in Japan and 8.4 ± 0.9g $100g^{-1}$ mushroom produced in Brazil) than those cultivated in the field (10.1 ± 2.1g $100g^{-1}$ mushroom). Zied [27] evaluated different cropping practices that influenced the amount of β-glucan in *A. subrufescens* mushrooms and found a variability of 35.8% of the value of β-glucan influenced by strain and 9.9% of the value of β-glucan influenced by the compound used.

According to the review carried out by Manning [28] on the chemical composition and nutritional value of cultivated mushrooms, carbohydrates are the main component of mushrooms apart from water, and account for an average of 4.2% of the fresh weight. Among them, glycogen and hemicellulose are the main polysaccharides found in mushrooms; contents of 8.18% (dry weight) of crude hemicellulose have been recorded in *A. campestris*, markedly lower than those obtained in this work with *A. subrufescens* (between 17.5 and 22.7%).

CONCLUSIONS

Despite the observed differences between composts, the best composting process for the cultivation of *A. subrufescens* is still unknown, requiring further research with management approaches, methods and formulations to be used for the commercial production of a selective substrate. The intercontinental hybrid resulted in an increase in the weight of basidiocarps, while still maintaining high yield close to its better parent. Breeding programs for improving mushroom quality and yield of *A. subrufescens* are worth being developed.

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RECYCLING OF MUSHROOM PEAT CASING SOIL THROUGH A PLASTIC MESH

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ABSTRACT

The objective of this research was to lower the cost of mushroom production through recycling peat soil by using a plastic mesh. Phase III compost was covered by a plastic mesh and the casing soil was spread on it. Mycelia penetrated the mesh and occupied casing soil as they did in casing without plastic mesh. Mean yield of mushroom collected over three flushes was not affected by plastic mesh. Piling of collected casing soil helped in decomposing of its remained mycelia from previous crop and bleaching it, brought down its EC to a desirable level. Reusing of treated peat soil did not have any effect on yield of next crop. Growers could therefore recycle it and use for next crop.

Keywords: Casing, Plastic mesh, Mushroom, Peat moss soil, *Agaricus bisporus*

INTRODUCTION

Casing the surface of composted substrate fully colonized by mycelium of mushroom (*Agaricus bisporus*) is an essential function in stimulating and promoting the development of fruit bodies. The key physical requirements of the soil used for casing include water holding capacity to supply water for the growth of mycelium and sporophores, sufficient porosity for mycelia respiration, and ability to resist structural breakdown following repeated watering, and suitable chemical and microbiological characteristics for sporophore initiation [1]. These characteristics are found in peat soil which is being used as casing material for several decades in many countries [2]. In many mushroom-growing areas of the world, there are no available sources of peat and in those available there is an environmental pressure against extracting peat for horticultural use [3].This has led to considerable research into possible peat alternatives for casing [4]. These alternatives include bark [5], spent mushroom compost [6], coconut fibre [7] and paper waste by-products [8]. However, none of these materials has replaced peat for casings on the market [9].

Some growers mix a percentage of spent mushroom compost with peat soil as casing material, but the yield of mushroom with this casing is not equal to that of peat. When casing the surface of composted material with peat soil, if we could prevent it from being mixed with compost, while it could perform its functions properly, it is possible to collect it after mushrooms being harvested and to reuse it. However it may need to be treated with some material and be bleached to regain some of its lost properties. The aim of this work was to find a way to collect and reuse the peat soil for mushroom production by recovering it from compost surface without being mixed by compost. This would bring down the cost of mushroom production by at least 25%.

MATERIAL AND METHODS

The blocks of phase II compost [10] were placed on the shelf of growing room with a temperature of 25 ˚C and 93% relative humidity for spawn running. Each block was considered as a unit of experiment. After 14 days, when the blocks were ready for casing, the surface of four blocks were evened and covered with a plastic mesh (the size of holes: 1 × 1 mm). Four neighboring blocks without being covered by the plastic mesh, were considered as control. Casing soil was applied as usual with 4.5 cm depth layer of original peat moss soil [9]. All other works including irrigation, ventilation and ruffling was the same for both controls and the blocks covered by plastic mesh. Mushrooms were harvested from each block separately. After harvesting the third flush, plastic mesh was lifted from the blocks with whole used casing soil on it.

The used casing soil was transferred to a container for measuring its properties such as EC (electrical conductivity), pH percentage of pore spaces and bulk density. These properties were determined by our soil lab according to the general protocols. The used soil was piled for three weeks for decomposition of mycelia remained from previous crop and was then leached with distilled water to bring down its EC to a recommended level. As pH was in the range of recommended, no attempt was done to adjust it. The used peat soil was then pasteurized at 60 ˚C for six hours to be reused as casing soil. At the next experiment four blocks of fully colonized phase III compost were cased with used peat soil and four others were covered with the original peat soil. The experiment was carried out as the first one. All factors affecting the yield of mushroom were the same for both controls and the blocks covered by plastic mesh and cased with reused peat. Data on yield of three flushes were collected separately, using each block as a unit of experiment or a replication. Means were compared using unpaired t-test.

RESULTS

Table 1 presents the mean and the t test statistic for comparison of yield of mushroom of control and the blocks covered with the plastic mesh. As it shows, there is not a significant difference between mean of the control and that of covered with plastic mesh. Plastic mesh did not prevent mycelia of penetrating the casing soil. When examining the mycelia penetration in casing soil on plastic mesh, it was observed that mycelia growth was the same in casing both with and without plastic mesh. The plastic mesh had not any inverse effect on both mycelium growth and yield. Plastic mesh, while being a cheap material, could help growers to collect peat casing soil without being mixed with compost material.

Table 1: Mean yield of blocks (20 kg with RE=67%) cased with original peat with and without plastic mesh

Type of casing soil	Mean of yield (kg/block)	t-test
Peat without plastic mesh	2.25	1.089
Peat with plastic mesh	2.14	Prob> |t|=0.3178

Peat collected on plastic mesh had retained its physical properties such as its pore space and bulk density (Table 2). The pH of used peat (pH=7.26) did not change significantly compared to the pH of original peat soil (pH=7.47) and it was in the range of recommended ones. The only chemical property which changed dramatically was EC due to evaporation of water

from the surface of casing soil. EC of used peat soil (1247 µs cm^{-1}) was about 9 times of that of the original peat soil (154 µs cm^{-1}). Bleaching of collected peat with distilled water lowered EC to 540 µs cm^{-1}. Needs for bleaching depend on the EC of casing soil, the amount of water used at each bleaching and its frequency. Growers should monitor EC after each bleaching to set it at a desirable level. If needed, pH also could be adjusted, by calcium carbonate. The yield of mushroom cased by used peat soil was not different from that obtained of original peat soil (Table 2).

Table 2: Mean yield of blocks (20 kg with RE=67%) with original and used peat along with some other important properties

Type of casing soil	EC µs cm^{-1}	pH	Pore spaces (%v/v)	Bulk density dry (gl-1)	Mean of yield (kg/block)	t-test
Original peat	154.3	7.47	85.4	83.7	2.24	-0.873
Used peat	540.4	7.26	239	227	2.11	Prob> |t|=0.4162

DISCUSSION

Casing soil costs about 25 -30% of total costs of mushroom growing in Iran. As physical properties of reused soil is not changed and its chemical properties could be recovered by some treatments, collecting and reusing it would bring down the cost of producing mushroom in those countries importing it from abroad. In countries having supply of peat, using plastic mesh would bring down the pressure on peat mines and would help the ecosystem from destroying.

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HYDROGEN SULPHIDE GAS PRODUCTION FROM SPENT MUSHROOM COMPOST UNDER FIELD AND LABORATORY CONDITIONS

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ABSTRACT

Hydrogen sulphide (H_2S) gas production was monitored during the disturbance of four heaps of Spent Mushroom Compost (SMC) that were stored for up to 12 months either outdoors or under cover (indoors). QRAE (www.raesystems.eu) and ITX (www.indsci.com) gas monitors with data logging facilities were used to measure the 10-second average concentration of H_2S released into the air above the heaps of SMC as they were being disturbed. The highest concentrations of up to 2083 ppm were detected for the outdoor stored material in comparison to concentrations of up to 687 ppm detected for indoor stored material. Outdoor stored SMC had higher moisture contents (66-72%) compared to indoor stored material (53-65%). Laboratory studies were conducted to study the effects of moisture content (69 – 85%) and temperature (35ºC or 45ºC) on H_2S production from fresh un-steamed and steamed samples of SMC. The H_2S gas concentration in the head space of the incubation vessels was measured every 24 hours for 3 days. Un-steamed SMC produced higher levels of H_2S than steamed SMC and in general the higher the moisture content the higher the H_2S concentration. Higher concentrations of H_2S were also produced with the higher incubation temperature of 45 ºC. These results suggest that the high levels of H_2S detected during disturbance of stored SMC are heavily influenced by moisture content. The higher rates of H_2S production at 45 ºC also suggest that thermophilic microorganisms are involved in the process. Health and Safety aspects of H_2S production from stored SMC are discussed

Keywords: Hydrogen Sulphide, Spent Mushroom Compost, Health and Safety

INTRODUCTION

Hydrogen sulphide (H_2S) gas is a by product of the anaerobic decomposition of organic matter and is produced when stored spent mushroom compost (SMC) is disturbed and removed for spreading on land or other uses[1]. It is a toxic gas which can cause respiratory paralysis and death within a few minutes following exposure to concentrations in the region of 1000 ppm. Occupational exposure limits (OEL's) are important regulatory instruments to protect employees from exposure to toxic chemicals in the workplace. The short term exposure limit (STEL) for H_2S is currently 10 ppm for a period of 15 minutes, and for no more than 4 times a day[2].

Organic sulphur can be found in amino acids and is usually attached to organic material through carbon or ester bonds [2]. In aerobic environments, the oxidation of organic sulphur to sulphates includes the production of sulphides as an intermediate step in the mineralization process. Sulphides are easily adsorbed to organic material and are oxidized in aerobic conditions by *Thiobacillus* bacteria. In extremely low oxygen environments, bacteria such as *Clostridium*,

Salmonella and *Desulfovibrio* spp. reduce sulphate to sulphides [2, 3]. If aerobic bacteria do not oxidize the sulphides then inorganic sulphur, such as hydrogen sulphide gas, can accumulate.

Hydrogen sulphide (H_2S) is a toxic and colourless gas, which has a characteristic odour similar to rotten eggs at low concentrations. At higher concentrations H_2S is odourless and, depending on the concentration, it can cause rapid loss of consciousness, neurological and respiratory impairment or sudden death [4]. H_2S is a common cause of fatal gas inhalation exposures in the workplace. In many cases, additional deaths of co-workers occur when attempts are made to rescue the first victim [5]. Occupational exposure limits (OEL's) are important regulatory instruments to protect employees from exposure to toxic chemicals in the workplace. In Europe, there are two OEL's in place for H_2S exposure to protect workers against adverse effects of this toxic gas: the time-weighted average (TWA), which is currently 5 mg kg^{-1} for a conventional 8 hour day/40 hour week and the short term exposure limit (STEL), which is 10 mg kg^{-1} over a period of 15 minutes for up to 4 times per day [6, 7, 8].

At the end of a mushroom crop most growers steam the compost at 60-70 ºC for 8-12 hours in order to kill off all pathogens and pests. Thus the microbial population of steamed SMC is considerably lower than un-steamed SMC. In addition, fresh SMC that has been steamed has a higher moisture content compared to fresh SMC that has not been steamed. The mushroom growing substrate is then removed and is stored as SMC until needed. During storage outdoors, the SMC is exposed to rain and wind (evaporation) so the moisture content may fluctuate depending on the weather during storage. SMC stored under cover is largely protected from rainfall but the open sides of the storage barns will allow evaporation to occur. Thus SMC stored indoors, under cover is likely to be drier than SMC stored outdoors. The primary objective of this work was to monitor the emissions of H_2S from stored SMC at existing commercial storage sites at the time when the SMC was being disturbed and removed for spreading on nearby farmland. A secondary objective was to identify what factors, if any, may influence the concentration of H_2S produced from SMC under controlled laboratory conditions.

MATERIALS AND METHODS

SMC Storage Sites. Four commercial SMC storage sites were selected and H_2S emissions were monitored when the SMC was being disturbed and removed for land spreading or other uses in 2009. Sites 1 & 2 were outdoors and consisted of heaps of SMC stored on a concrete platform with retaining side walls to approx 3 m. Sites 3 & 4 were indoors and consisted of heaps of SMC stored under cover in an open-sided Apex-roofed barn with concrete floor and retaining side walls to approx 3 m. SMC was delivered weekly to each site and, on the day of H_2S measurement, the heaps consisted of material varying in age from 1-8, 1-12, 1-6 and 1-12 months for Sites 1- 4, respectively. Average monthly rainfall during the storage period was 97, 116, 102 and 90 mm, for Sites 1-4, respectively (some rainfall lands on SMC stored undercover via the open sides of the storage barns). On the day when H_2S was measured, as the day progressed the SMC was removed from older to younger material at the outdoor sites and from younger to older material at indoor sites. The age of the SMC was estimated based on heap dimensions. SMC samples (3-5 kg) were taken throughout the day at middle depths of each heap for moisture content determination (n = 25, 9, 45 and 20 for Sites 1-4, respectively). The temperature of the SMC heap at middle depths was measured throughout the day at 28, 18, 25 and 25 locations for Sites 1-4, respectively, as the SMC was being progressively removed.

H_2S Measurement. Hydrogen sulphide (H_2S) gas production was monitored during the disturbance of the heaps of SMC which had been stored for up to 12 months. QRAE (www.raesystems.eu) and ITX (www.indsci.com) gas monitors with data logging facilities were

used to measure the 10-second average concentration of H_2S released into the air above the heaps of SMC as they were being disturbed.

Laboratory Studies. A 6 kg sample of fresh un-steamed SMC was collected in June 2009 from the mushroom research unit at Teagasc at the end of a mushroom crop. The crop was then steamed at 70 ºC (compost temperature) for 8 hours to kill off any pests and pathogen and, when it had cooled down, a further 6 kg sample of fresh steamed SMC was then taken. . The moisture content of the fresh un-steamed SMC was 69% and of the steamed SMC was 74%. Each batch of SMC was subdivided into 3 x 2 kg sub-samples and the moisture content of two of the subsamples was adjusted to give higher moisture contents as indicated in Table 1.

Table 1. Moisture content of SMC samples used in laboratory experiments

	% moisture content		
	Sub sample 1	**Sub sample 2**	**Sub sample 3**
Un-steamed SMC	69 (fresh)	75	80
Steamed SMC	74 (fresh)	80	85

The SMC samples were then stored at 4 ºC until needed for laboratory experiments in June and July 2009. Four separate experiments were conducted:

- un-steamed SMC at different moisture contents incubated at 35ºC,
- un-steamed SMC at different moisture contents incubated at 45ºC,
- steamed SMC at different moisture contents incubated at 35ºC and
- steamed SMC at different moisture contents incubated at 45ºC.

In each experiment SMC (140 g) was placed in a sterile 500 ml glass bottle. The cap of each bottle was fitted with an airtight tube that could be connected directly to an ITX gas monitor. Three replicates were prepared for each SMC at each moisture content The prepared bottles of SMC were incubated for a total of three days. An ITX gas monitor was used to measure the H_2S gas concentration in the head space of the bottles once every 24 hours over 3 days.

Personal Safety. A full face gas mask (EN 136:1998 CL 1) fitted with a H_2S filter code EN 141 A1B1E1K1 (www.northsafety.com) was worn during H_2S measurements.

RESULTS AND DISCUSSION

Maximum peaks of H_2S concentration of 680, and 2083 ppm were detected during disturbance of 4-12 month-old, outdoor-stored SMC (Sites 1, and 2, respectively), in comparison to maximum peaks of 687, and 89 ppm for indoor-stored material of the same age (Sites 3, and 4, respectively) (Table 2). Most SMC in Ireland is stored for between 4-12 months so these results highlight a significant risk of H_2S exposure for those working with SMC during its disturbance and removal from the storage site. Younger SMC (1-3 months) generally produced lower H_2S readings (280-610 ppm) compared with older material but exposure to these concentrations would still be considered a health and safety risk. The lowest concentrations of H_2S were detected at indoor Site 4, which was a much smaller heap of SMC compared to the others, and this would suggest that smaller heaps are better from a health and safety perspective.

Outdoor stored SMC had a higher average moisture content (69-70%) compared to indoor stored material (61-65%), reflecting the fact that it would have absorbed any rainfall during the storage period. This is likely to have had a significant influence on the peaks of H_2S detected at the outdoor locations as H_2S would be produced more readily in the wetter, more

anaerobic, SMC. The SMC heap that had been exposed to the highest monthly rainfall (Site 2) had the highest H_2S levels.

The average internal temperature of the outdoor-stored SMC heaps was lower at 28-30 ºC compared to the indoor stored heaps at 37-46 ºC. This suggests that there may have been greater microbial activity occurring in the indoor stored SMC, compared to the outdoor stored material, or that more heat was retained within the heap when there was a protective roof overhead. The large indoor-stored SMC heap at Site 2 had a much higher temperature than all other heaps and this factor may have contributed to the high levels of H_2S detected during its disturbance

Further studies on outdoor and indoor stored heaps of SMC, where heap size, storage conditions and SMC age are controlled, would highlight more clearly the relative importance of individual factors on H_2S emissions.

Table 2. Characteristics of SMC heaps and peaks of H_2S detected during disturbance and removal.

Site No	Type of storage	Heap size (approx) m^3	Age of SMC (months)	Date of Visit	Average [1] % moisture (Min-Max)	Average[1] Temperature (Min-Max)	Max H_2S conc. (ppm) SMC Age: 1-3 months	Max H_2S conc. (ppm) SMC Age: 4 -12 months
1	Outdoor	2000	1 - 8	Feb-09	69 ± 1.9 (66-72)	30 ± 6.7 (22-41)	280	680
2	Outdoor	2300	1 - 12	Feb-09	70 ± 1.4 (68-72)	28 ± 3.3 (23-33)	610	2083
3	Indoor	1800	1 - 6	Oct-09	65 ± 3.7 (55-68)	46 ± 3.3 (41-54)	395	687
4	Indoor	600	1 - 12	Oct-09	61 ± 7.3 (43-68)	37 ± 3.7 (30-43)	49	89

[1] Mean ± standard deviation; (Min-Max) = minimum and maximum values.

In the laboratory studies, H_2S gas was detected in the head space of the sealed bottles after 24 to 48 hours. More H_2S was produced from un-steamed SMC incubated at 35°C, compared to steamed SMC (Fig. 1), suggesting that the steaming process reduced the sulphate-reducing bacteria (SRB) population significantly. The level of H_2S produced from both the un-steamed and steamed SMC incubated at 45°C were more similar, suggesting that although the numbers of sulphate reducing bacteria may have been reduced by steaming, they survived the steam treatment through the formation of endospores, which would allow the SRB population to rapidly re-establish itself in the SMC under favourable thermophilic growth conditions.. The fact that H_2S production from steamed SMC was highly enhanced at 45°C compared with 35°C confirms (a) the thermophilic nature of the SRB population and (b) that the SRB population responds favourably to the process of steaming.

Increasing the moisture content of the fresh SMC (un-steamed or steamed) to 80% significantly increased the H_2S production by approximately three to eight fold. This highlights the fact that by increasing the moisture content, the SMC becomes more anaerobic, resulting in higher levels of H_2S production. However, when the moisture content was increased to 85% the H_2S accumulation in the head space of the incubation vessels was reduced to very low levels. Although it might have been expected that H_2S production at 85% moisture content would be

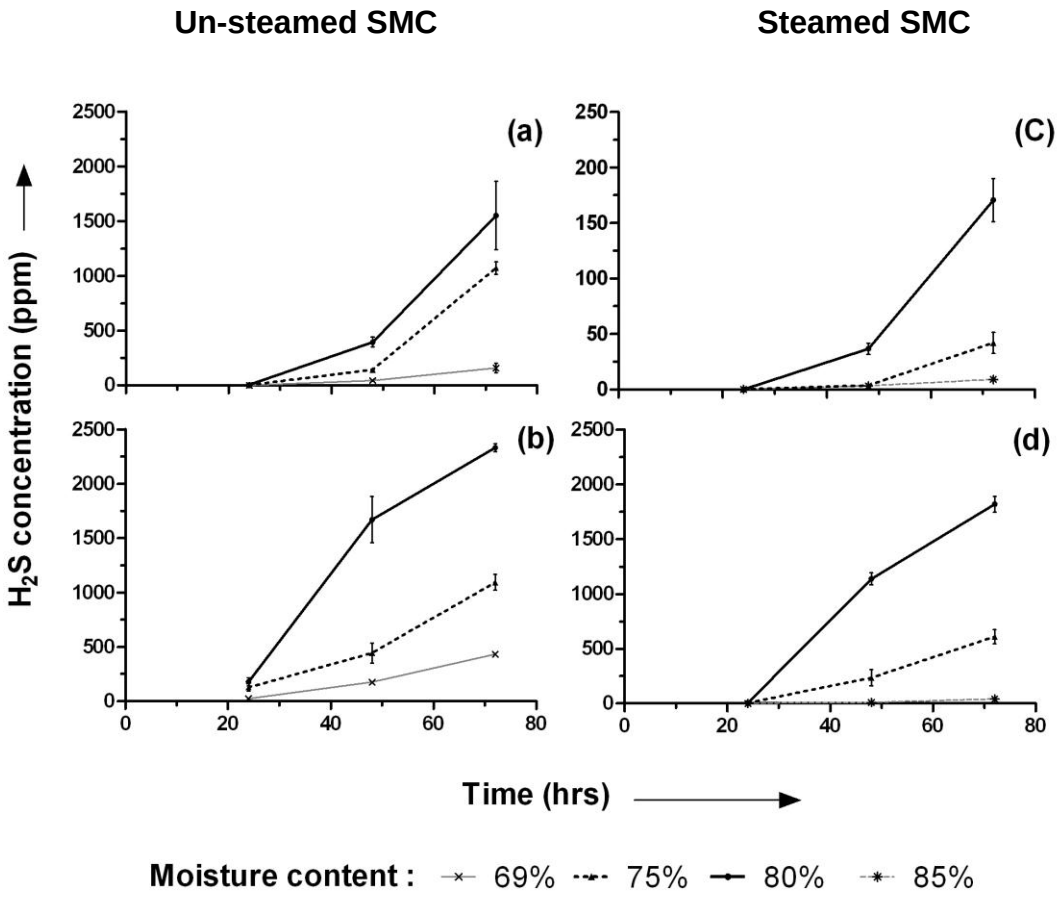

Figure 1. H_2S production from un-steamed and steamed SMC during incubation at 35°C (a and c) and 45°C (b and d).

very high, the SMC in this treatment was semi-liquid in consistency. It is likely therefore, that any H_2S, produced by the 85% SMC, was trapped within the semi-liquid substrate and not released into the head space of the incubation vessel. It is possible that high H_2S concentrations would have been detected if the 85% SMC had been physically disturbed, allowing the release of H_2S.into the atmosphere.

Further research is required to confirm the role of thermophilic SRB in H_2S emissions from stored SMC

CONCLUSION

The level of H_2S released from stored SMC during its disturbance is influenced by its age as well as its moisture content and temperature. The practise of steaming mushroom compost, before its disposal and storage as SMC, does not eradicate populations of SRBs; they recover rapidly, especially if the SMC temperature increases above 35°C. The best conditions for SMC storage to minimise high levels of H_2S during disturbance would appear to be in small heaps under cover where the lower moisture content and moderate temperature do not enhance H_2S production.

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TRICHOLOMA GIGANTEUM- A NEW TROPICAL EDIBLE MUSHROOM FOR COMMERCIAL CULTIVATION IN INDIA

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ABSTRACT

The varied agro climatic conditions of India offer greater scope for cultivation of a variety of mushrooms. During the year 2007, two milky mushroom (*Calocybe indica)* strains- Ci (P), Ci (N), and *Tricholoma giganteum* were collected from Coimbatore and Erode districts of Tamil Nadu. Trials conducted during 2008-10 showed that *Tricholoma* recorded significantly higher yield of 177 % bioefficiency compared with existing commercial isolate of *Calocybe indica*. The mushroom *Tricholoma* has been partially sequenced using ITS primers 1 and 2 and it shares 91 % homology with *Tricholoma giganteum* and is given with Gen bank accession number 120872. This mushroom can be best cultivated using paddy straw substrate through out the year in sunken blue poly houses (250 gauge thickness of siplaulin) with three feet depth to maintain a temperature range of 30-35°C and relative humidity of more than 70 % during the cropping period. This mushroom contains the essential macro, and micronutrients, carbohydrates, protein, fibre content and also possess antioxidant activity. Taste of the mushroom is excellent, with good odour and aroma and has shelf life of 3-4 days under room temperature and 5-6 days under refrigerated conditions. Six trials were conducted during the year 2010, where *Tricholoma giganteum* recorded significantly higher yields with bioefficiency of 164 to 174 %. Identification and domestication of wild mushroom strains for commercial production offers great scope for increasing the mushroom productivity of the country.

Key words: *Tricholoma*, bioefficiency, blue silpaulin polyhouse

INTRODUCTION

Mushroom production represents one of the most commercially important steps towards diversification of agriculture based on microbial technology for large-scale recycling of agro-wastes in an agricultural country like India. Commercial production of edible mushrooms represents unique exploitation of the microbial technology for the bioconversion of the agricultural, industrial, forestry and house-hold wastes into nutritious food. Out of about 2,000 edible fleshy fungi, 20 types are artificially cultivated and about 10 are being produced and marketed in sizeable quantities across the world [1]. In India, at present, four mushroom varieties viz., *Agaricus*

bisporus, Pleurotus spp., *Volvariella* spp. and *Calocybe indica* have been recommended for the year round cultivation. The Indian subcontinent is known worldwide for its varied agro climatic zones with a variety of habitats that favour rich mushroom biodiversity [2]. About two decades ago, *Calocybe indica* P. & C. was identified as a wild edible mushroom in India. . Only limited attempts were made for its cultivation until 1998 [3, 4, 5, 6]. However, complete commercial production techniques were evolved for the first time in Tamil Nadu [7].

Tricholoma giganteum Heim, a new edible mushroom pure white in colour resembling the morphology of *Calocybe indica,* was reported growing widely in summer in Indo-gangetic plains of Howrah district, Hooghly in India [8]. The possibility of commercial cultivation of *Tricholoma lobayense* was already explored in Tamil Nadu during 2002 [9]. The simple production techniques, substantial and sustainable yield, increased shelf life, attractive color, flavor and shape are the attractive features of this new edible mushroom. As a new introduction to the edible mushroom world, no doubt that our country has greater prospects and potentiality to exploit this mushroom. Development of mushroom strains well adapted to the hot climatic plains of India with suitable simpler cultivation technology, higher yield potential and prolonged shelf life are the present day needs of commercial cultivation. Keeping all these things in mind, the research work was initiated to find out the variations on the yield performance(*i.e.* bioefficiency) existing among the different strains of *C.indica* and *T. giganteum* under field conditions and to identify a better strain/species for commercial cultivation that would suit the hot climatic zones of our country. In order to identify a better strain well adapted to the hot climatic plains of India with suitable simpler cultivation technology, higher yield potential and prolonged shelf life, the present investigation was conducted.

MATERIALS AND METHODS

Collection of mushroom strains. During the year 2007, two milky (*Calocybe indica*) mushroom strains Ci (P) and Ci (N) and *Tricholoma* were collected from Erode and Coimbatore districts of Tamil Nadu. The mushrooms were pure cultured from the cap using tissue culture method and maintained on Potato Agar slants and used for further studies.

Growth and yield performance studies of wild mushroom strains. The Growth and yield performance studies of wild mushroom strains was conducted at the Mushroom Research and Training Centre, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. A milky mushroom variety- *Calocybe indica* var.APK 2 was identified and released as a commercially cultivated species from Tamil Nadu Agricultural University, Coimbatore during 1998. It was used for comparison. The cultures of different strains of wild *C.indica* Ci (P) and Ci (N) collected from different places and *Tricholoma giganteum* were inoculated onto sorghum grains for spawn production. Using the sorghum-based spawn, cylindrical beds were prepared using paddy straw as substrate (1 kg of paddy straw/bed) .Four to six holes were made on the sides of the beds for aeration. The beds were incubated at room temperature of 28$\pm$2˚C for spawn running. After complete spawn run, the beds were cut into two equal halves and steamed casing soil (garden soil) was applied uniformly over the spawn run beds. The moisture level was maintained by regular water spraying on beds. The cased beds were incubated in partially sunken poly houses roofed with blue coloured high density polythene sheet, where a temperature of 30-35˚C with 75-80% relative humidity was maintained. The observations on Days For Spawn Run (DFSR), Days

For Pin head Formation (DFPF), Days For First Harvest (DFFH), Days to complete three harvests (total cropping period), total yield, and bio efficiency was recorded or calculated.

$$\text{Bio efficiency (\%)} : \frac{\text{Total weight of harvested mushrooms}}{\text{Dry weight of the substrate used}} \times 100$$

Molecular characterization

Isolation of DNA from *Tricholoma.* The mushroom was grown in malt extract broth and the mycelial mat was collected and ground with lysis buffer. After maceration the tube was kept in room temperature for 30 min and 150 µl of potassium acetate was added, vortexed for 2-3 seconds and kept in freezer for 30 min. The tubes were centrifuged at 15, 000 rpm for 5 min. The supernatant was transferred to another tube and equal volume of isopropyl alcohol was added. The tube was mixed by inversion and centrifuged at 15,000 rpm for 2 min. and the supernatant was discarded. The DNA pellet was washed in 300 µl of 70 % ethanol and centrifuged at 10,000 *g* for one min. and the supernatant was discarded. The pellet was air dried and dissolved in 50 µl of 1X TE buffer and used as genomic DNA for PCR reaction [10].

Sequencing of *Tricholoma* . The genomic DNA extracted from the pure culture of *Tricholoma* was used for PCR studies. The Polymerase Chain Reaction primers, ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were used to amplify the ITS of ribosomal DNA which encompasses the 5.8 sRNA gene and both ITS -1 and ITS-2 regions [11]. The PCR reaction mixture (50 µl containing 1 U of Taq DNA polymerase, 5 µl of 10X PCR buffer,160 µM each of dATP, dCTP, dGTP, dTTP (MBI Fermentas), 50 pM each of ITS-1 and ITS-4 primers, 2 µl of 5 % glycerol and 40 ng of genomic DNA) were performed in a mater cycler with PCR conditions consisting 34 cycles of 1 min denaturation at 95°C, 30 s annealing at 50˚C, 1 min 20s elongation at 72˚C and ending by 10 min final elongation step at 72˚C with lid heating option at 110˚C [10]. Amplified products were run on 2 % agarose gel, stained with ethidium bromide and visualized under UV illumination. The sequencing was done using ITS-1 (forward) and ITS-4 (reverse primer) and the nucleotide sequence comparisons were performed using Blast Multiple Alignment Tool (BLAST) network sequences against the National Centre for Biotechnology Information (NCBI) database.

Studies on the nutritive value of *Tricholoma giganteum.* Moisture contents of mushrooms were estimated by drying 50 g of fresh mushrooms in an oven at 80^{o}C for three consecutive days. It was later cooled in a desiccator and reweighed. The moisture content was arrived from the differences in the weight [12]. The crude protein content of the mushroom was estimated by Micro Kjeldahl method [13]. The total carbohydrate content was determined by following anthrone method [14]. Estimation were made of ascorbic acid [15], crude fat [12], total phenolic content using the Folin-Ciocalteu method [16], crude fibre [17], total ash content (18), total nitrogen analyzed by Diacid extract method-semiautomatic Kjeldahl distillation, total phosphorous by Triacid extract method- vanodamolybdate calorimetric method, total potassium and total calcium [19]. Antioxidant activity was measured using Ferric reducing antioxidant power (FRAP) assay [20] as described previously. All nutrients and ingredients were analyzed on dry weight basis. The micro nutrients were expressed in mg /kg. The experiments on the

estimation of nutritive values were performed at the Post harvest Technology Centre at Tamil Nadu Agricultural University, Coimbatore-3.

Performance testing of *T. giganteum* in Farmer's field at different locations. Different strains of *C. indica* and *T.giganteum* were subjected to growth and yield performance trials at three commercial mushroom farms *viz.,* (Farm 1 -Sujiie Mushroom Farm, Farm 2 -Sun Mushroom Farm, Farm 3- Kongu Mushroom Farm of Erode district) as per the method described earlier. The cropping room conditions of 30-35˚C with 75-80 % relative humidity were uniformly maintained in all the farms. The performances of the mushrooms were tested in different commercial farms at different locations so as to test/judge the acceptance of the consumers. The observations on days for spawn run (DFSR), days for pin head formation (DFPF), days for first harvest (DFFH), days to complete three harvests (total cropping period), total yield, and bioefficiency were recorded. Harvested mushrooms were cleaned and packed (250 g) separately in perforated polythene bags and placed under natural conditions (room temperature).Under refrigerated condition they were placed in non-perforated polythene bags and observations on keeping quality were recorded.

All the experiments conducted during the study were laid out based on completely randomized block design (CRD). Statistical software (AGRES) was used for the analysis of the data.

RESULTS AND DISCUSSION

Collection of mushroom strains and their morphology. The *Calocybe indica* strains *viz.,* Ci (P), Ci (N) had long stipe measuring 3-6.7 cm in diameter with centrally attached stipe similar to the commercially cultivated *Calocybe indica* var.APK 2. The stipe of *Tricholoma* is sub globose, short, 3.9- 4.2 cm with small hair like white outgrowths on the stipe initially and in matured mushrooms the stipe looked smooth at the time of harvest (approximately five days after pin head formation) (Fig. 1).

Figure 1. Morphology of *Tricholoma giganteum*

Yield performance studies of wild mushroom strains. The mushroom was cultivated in partially sunken polyhouses roofed with blue coloured high-density polythene sheet, where a temperature of 30-35˚C with 75-80 percent relative humidity was maintained (Figure 2.) A maximum mean yield of 884.9 g/bed with a biological efficiency of 177% was observed for various strains of *T. giganteum* and is compared to *Calocybe indica* var. APK2 (Table 1).

Figure 2. Cultivation of *T. giganteum*

Table 1. Performance of wild *C. indica* isolates and *T. giganteum* at the Mushroom Research Laboratory, TNAU, Coimbatore.

Strain	DFSR	DFPF	DFFH	Yield (g/500g dry paddy straw)	Bio efficiency (%)
C. i(APK2)	14.6	8.3	13.3	727.0	145.3
C. i(N)	16.6	10.3	15.3	662.3	132.4
C. i(P)	14.6	7.3	12.3	861.2	172.4
T. giganteum	14.3	7.6	12.6	884.8	176.9
CD at (0.05)				12.28	
SEd				5.92	

Pooled mean of two trials, SEd: Standard Deviation

Molecular characterization. Amplification of the ITS regions of *Tricholoma* with IT -1 and ITS-4 primers showed 91 % homology with *T.gignateum.* The sequences were submitted to NCBI and given Gen Bank accession number 120872.

Performance testing of *T. giganteum* at farmer's location. This study was conducted in different farms at different locations under controlled conditions (with temperature range of 30-

35˚C with 75-80 % relative humidity) so as to test the yield performance and the consumers acceptability of the mushroom species.

The results of yield trials conducted at Farm 1 indicated *T. giganteum* as the best performer based on maximum mean yield (820 g /bed) and bio efficiency (164%) compared to *C. indica* var. APK2 with 172% bioefficiency (Table 2). Also, at Farm 2, *T.gignateum* performed with significantly higher yield of 847 g/ bed and bioefficiency of 169% compared to *C. indica* var. APK 2 (827 g/ bed; 165% bioefficiency) (Table 3). At Farm 3, T. *gignateum* recorded a yield (870 g/ bed; 174% bioefficiency) on par with *C. indica* var. APK 2 (Table 4). In all three trials, there was no variation in DFSR, DFPF and DFFH. The nutritive values analyzed for *P. djamor* showed the presence of all essential nutrients with a calorific value of 19.8 Kcal/100 g of fresh mushrooms (Table 5).

No incidence of pest and disease was recorded. The mushrooms could be stored under room temperature for two days and under refrigerated storage for 6 days without any microbial spoilage and liquefaction. The mushroom *T. giganteum* resembles milky mushroom in morphology. However, the stipe is sub-globose in *T. giganteum* where as in milky mushroom the stipe is elongated. The possibility of commercial cultivation of a new edible mushroom, *Tricholoma lobayense* closely resembling *C. indica* was reported [21].

Table 2. Yield performance of wild *C. indica* isolates and *T.giganteum* at Farm 1**.**

Strain	DFSR	DFPF	DFFH	Yield (g/500g dry paddy straw)	Bio efficiency (%)
C. i (APK2)	15.3	8.6	13.3	804.0	160.8
C. i (N)	16.3	10.3	15.3	682.6	136.5
C. i (P)	14.6	7.6	12.00	730.49	146.0
T. giganteum	14.3	7.3	11.3	820.0	164.0
CD at (0.05)				14.25	
SEd				6.80	

Mean of four replications , SEd: Standard Deviation

Table 3. Yield performance of wild *C. indica* isolates and *T.giganteum* at Farm 2.

Strain	DFSR	DFPF	DFFH	Yield(g/500g dry paddy straw)	Bio efficiency (%)
C. i (APK2)	14.0	8.6	13.00	827.0	165.4
C. i (N)	16.0	10.3	15.3	693.8	138.8
C. I (P)	14.3	8.3	13.3	740.7	148.1
T. giganteum	13.6	7.3	12.3	847.0	169.4
CD at (0.05)				12.33	
SEd				5.23	

Mean of four replications, SEd: Standard Deviation

Table 4. Yield performance of wild *C. indica* isolates and *T.giganteum* at Farm 3.

Strain	DFSR	DFPF	DFFH	Yield(g/500g dry paddy straw)	Bio efficiency (ù)
C. i (APK2)	14.6	8.6	13.6	866.0	171.2
C. i (N)	17.0	10.6	15.3	617.0	123.5
C. I (P)	14.6	7.6	12.0	771.0	154.2
T. giganteum	14.3	7.3	11.6	870.0	174.0
CD at (0.05)				9.91	
SEd				4.68	

Mean of four replications, SEd: Standard Deviation

Table 5. Nutritive value of *Tricholoma giganteum*

Parameter	Nutritive values on dry weight basis
Moisture$	86.20
Crude protein#	32.9
Carbohydrate#	11.8
Crude Fat#	0.91
Crude fibre#	20.71
Ash#	8.32
Iron*	5.60
Manganese*	1.18
Zinc*	1.38
Copper*	1.10

$ - Presented in fresh weight basis
# - % dry weight basis
* - Presented in mg/kg (dry weight basis)

CONCLUSION

Development of mushroom strains well adapted to the hot climatic plains of India with suitable simpler cultivation technology, higher yield potential and prolonged shelf life are the present day needs of commercial cultivation. Being a tropical mushroom, *T. giganteum* has greater scope for commercial exploitation throughout the globe. The simple production techniques with sustainable yield, increased shelf life, attractive color, flavor and shape are the special features of this new edible mushroom.

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NEW CULTIVATION TECHNOLOGY FOR PADDY STRAW MUSHROOM (*Volvariella volvacea*)

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ABSTRACT

Paddy straw mushroom (*Volvariella volvacea*) is a world famous edible mushroom variety that has high demand due to its deliciousness and nutritive value. Although there is a big demand in Sri Lanka, there was no proper method of cultivation since it needs high temperature (33-35 $^{\circ}$C) and 85-90 % relative humidity in the growing environment.

Therefore, an experiment was designed at Regional Agricultural Research and Development Centre, Department of Agriculture – Makandura during the year 2009 to develop an indoor (poly house) cultivation method that would maintain above environment requirements. The outdoor method was compared with an indoor method in RCBD. Paddy straw and cotton waste were used separately as main composting materials.

The yield of indoor cultivation method of cotton waste and paddy straw were 5.38 kg/m^2 and 4.71 kg/m^2 respectively, while yields for the same parameters in outdoor cultivation method were 1.79 kg/m^2 and 1.73 kg/m^2. There were significant yield differences between indoor and outdoor method in both composting materials.

It is concluded that the indoor method can be successfully used to cultivate paddy straw mushroom profitably in Sri Lanka.

Key words: Indoor cultivation; Paddy straw mushroom

INTRODUCTION

The mushroom defined as “a macro fungus with a distinctive fruiting body, large enough to be seen with the naked eye and to be picked up by hand” (Chang and Miles, 1992). In a narrow sense, the world mushroom also refers only to the fruit body. Unlike green plants, mushrooms are heterotrophs. Not having chlorophyll, they cannot generate nutrients by photosynthesis, but take nutrients from outer sources. Fungi cultivation develops with the advance of science and technology. In history, human beings only hunted and collected wild mushrooms at the beginning. After a long period they observed and learned how to cultivate fungi. Presently, mushrooms have become popular throughout the world since they have wonderful food and medicinal values. The local demand for mushrooms is also steadily increasing [2].

Population increases are creating an alarming situation in the food supply in Sri Lanka. Malnutrition in terms of 'protein' deficiency is becoming a major hazard in developing countries. Exploiting non-traditional food resources can make a substantial breakthrough to meet the serious food deficit. In this circumstance, popularizing mushroom as part and parcel of every day food is

of paramount importance. No food is so wrapped in mystery as mushroom. It is amazing to see tiny pin heads on a composting medium growing into buttons rich in protein, vitamins and minerals. Not only because that mushrooms have a marvelous medicinal values. Mushroom is a protein source without cholesterol. On the other hand, they have an ability to maintain the blood cholesterol at the optimum level. In addition to that there are some other medicinal values that also can be found in mushrooms [3].

Mushroom farming is becoming successful because of its very low inputs. In Sri Lanka, mushroom cultivation is highly rewarding because of the prevailing climatic conditions. The technology profitably is located in areas where land is limiting factor and agricultural residues are abundantly available.

Although the history of the world mushroom cultivation goes back to thousands years the commercial mushroom cultivation in Sri Lanka was started in the middle of 1980's with the cultivation of Oyster (*Pleurotus ostreatus*). However, compared to other countries technological improvements of mushroom cultivation are not satisfactory in Sri Lanka. Oyster, Paddy straw (*Volvariella volvacea*) and Milky (*Calocybe indica*) mushrooms are some of the high potential cultivable mushrooms in Sri Lanka since they can grow well under tropical and subtropical condition.

Paddy straw mushroom is a popular variety among people because of its distinct flavor, pleasant tastes, higher protein content and shorter cropping duration compared to other cultivated mushrooms. Presently, Sri Lanka imports canned paddy straw mushrooms from China and this mushroom is available in most of the supermarkets. It originally grows in rice straw stack in tropical and sub tropical zones that have high temperature and a rainy climate. Chinese growers developed its cultivation more than 300 years ago. Therefore, it was named "Chinese Mushroom" (Zhanxi and Zhanhua, 2000). *Volvariella* requires a high temperature (35 ± 2 ºC) for better and early hyphal growth. Also 32±2 ºC and 80-90 % RH (relative humidity) are needed for the formation of fruiting bodies [4]. The yield of straw mushroom depends on the cultivation methods and compost (growing) medium. Prior to 1970, rice straw was practically the only material used for preparing the growing medium for *Volvariella volvacea.* Straw alone is not sufficient as a composting material as it contains a little quantity nutrients and has a slow rate of decomposition [5]. Therefore, straw mushrooms presently are grown in some other materials such as cotton waste, sugar cane bagasse, dried banana leaves, oil farm bunch waste etc [4]. However, paddy straw is the material freely available in Sri Lanka and therefore, this cultivation is ideal in rural area where paddy straw is abundant after each paddy harvest and it can provide additional income.

An outdoor cultivation method was introduced for paddy straw mushroom by the Department of Agriculture in the middle of the 1980's. However, farmers are reluctant to produce mushrooms using this method because of the uncertainty of production with irregular and low yield, due to difficulties to control environment factors such as temperature, RH and pest problems. Unlike the oyster mushroom, the straw mushroom is highly sensitive to the climatic conditions and their fluctuation. To overcome these problems, indoor cultivation method under controlled environment may be an effective alternative [6].

Therefore, an experiment was conducted to identify the suitability of an indoor cultivation method for paddy straw mushroom under Sri Lankan conditions, using locally available low cost raw materials.

MATERIALS AND METHODS

The experiment was conducted at the Regional Agricultural Research and Development Centre, Makandura, Sri Lanka from January to July 2009. The site belongs to Law Country Intermediate Zone of Sri Lanka where the average day and night temperatures are 31.9 ºC and 22.4 ºC respectively with an annual rainfall of more than 1,400 mm. Two types of growing media were prepared using paddy straw and cotton waste. Both growing media were tested in indoor and outdoor (existing method) systems. Cost was also calculated for each treatment.

Indoor cultivation. . Composts (growing media) were prepared using paddy straw and cotton waste. Paddy straw composting was done by soaking dried paddy straw (50 kg) in a 2% lime solution for four hours and excess water was allowed to drain. Paddy straw was spread on a clean cement flow (platform) as a thin layer. Urea (1%), gypsum (5%), measured on dry weight of paddy straw were evenly spread on the wetted straw and mixed well to prepare compost medium. A heap was built using the compost medium and it was covered by a black polythene tarp having 300 gauge. The heap was turned twice in two days intervals. Therefore, compost was ready to use after six days. The method of composting of cotton waste was similar to paddy straw and the excess water in soaked cotton waste was removed manually by squeezing.

A barrel-roof-type polythene house (length – 8 m, width – 3 m, height – 3m) was constructed using transparent polythene having 300 gauge. Pre-prepared compost layer was packed (15 cm thick) into a wooden frame (90 x 30 x 30 cm) and surface of the compost was inoculated with 125 g of spawn. The remaining 15 cm of the wooden frame was filled with same amount of compost and another 125 g of spawn was applied on the surface. Then the wooden frame was removed by pulling upwards remaining a block of inoculated compost. The same method was used for both paddy straw and cotton waste compost. The blocks were prepared keeping a 30 cm space between each bed. Finally, the blocks were covered with black polythene (300 gauge) for five days and afterwards removed for fruiting.

Outdoor cultivation. The same composting and block preparation methods were practiced for the outdoor cultivation method. However, the blocks were kept in an open area close to the polyhouse. The blocks were covered with black polythene (300 gauge) for five days as was done also in the outdoor method and afterwards removed for fruiting.

The experiment was arranged in a RCBD (Randomize Complete Block Design) with three replicates and treatments were as follows. Five beds (blocks) were in each treatment.

Treatment 1 – Paddy straw compost in poly house
Treatment 2 - Cotton waste compost in poly house
Treatment 3- Paddy straw method in outdoor (control)
Treatment 4– Cotton waste compost in outdoor

Mushrooms were harvested at the egg stage before opening in the morning and afternoon. Beds were sprayed with sufficient water twice a day (8 a.m. and 4 p.m.). Minimum and maximum temperatures inside the poly house and open area (outdoor) were recorded and bed temperatures (just below the surface) and RH were also recorded daily at 3 p.m. after removing the black polythene for fruiting. Total number of mushrooms, number of marketable mushrooms, total weight of mushrooms, and weight of marketable mushrooms were recorded daily.

RESULTS AND DISCUSSION

Temperature and relative humidity. Maximum and minimum temperatures inside the polythene house were high compared to the open area (Fig. 1). Similarly, relative humidity was at fairly suitable level for paddy straw mushroom throughout the fruiting period, (Table 1).

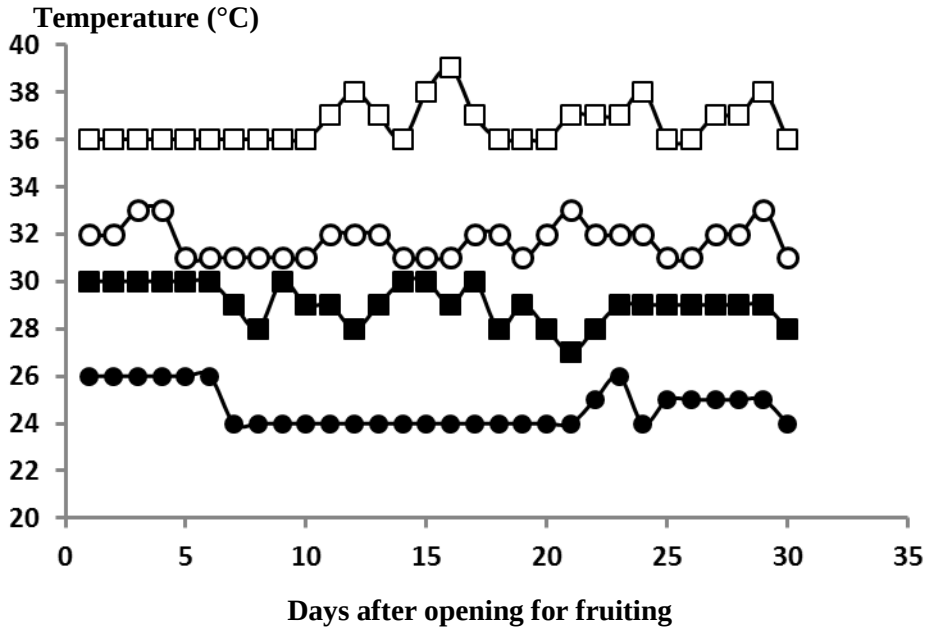

Figure 1: Maximum and minimum air temperatures.
■: indoor, minimal temperature, □: indoor, maximal temperature, ●: outdoor, minimal temperature, ○: outdoor maximal temperature

Table 1: Average minimum and maximum temperature and relative humidity observed during the cultivation period.

Item	Indoor	Outdoor
Maximum temperature	36.53	31.53
Minimum temperature	28.46	23.86
Relative humidity	84%	65%

Bed (block) temperature. Bed temperatures inside the polythene house were high compare to the open area. Increase of the temperature of the beds after the watering was slow in open area compare to the polythene house (Fig. 2).

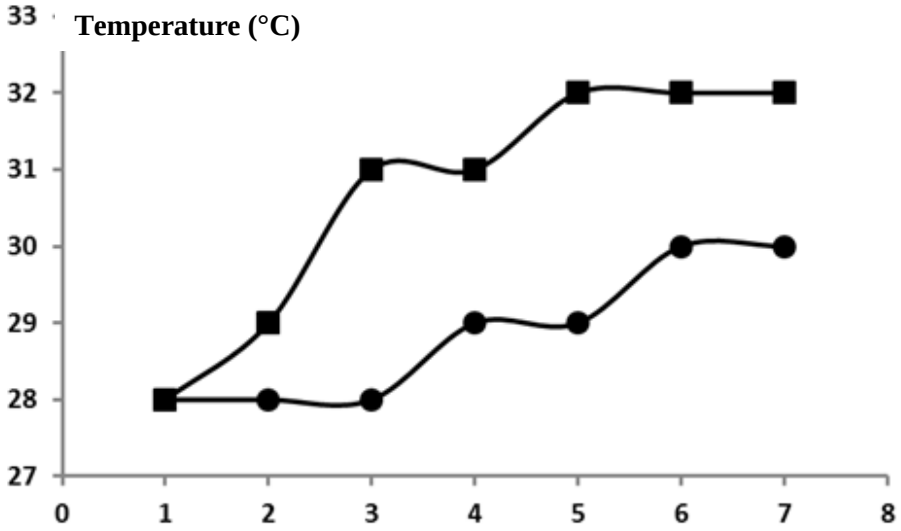

Figure 2: Bed (block) temperature after watering
■: indoor, ●: outdoor

Yield. A significantly higher yield (5.38 kg/m^2) was observed in cotton waste medium in indoor method (T-2) while the lowest value (1.73 kg/m^2 block) was observed from paddy straw in outdoor method (T-3) (Table 2).

Table 2. Fruit bodies (egg stage mushroom) weight of each treatments in (kg/m^2)

Treatments	R-1	R-2	R-3	R-4	Means
T-1	4.8	4.6	4.7	4.7	4.71 [a]
T-2	5.3	5.9	5.1	5.2	5.38 [a]
T-3	1.8	2.1	1.6	1.4	1.73 [b]
T-4	1.9	1.7	1.8	1.7	1.79 [b]

Means followed by the same letters in each treatment are not significant at 5% probability level

Significant differences in the number of fresh mushrooms were observed among four treatments at 5% probability level. The highest number of fruits (49.7 kg/m^2) was recorded in cotton waste medium in indoor method while the lowest (17 kg/m^2) was given by the paddy straw compost in out door method. (Table 03)

Table 3. Number of fresh mushroom harvested per m^2

Treatments	R-1	R-2	R-3	R-4	Means
T-1	48	52	45	31	44.0 [a]
T-2	46	54	50	49	49.7 [a]
T-3	17	14	10	27	17.0 [b]
T-4	20	13	14	13	15.0 [b]

Means followed by the same letters in each treatment are not significant at 5% probability level

Economical balance. The study reveals that the indoor cultivation method with cotton waste compost substrate gives the highest yield and indoor cultivation method with paddy straw compost also resulted higher yield compared to the existing outdoor cultivation method. But, highest benefit:cost ratio was observed with the paddy straw in indoor method since additional labor cost needed to prepare the cotton waste medium (Table 4).

Table 04 Cost of different treatments and income related to the yields

Treatments	Material cost in Rs	Labor cost in RS	Depreciation value of poly house in Rs	Total cost in Rs	Income in Rs	Economical balance : income/costs
T-1	3500	5000	2000	10 500	29 437	2.80
T-2	4500	6000	2000	12 500	33 625	2.69
T-3	3500	5000	0	8 500	10 812	1.27
T-4	4500	6000	0	10 500	11 187	1.06

- Labour charges 500 Rs / day
- Two years warranty for the growing house
- Price of 01 kg of fresh mushroom – 250 Rs
- 115 Rs equal to one USD

The poly house was able to maintain day temperature around 36 °C, relative humidity at 84% and provided shade needed for mushroom production during the experimental period giving ideal conditions for straw mushroom cultivation. Outdoor cultivation does not provide the necessary environmental controls as indoor cultivation method. Requirement of the additional initial cost to construct growing house is the main drawback of indoor cultivation method.

CONCLUSION

Indoor cultivation method can be used to cultivate paddy straw mushroom profitably in Sri Lanka using both paddy straw and cotton waste growing media.

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RECENT ADVANCES ON BACTERIAL DISEASES OF CULTIVATED MUSHROOMS

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ABSTRACT

Recent studies on bacterial diseases of cultivated mushrooms in southern Italy showed that brown blotch of *Agaricus bisporus* and the yellowing of *Pleurotus ostreatus,* caused by *Pseudomonas tolaasii,* are actually complex diseases since, besides *P. tolaasii,* which may be considered the main causal agent of the above diseases, also *P. reactans* and not yet characterised fluorescent pseudomonads appear to participate to the expression of the diseases symptoms. Furthermore, *P. reactans* appears to be the causal agent of *P. eryngii* yellowing. Strains of *P. tolaasii* produce tolaasin I and II (Tol I and Tol II) and five minor analogs called tolaasins A-E). Tolaasins are involved in the pathogen virulence causing membranes lysis through biosurfactant activity and transmembrane ion channel formation for which a barrel-stave mechanism has been proposed. Furthermore, strains of *P. tolaasii* produce *in vitro* an array of volatile substances which are apparently involved in the virulence of the producers and the pathogen/mushrooms interaction. Strains of *P. reactans* produce the White Line Inducing Principle (WLIP) which causes the brown discoloration of *A. bisporus* tissues though at lesser extent than tolaasin I. The loss of WLIP production by avirulent morphological variants of *P. reactans* supports its role in the pathogen virulence. The formation of avirulent variants in the cultures of *P. reactans* strains appears to be responsible for the attenuation/loss of virulence and this may tentatively explain why the pathogenicity of *P. reactans* was neglected and/or not well understood in the past. A comparative evaluation of Tol I and WLIP on blood red cells and artificial lipid vesicles demonstrated a detergent-like mechanism for WLIP. REP-PCR analysis showed that *P. reactans,* on the contrary of *P. tolaasii,* is not a genetically uniform group.

Keywords: *Agaricus bisporus*; *Pleurotus ostreatus*; *Pleurotus eryngii*; *Pseudomonas tolaasii*; *Pseudomonas "reactans".*

INTRODUCTION

World production of cultivated mushrooms is growing and, in fact, from 1,500,000 tons in 1985 [1] it rose to 3,206,738 tons according to FAO data in 2007, with China as the first world producer followed by United States of America and several European Countries. The Italian annual production of cultivated mushrooms is about 90.000 tons [2], and the most cultivated mushroom is the button mushroom *Agaricus bisporus* (Lange) Imbach followed by the oyster mushroom *Pleurotus ostreatus* (Jacq. ex Fr) Kum and a few other mushroom species including the king oyster mushroom (cardoncello) *P. eryngii* (DC ex Fr).

Cultivated mushrooms are attacked by a number of fungal and bacterial diseases that may cause significant production losses [3, 4, 5]. These occurrences are due to environmental conditions to which the mushroom cultivation is generally carried out. Though reliable data in this respect are not available, it is believed that the bacterial diseases are the main causes of product loss [3]. Several bacterial diseases of cultivated mushrooms of the genus *Agaricus* and *Pleurotus* are caused by fluorescent pseudomonads [5]. This is the case of brown blotch

disease of *A. bisporus* [6] and the yellowing of *P. ostreatus*, both caused by *P. tolaasii* [5]. Brown blotch symptoms on *A. bisporus* are also caused by *P. reactans* [7, 8, 9, 10], *Pseudomonas* sp. strain NZ17 apparently related to *P. syringae* [11] and *P. costantinii* sp.nov. [10]. The ginger blotch disease of *A. bisporus* is caused by *P. gingeri* [12], the mummy disease of *A. bisporus* is caused by *Pseudomonas* spp. [4, 13] and the drippy gill of *A. bisporus* [14] is caused by *P. agarici.* The latter pathogen was also reported as the causative agent of the yellowing of *P. ostreatus* observed for the first time in California [15] and, more recently, of the brown discoloration of mushrooms [8, 16, 17] observed in The Netherlands and Italy.

Also the yellowing of the king oyster mushroom (cardoncello), a mushroom whose cultivation was initially limited to some regions of southern Italy but nowadays cultivated worldwide, was reported to be caused by *P. tolaasii* [18, 19] or by *P. reactans* and fluorescent Pseudomonads [8, 9 ,20]. The etiology of this disease is not yet completely established.

Bacterial disease of cultivated mushrooms caused by bacteria other than fluorescent pseudomonads are the soft rot of *Agaricus* spp. caused by *Burkolderia gladioli* pv. *agaricicola* (ex *Pseudomonas gladioli* pv. *agaricicola*) and *Janthinobacterium agaricidannosum* sp. nov [21]. *B. g.* pv. *agaricicola* is also responsible for the cavity disease. *Ewingella americana* is responsible for the internal stipe necrosis of *A. bisporus* [22, 23].

The frequent occurrence of a variety of the mushroom alterations, probably caused by bacterial infections, in cultivation of *A. bisporus*, *P. ostreatus* and *P. eryngii* in several mushroom farms located in Apulia and Basilicata, Southern Italy, prompts a series of studies with the final aim to define the agent(s) responsible for the alterations observed, as well as to study the mechanisms of host pathogen interactions. Here, we present some results on the etiology of brown blotch disease of *A. bisporus* and yellowing of *P. ostreatus* and *P. eryngii* as well as some aspects of *P. tolaasii* and *P. reactans* interaction with the mushroom hosts.

ETIOLOGICAL ASPECTS

Brown blotch disease. The brown blotch disease of the button mushroom *Agaricus bisporus*, caused by the bacterium *Pseudomonas tolaasii* [5], was observed for the first time in some mushroom farms in the United States of America [24] and its etiology was defined a few years later [25]. In Italy, the disease has been reported for the first time in 1970 in a mushroom farm in Apuglia [26]. The disease is characterized by brown, irregular, sunken lesions on the pileus and/or stipe. Under favorable environmental conditions the lesions, initially small and separated, coalesce affecting large areas of the pileus which may gradually decay with the formation of a strong and disagreeable smell. This may occur also after harvest [7].

In our experience from *A. bisporus* sporophores showing brown blotch lesions, bacterial **c**olonies with different morphologies were ever obtained [8, 9]. In particular, from the altered pseudotissues were isolated bacteria that were positive in the white line assay (WLA) [12] when grown on agar media near to strain NCPPB1311 of *P. reactans*, and these isolates, as expected, showed the nutritional, biochemical, and pathogenicity characters of *P. tolaasii* strains [27, 28]. In the same isolation plates in addition to *P. tolaasii*, other bacteria with the biochemical characteristics of the fifth group of fluorescent pseudomonads [28] were ever obtained. Some of these isolates were identified as strains of *P. reactans* since they were positive in the WLA when grown on agar media near to type strain of *P. tolaasii* NCPPB2192 and showed the nutritional characteristics of *P. reactans* [27]. The simultaneous presence of *P. tolaasii* and *P. reactans* in altered mushroom tissues very often gave rise to white line precipitates between colonies of different morphologies. *P. reactans* strains caused the browning of *A. bisporus* tissue blocks, although at different levels depending on the strains, but the effect was lower when compared to *P. tolaasii*. The suspensions still capable to cause visible alterations of tissues was 10^6 c.f.u./ml or less in the case of P. t*olaasii* and 10^7-10^8 c.f.u./ml for *P. reactans* [8, 9]. Similar results were

independently obtained by other authors [7, 29].

Other bacteria with the biochemical characteristics of the fifth group of fluorescent pseudomonads [28], not yet characterized but negative in the WLA, caused alterations of the tissue blocks in a comparable way to those of certain strains of *P. reactans* [8, 9].

Yellowing of oyster mushrooms. The yellowing of the oyster mushrooms *Pleurotus ostreatus*, a disease caused by *Pseudomonas tolaasii*, may interest all stages of development of the mushroom sporophores [3, 5, 18].When the disease occurs at the early differentiation stage the young sporophores turn to a yellowish-reddish color, show a slow development followed by a rapid wilting. The alteration can affect the whole bunch or parts of it. On developed sporophores depressed, yellowish-reddish lesions, sometimes surrounded by yellow-reddish halos may interest pilei and/or stipes. Under high temperature and humidity sporophores rapidly rot with the production of an unpleasant smell. Sometimes, yellow superficial discolored lesions may interest the whole sporophore or part of it. The disease has an unpredictable course. In the same cultivation all the substrate bags in production or only part of them may be interested by the above symptoms. In some bags the disease appears severe with a significant loss of production while in others only a change in color of sporophores is observed. The disease may affect the first flush and then disappear or vice versa; in other occasions the disease interest the cultivation throughout the period of production. Nothing is known about the factors that determine the strange course of the disease.

In our experience from *P. ostreatus* sporophores showing depressed, yellowish-reddish lesions bacterial **c**olonies with different morphologies were ever obtained. In particular, from the altered pseudotissues were isolated bacteria that were positive in the white line assay (WLA) [12] when grown on agar media near to strain NCPPB1311 of *P. reactans* and these isolates, as expected, showed the nutritional, biochemical, and pathogenicity characters of *P. tolaasii* strains [27, 28]. In addition to *P. tolaasii*, other bacteria with the biochemical characteristics of the fifth group of fluorescent pseudomonads [28] were obtained. Some of these isolates were identified as strains of *P. reactans* since they were positive in the WLA when grown on agar media near to type strain of *P. tolaasii* NCPPB2192 and, furthermore, they showed the biochemical and nutritional characteristics of *P. reactans* [27]. As expected *P. tolaasii* and *P. reactans* strains, in the pathogenicity assays on *A. bisporus* blocks, confirmed the higher virulence of the former when compared to the latter strains which in addition showed also a higher variability in this feature. Strains of *P. tolaasii* assayed for the virulence on *P. ostreatus* sporophores caused different symptoms depending on the inoculation procedure. In particular, they caused deep depressed lesions when their suspensions were injected by hypodermic syringes in the pileus flesh. In contrast, when drops of the same suspensions were deposited on the sporophore surface, only slight depressed yellow lesions were observed. When suspensions were sprayed on young not fully developed *P. ostreatus* sporophore bunches, they turned yellow, stunted and wilted. The same assay on more developed sporophore bunches caused the general yellowing followed by wilting. Only in some cases also depressed brownish-orange lesions were observed. It is not excluded these latter symptoms are consequence of bacteria penetration through micro wounds on the sporophore surface.

Strains of *P. reactans* in the above pathogenicity assays on *P. ostreatus* sporophores caused the bunches yellowing as well as sporophore treated tissues; they never caused depressed lesions. In general the virulence was lower when compared to the one caused by *P. tolaasii* strains.

Other fluorescent pseudomonads obtained in the same isolation plates beside *P. tolaasii* and *P. reactans* and negative in WLA showed a pathogenic feature similar to that of *P. reactans* strains and differed for some nutritional features from both *P. tolaasii* and *P. reactans.* They shared more nutritional characters with strains of *P. tolaasii* than with those of *P. reactans, P.*

agarici, P. gingeri and *Pseudomonas* spp. causal agents of the mummy disease used for comparison [30]. On the contrary, some of the above described fluorescent pseudomonads showed the nutritional feature of *P. tolaasii.* Apparently, the latter WLA negative isolates appeared to be hypovirulent strains of *P. tolaasii* which have lost production of the tolaasins. It is not clear if this happened being associated with the mushroom or during the isolation plate procedure.

These results indicate that all the above described bacteria appear to contribute, though at different degrees, to the development of symptoms of the yellowing of *P. ostreatus*. In particular, *P. tolaasii* cause depressed yellowish-reddish lesions. On the contrary *P. reactans* and the other fluorescent pseudomonads appear mainly responsible for the yellowish discoloration of whole or part of the sporophores. This evaluation is supported by the fact that bacteria with the feature of *P. reactans* and/or the negative WLA above described fluorescent pseudomonads were mainly obtained from superficial yellow discolored lesion on full developed sporophores or from yellow and mummified young sporophores.

Yellowing of cardoncello. The yellowing of the king oyster mushrooms (cardoncello) *Peurotus eryngii* is a disease whose etiology was not well defined for many years. Initially it was attributed to fluorescent pseudomonads related to *P. tolaasii* [18], but other studies indicated that fluorescent pseudomonads different from *P. tolaasii* were associated to the altered mushrooms [20]. The first symptoms of the disease are light brown discoloration of the pilei which then turn into reddish-brown. Stem symptoms are represented by hydropic areas, often elongated, which may coalesce, interesting the whole stems. Symptoms of the disease may interest primordia still in the casing soil layer or on fully developed sporophores. Mushroom sporophores turn stunted and wilted and then, under high temperature and humidity condition, bacterial exudates form on the sporophores which rapidly rot with the production of an unpleasant smell. The disease shows very unique features since it may occur in a disruptive way in the first flush and then it disappears resulting in normal production and vice versa; in other cases the disease may lead to the entire production loss. The disease initially can be localized to a substrate bags in production possibly interesting only a part of a mushroom bunch and then shortly the whole cultivation.

In our experience the isolation from the king oyster mushrooms sporophores showing the yellowing symptoms gave rise to different fluorescent pseudomonads. Most of them were positive in the *A. bisporus* tissue block assay [26] though the intensity of the alteration was lower than the one caused by strains of *P. tolaasii* used for comparison. Some of these bacterial isolates, obtained repeatedly in different isolations, were positive in the WLA when grown near type strain NCPPB2192 of *P. tolaasii,* belonged to the group V of fluorescent pseudomonads and showed the biochemical and the differential nutritional characters of *P. reactans* [27].

Pathogenicity assays *in vivo* on bags of *P. eryngii* commercial substrate by adding in the casing soil suspensions of strain P. NCPPB1311 of *P. reactans* naturally resistant to rifampicin allowed to demonstrate, at least in part, the Koch's postulates. From sporophores showing the classical yellowing symptoms bacteria resistant to rifampicin with the same characteristics of the *P. reactans* strain used in artificial inoculations and other not rifampicin resistant bacteria with the features of *P. reactans* as well as other bacteria belonging to group V of fluorescent pseudomonads were obtained [9,31]. In some occasion, the artificial inoculation with *P. reactans* strains was unsuccessful for so far unknown reasons.

These results indicate that *P. reactans* can be considered responsible for yellowing of cardoncello. The fact that bacteria with the feature of *P. reactans* were obtained from a wild cardoncello sporophore showing a large area of yellowing on the pileus reinforces this possibility (unpublished results). It is not excluded, however, that also the fluorescent pseudomonads, isolated together with strains of *P. reactans* may be responsible and/or contribute to the development of' the yellowing of cardoncello. In our experience from the cardoncello

sporophores with symptoms of yellowing, bacteria with the feature of *P. tolaasii* have never been obtained. In this regard other authors reported the latter pathogen as the causal agent of the "batteriosi del cardoncello" [18] or bacterial blotch of *P. eryngii* [19]. However, these authors refer to yellowish depressed blotch as the main symptom. Although further evidences are necessary it is not excluded that, as in the case of *P. ostreatus*, the different symptoms are related to the different pathogens involved. In fact, in pathogenicity assays when drops of strains of *P. tolaasii* suspensions were deposited or injected on *P. eryngii* sporophores dark brown-reddish depressed lesions were observed. In the same assays strains of *P. reactans* caused brown-reddish and superficial lesions. The latter symptoms resemble the natural ones caused by *P. tolaasii* and *P. reactans*, respectively.

ASPECT OF MUSHROOM-PATHOGENS INTERACTIONS

***Pseudomonas tolaasii* lipodepsipeptides.** *P. tolaasii* is a common pathogen of cultivated mushrooms such as *Agaricus bisporus*, *Pleurotus ostreatus* [5] and apparently *P. eryngii* [18, 19]. Virulent strains of *P. tolaasii* produce the lipodepsipeptides (LDPs) tolaasins which appear to be responsible for symptom development [32, 33].

Two components of the toxin preparations, designed tolaasin I (Tol I) and tolaasin II (Tol II), were first identified [34]. The primary structure of Tol I bears a β-OH octanoic acid blocking group at the N-terminus, a sequence of seven successive D-amino acids at the N-terminal region of the peptide (Pro 2 –Val 8), with a Ser-Leu-Val repeat, and then alternate L- and D-amino acids. It also contains a 2,3-dehydro-2-aminobutyric acid □(ΔBut) residue at positions 1 and 13, a D-homoserine (Hse 16) and a D-2,4-diaminobutyric acid (D-Dab 17). Finally, a lactone ring is formed between the hydroxyl of D-Thr 14 and the C-terminal L-Lys 18. Tol II differs from Tol I for the amino acid in position 16th in the peptide moyety: homoserine in Tol I and glycine in Tol II. More recently five other tolaasin analogs, called tolaasins A–E, have been isolated from the culture filtrate of type strain NCPPB2192 of *P. tolaasii* [35]. These tolaasin analogs showed chemical modifications in the fatty acid residue or in the lactone macrocycle. In particular, Tol A, D and E as well as tol II showed a modification in one or two aminoacidic residues at position 15 and/or 16 in respect of Tol I while tolaasin B exhibited a different fatty acid residue linked to the N-terminal residue. Tol C, showing the opening of the macrocyclic lactone ring, could be an artifact hydrolysis product of Tol I. However, this hypothesis is not confirmed, as the corresponding linear product originated from the other tolaasins was not found. The antimicrobial activity of HPLC grade tolaasins A-E, assayed in comparison with tolaasin I and II showed the gram-positive bacterium *Bacillus megaterium* as the most sensible test micro-organism used in the study [35]. In fact, all the analogs, but not tolaasin C, inhibited the grown of *B. megaterium* though differences among their specific activities were observed. The most active analogs appear to be tolaasin D followed by tolaasin I and II with a minimal inhibitory quantity of 0.16, 0.32 and 0.64 μg. Tolaasins A, B, and E resulted less active, with a minimal inhibitory quantity of 1.28 and 2.56, respectively. The antimicrobial activity of tolaasins appeared to be modulated by the structural modifications present in the different natural analogs of either Tol I or II. In fact, the opening of the lactone ring occurring in the case of Tol C eliminated the antibacterial activity [35].

Tolaasin appeared to be synthesized, as several other microbial LDPs, by a peptide synthetase complex [36] and its production and efficiency is mediated by an extragenomic factor [37].

Tolaasins disrupt the *A. bisporus* plasma membrane and vacuole membranes through the ion channel forming activity [32, 38] and the biosurfactant activity [39]. Two types of ion channels both inhibited by Zn^{2+} were identified by the incorporation of tolaasin into lipid bilayer [40]; Ni^{2+} inhibited the pore activity of Tol I but the mechanism is not yet determined [41].

Using model membranes of different lipid compositions a barrel-stave mechanism of action of Tol I, based on a valuable increment in the helical content of the LDP which was inserted in the membrane core and oriented parallel to the lipid acyl chain, was proposed [42]. Tol I and Tol II showed similar activities. The other tolaasin analogues were instead remarkably less active (up to 15 times when compared to tolaasin I) both on red blood cell and model membranes [43].

***Pseudomonas reactans* lipodepsipeptide.** *P. reactans*, beside *P. tolaasii*, is responsible for the brown blotch of *Agaricus bisporus*, the yellowing of *Pleurotus ostreatus* [5] and *P. eryngii* [8, 9]. Virulent strains of *P. reactans* produce an extracellular substance called the white line-inducing principle (WLIP) a LDP composed by the N-terminal β-hydroxydecanoic acid and a peptide moiety composed of nine aminoacids, six of which are in the D-form. The molecule contains a lactone ring between D-allo-threonine and N-terminal L-isoleucine [44]. Comparative antimicrobial assays of WLIP, produced by *P. reactans* strain NCPPB1311, and tolaasin I, produced by *P. tolaasii* type strain NCPPB2192, showed that both LDPs inhibited the growth of fungi - including the cultivated mushrooms *A. bisporus, Lentinula edodes* and *Pleurotus* spp. - chromista and Gram-positive bacteria. Assays of the two LDPs on blocks of *A. bisporus* showed their capacity to alter the pseudo-tissues of the mushrooms though WLIP was less active than Tol I. Nevertheless, *P. reactans* NCPPB1311, at least *in vitro*, produced more WLIP than the quantity of Tol I produced by type strain NCPPB2192 of *P. tolaasii*, suggesting that the lower antimicrobial activity of WLIP was compensated by the higher quantity produced in culture [45]. Contrary to previous studies, Tol I was found to inhibit the growth of Gram-negative bacteria belonging to the genera *Escherichia*, *Erwinia*, *Agrobacterium*, *Pseudomonas* and *Xanthomonas*. The only Gram-negative bacterium affected by WLIP was *E. carotovora* subsp. *carotovora*.

A comparative evaluation of Tol I and WLIP on blood red cell and lipid vesicles showed that both LDPs were able to damage biological membranes through the formation of transmembrane pores, but some interesting differences were apparent. The conformation of WLIP changed slightly when it passed from the buffer solution to the lipid environment. The LDP had an insufficient length to pass through the entire membrane and exhibited a permeabilizing activity in the same range of that of detergents, suggesting a detergent-like activity for WLIP [42].

Further, though preliminary, experiments on lipid vesicles using solutions of Tol I and WLIP in different ratio provide evidences of an interaction of the two LDPs leading to an apparent antagonistic action [43].

The pathogenesis of *P. reactans* on the host mushrooms (*A. bisporus*, *P. ostreatus* and *P. eryngii*) is far to be understood but the antifungal activity of WLIP together with the finding that avirulent morphological variants of *P. reactans* lack WLIP production [9] suggest that WLIP may play an important role in the interaction of the producing bacterium *P. reactans* and cultivated mushrooms [45].

***Pseudomonas tolaasii* volatile substances.** Strains of *P. tolaasii*, besides tolaasins, produce *in vitro* other biological active substances which appear to be important in the biology of the pathogen and in the *Pseudomonas tolaasii*/mushrooms interactions. In fact, it was demonstrated that virulent strains of *P. tolaasii* produce *in vitro* an array of volatile substances (VOCs) which inhibit the growth of *Agaricus bisporus* and *Pleurotus ostreatus* mycelia as well as they cause the brown discoloration of *A. bisporus* and *P. ostreatus* pseudotissues blocks. Furthermore, this pool of volatile substances inhibited the seed germination and/or seedling growth of several cultivated herbaceous plants (lettuce, broccoli) and several word-wide diffused weeds (unpublished results). GS-MS analysis of the VOCs showed the complexity of the volatile mixture. On average dimethyl sulfide and methanethiol were found the main compounds of the mixture. These pure substances in a concentration/effect figure, though with at a different

concentration range, reproduced the above toxic effects on either mycelia, pseudotissue blocks of *A. bisporus* and *P. ostreatus* as well as on the seed germination and/or seedling growth of several cultivated herbaceous plants. The antifungal activity of these substances clearly suggested a potential role of these substances in the *P. tolaasii* mushrooms interactions in different phases of *A. bisporus* and *P. ostreatus* cultivation. Furthermore, the possible technological exploitation of this feature is not excluded. However, other studies appear necessary.

CONCLUSION

The results here reported clearly indicate that brown blotch of *Agaricus bisporus* and yellowing of *Pleurotus ostreatus*, reported to be caused by *P. tolaasii*, appear to be complex diseases caused by several bacteria. In particular, *P. tolaasii*, to be considered the main and more aggressive pathogen, as well as *P. reactans* and other not yet characterized fluorescent pseudomonads, appear to contribute, though at different degrees, to the expression of the above disease symptoms. So far, it is not yet clear whether the two pathogens are the causal agents of the yellowing of *P. eryngii*. It is not excluded that in the different cultivation conditions (mushroom strain, cultivation procedure, substrate composition, environmental condition, etc) one of the two pathogens may be prevalent and responsible for the yellowing caused by *P. reactans* and blotch disease caused by *P. tolaasii*. As a matter of the fact in our experience never *P. tolaasii* was isolated from *P. eryngii* specimens showing the symptoms of the yellowing. Of interest is the definitive characterization of *P. reactans*, only in part addressed in the past, which appears, on the basis of preliminary RE-PCR analysis, not to be a homogeneous genetic group. Further biochemical, nutritional and genetic characterization of a collection of *P. reactans* strains in comparison to *P. tolaasii* are in progress. Efforts leading to the pathogen classification appear necessary too.

The different level of virulence in *P. tolaasii* and *P. reactans* strains seems to parallel the different level of biological activity of tolaasins and WLIP which, though with a different mechanism, have cell membranes as target for their toxicity.

Of interest is the evaluation of the role of volatile substances produced by strains of *P. tolaasii* in the blotch symptom expression and in the pathogen-mushrooms interactions.

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MUSHROOM VIRUS X – THE IDENTIFICATION OF BROWN CAP MUSHROOM VIRUS AND A NEW HIGHLY SENSITIVE DIAGNOSTIC TEST FOR PHASE III COMPOST

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ABSTRACT

Mushroom Virus X (MVX) first observed in 1990 causes a range of yield and quality symptoms and is associated with double-stranded RNA (ds-RNA). Evidence is accumulating that MVX is caused by a collection of different viruses. Previous research has centred largely on ds-RNAs; this paper presents research based on transcript changes during MVX infection.

Suppression subtractive hybridization and microarrays identified 25 up-regulated and 32 down-regulated genes during MVX infection. Sixteen highly up-regulated transcripts were absent from the *A. bisporus* genome and hybridize to a subset of MVX ds-RNA elements and so are inferred to be viral. It is hypothesized that the mushroom cap browning symptom is caused by a distinct *Partitivirus*-like virus of two ds-RNAs, tentatively named Brown Cap Mushroom Virus (BCMV).

A second project was aimed at developing a highly sensitive detection method for MVX at low levels in compost, casing, and potential sources of infection. The current test based on separation of ds-RNAs on gels is insensitive requiring whole mushroom fruitbodies. All known MVX and mushroom genes were screened using microarrays and Quantitative PCR to identify the best indicator genes. This resulted in a combined PCR test based on two sequences which detects both forms of MVX (browning and pinning disruption symptom) at low levels in compost.

These quantitative results revealed that small amounts of early infection leads to 100-fold increases of transcript levels in the compost during cropping. Viral transcript levels were lower in the casing indicating low viral activity compared with compost.

Keywords: MVX; ds-RNA; Virus; Diagnostic test; Transcription

INTRODUCTION

The Mushroom Virus X (MVX) syndrome has been present in the industry for at least 20 years and reached epidemic levels some 5-6 years later. It was documented in 2000 by Gaze [1] who described the spread of the disease from 1996. However Gaze has since found earlier detailed notes of the description of disease symptoms which accurately match those of MVX from August 1990 (Richard Gaze, personal communication). Gaze observed that the symptoms were found on shelves, were specific to bulk compost and that similar symptoms had been observed previously. MVX disease causes a diverse a range of symptoms, including pinning disruption, crop delay, premature veil opening, various fruitbody abnormalities, and discoloured mushroom

caps, ranging from off-white to brown (which for the sake of clarity will be referred to here as the brown symptom). These effects on yield and quality have resulted in economic difficulties and even farm closures. The disease symptoms differ by geographical location – the 'patch' problem (discrete areas without mushrooms) more prevalent in the UK while browning symptoms are seen more in Ireland, Belgium, The Netherlands and Poland. This suggests that the term "MVX" is a catch-all term to describe more than one disease with uncharacterised causal agents of unknown origin.

The association between MVX disease and double-stranded RNA (ds-RNA) led to the hypothesis that a novel ds-RNA virus may be the cause of MVX disease however viral particles could not be observed by electron microscopy [1], [2]. Grogan identified 26 ds-RNA bands separated by gel electrophoresis with different banding patterns occurring in different samples [2]. The maximum number of ds-RNA bands from a single sample was 17. Two research groups found an association between the four low molecular weight ds-RNA bands (0.6- 2 kbp) and the browning symptom but no obvious correlation between the banding patterns and other symptoms [2], [3]. The authors conclude that it was not possible to prove Koch's postulates and so it was not possible to prove that the disease was caused by the ds-RNA molecules [2]. As transmission electron microscopy failed to reveal viral particles consistently associated with the disease, it was concluded that the infective agents are ds-RNAs, non-encapsidated, naked genetic elements [2].

Rao *et al* proposed a bacterial explanation for the cause of the brown symptom of MVX disease [4]. These authors suggest that *Pseudomonas tolaasii* may be the cause for the symptom as this bacterial species was detected on the skin of diseased mushrooms. *P. tolaasii* is the causative organism of 'brown blotch' disease on mushrooms manifesting as discrete sunken lesions with brown discolouration. This bacterium produces an 18 amino acid peptide called tolaasin which forms pores in plasma membranes which allow mixing of the contents of intra-cellular compartments [5], [6]. Tissue browning caused by disease such as brown blotch disease, mechanical damage or senescence is the result of oxidation of phenolics by the enzymes tyrosinase which is subject to activation by proteinases. The phenolics, tyrosinase and proteinase are believed to be located in different cellular compartments and the tolaasin or mechanical damage and ageing allow these components to mix and so the browning reaction takes place [7]. However, the Rao hypothesis was questioned on the grounds that the symptoms of bacterial blotch disease caused by *P. tolaasii* and MVX disease are very different in several aspects and that the association between MVX disease symptoms and the presence of *P. tolaasii* is from a limited sample size [8].

It is now clear that the collection of MVX ds-RNAs represents a complex of different viruses which accounts for the diversity of symptoms. Sonnenberg and Lavrijssen found that the sequence of a portion of the 17 kbp band shared significant similarity with *Cryphonectria parasitica* hypovirus [3]. The 12.75 kbp band of MVX has also been sequenced and described as an Endornavirus, AbEV1 [9]. Strong associations were made between the 4 smaller ds-RNAs and the browning symptom, and it was speculated that these bands may represent a single virus [2], [3].

Transmission of the MVX viruses is thought to be via infected spores and/or infected mycelium and symptom expression from mycelial infection appears to depend on the time and degree of infection and type of the infecting mycelium [2], [10]. Hygiene measures involving these principles have resulted in some limited success in controlling the disease. However MVX still represents a largely uncharacterised disease with little known about the causative agents. The current detection method for MVX by visualizing gel separated double-stranded RNA bands has shed some light on the disease and its epidemiology. However the method has low sensitivity (requiring whole mushroom fruitbodies) and so it is too late in the cropping cycle to be commercially useful and is unable to identify low level sources of infection.

This paper describes two research projects aimed at further understanding of the biology of MVX by examination of the transcript changes (single-stranded RNA) produced during infection, and then using the findings to develop a highly sensitive Quantitative PCR test to detect MVX at low levels in compost.

MATERIALS AND METHODS

To examine the transcripts changes in the mushroom after MVX infection and to develop a high-sensitivity detection method, five molecular methodologies were employed.

Suppression Subtractive Hybridization (SSH). RNA was isolated from non-infected, and brown coloured MVX-infected mushroom fruitbodies (strain A15) using TRI reagent, and enriched for poly A m-RNA. Subtractive hybridisation was performed followed by suppressive PCR to enrich for differentially expressed transcripts. Comparisons were made between RNA from non-infected and infected fruitbodies in both directions to identify transcripts which increase and decrease upon infection. Each gene fragment putatively identified as differentially expressed by SSH was Sanger sequenced.

Custom Microarrays. Two custom microarray designs were used in these studies. The first of these (version 3) contained probes to 1,300 genes/transcripts identified from the SSH work (above), other research projects from the Burton research group (from initiation, diooxygenase and post-harvest studies) and 550 *Agaricus bisporus* genes from the EMBL data-base. Version 4 microarrays were based on the 60,000 probe format and consisted of probes to the above 1,300 genes (replicated 3 times) and the 10,438 Open Reading Frames ORFs, (5 oligo probes per ORF) identified from annotation of the *A. bisporus* genome sequencing project (public release in May 2010). The microarrays were manufactured by Agilent Technologies and the probes were 60-mer *in situ* synthesized on the array. RNA was isolated from mushroom samples, converted to cRNA, fluorescently-labeled and then hybridized to a microarray. To determine the amount of hybridization to each probe the arrays were laser scanned and the resulting images analysed using commercially available image analysis software. Comparisons between treatments were made and statistically analysed using GeneSpring software (Agilent Technologies).

For crop experiment one ('Identification of transcripts changing during MVX infection'): 3 batches of non-infected mushrooms were compared with infected mushrooms from 5 different commercial farms. Four sample replicates were used for each batch/treatment.

For experiment two ('Development of a PCR-based test to detect MVX infection in compost'): microarray screening examined 4 different developmental states of the mushroom (i.e. as fruitbodies and as mycelium growing in axenic culture, compost and casing) and 2 infection states (MVX infected and non-infected). Four replicates per treatment.

Quantitative reverse transcriptase-PCR (Q-PCR). RNA samples were DNase treated (RQ1 RNase free DNase, Promega) according to manufacturer's instructions. DNase treated samples then underwent reverse transcription using Superscript II (Invitrogen) reverse transcriptase according to manufacturer's instructions producing cDNA samples ready for PCR analysis.

Primers were designed, using Primer Express (Applied Biosystems), to specifically amplify sections of each of the identified bands/transcripts based on their previously established sequence. These primers were then optimised using Sybr Green for detection and the ABI 7900HT thermocycler (Applied Biosystems). Standard curves were run, which were assessed for linearity and efficiency to ensure accurate quantification.

Bioinformatics. Transcript sequences identified during the course of these experiments were compared with known sequences available on open access and proprietary data-bases. Comparisons were made with *A. bisporus* genome sequence after its public release in May 2010.
Autogradiograph Northern methodology is described in the text.

RESULTS AND DISCUSSION

Identification of transcripts changing during MVX infection

Suppression Subtractive Hybridization (SSH). SSH analysis successfully generated putative differentially expressed transcripts associated with MVX disease. These transcript fragments were sequenced and a total of 197 unique sequences were identified. Probes were designed to the sequences and incorporated onto the microarray.
Custom Microarrays. Changes in gene expression from infected mushroom from 5 different farms were compared with non-infected mushrooms. Statistical analysis of all the microarray data identified 25 transcripts up-regulated during MVX infection and 32 down-regulated during infection. Of the 25 up-regulated transcripts, 16 were found by comparison with the *A. bisporus* genome sequence to be not derived from the *A. bisporus* host and are therefore to be presumed viral. All 32 down-regulated transcripts were host genes.

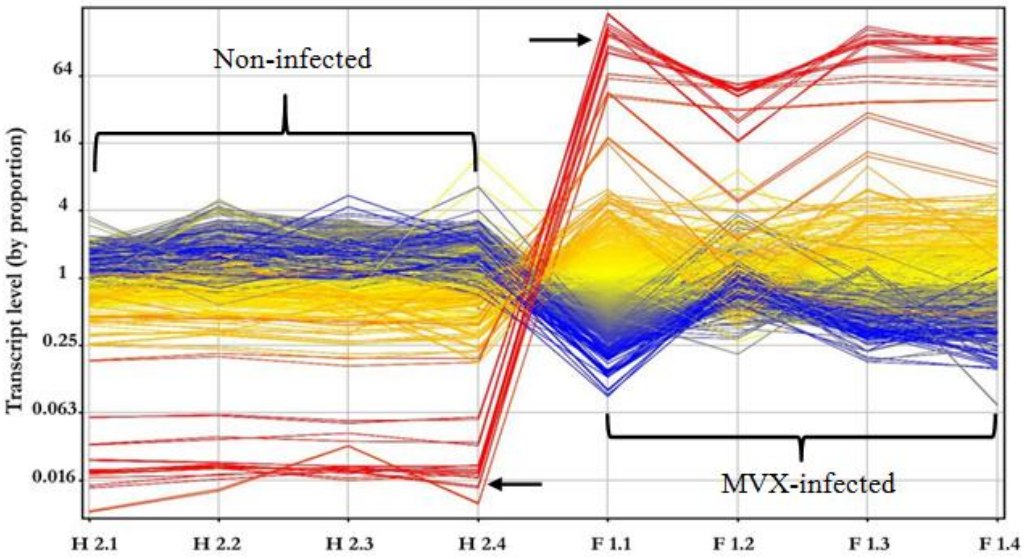

Figure 1: The expression of transcripts in non-infected and MVX-infected mushrooms determined by version 3 microarrays. Four separate non-infected samples were compared with four MVX-infected samples. Expression values are shown as normalised values relative to average expression. Y-axis is shown on $\log_2$ scale.

A typical microarray result is shown in Figure 1 which presents gene expression of four control (non-infected) mushrooms with four infected mushrooms from farm number one. The figure also demonstrates that most transcripts show little or no change in expression levels between infected and non-infected states. However there is a group of presumed viral transcripts (indicated with arrows in figure 1) which are at very low levels in non-infected mushrooms and very high levels in infected mushrooms. These up-regulated transcripts show average increases in infected fruitbodies from 150 to 4,000 fold and were found to accumulate to very high levels

in MVX-infected brown mushrooms and at lesser levels in white infected mushrooms. There appeared to be a correlation between the degree of tissue discoloration and the level of transcript.

Quantitative reverse transcriptase-PCR. The microarray analysis of the 10 transcripts showing the greatest change was confirmed independently by Q-PCR, however the fold change between non-infected and infected samples was much higher for the Q-PCR data compared with microarrays reflecting the greater dynamic range of PCR (Table 1).

Table 1: Comparison of the average fold-change values (MVX-infected relative to non-infected) of transcript levels between microarray and Quantitative PCR (Q-PCR) analyses

Viral transcript	Fold-change values	
	Microarray	Q-PCR
1	4,020	240,100
2	3,700	88,000
3	3,470	24,200
4	2,790	240,400
5	2,770	1,060,000
6	2,650	106,000
7	2,550	179,000
8	1,730	3,010,000
9	1,130	83,000
10	150	105,100

Autoradiograph Northern blots. Experiments were then performed to ascertain whether the 16 up-regulated and presumed viral transcripts originated from ds-RNA bands. Double stranded-RNA was extracted from a number of mushrooms showing a diversity of symptoms (to maximise the number of ds-RNA bands). The ds-RNAs were then gel separated in a number of identical tracks and blotted and fixed onto a membrane. Each track was then dissected out and separately hybridised with a radiolabelled probe specific to each of the 16 transcripts. The position of the probe was detected by autoradiography and compared against reference gel and markers.

All 16 probes hybridised onto ds-RNA bands as discrete bands. This strongly suggests that these transcripts are encoded by ds-RNA and so are presumably of viral origin. All of the hybridisations were to bands of size of 2 kbp (band 18) or less. These include the ds-RNA bands previously associated with tissue browning and 5 additional bands revealed by the greater sensitivity of radioactive detection used in autoradiography. These transcripts were originally identified from the SSH experiment comparing fruitbodies non-infected and infected with the browning form of MVX. Twelve of the 16 probes hybridised to two or more bands, indicating close sequence similarity between many of the bands and suggesting that the smaller ds-RNAs probably originate from the larger ones.

The hybridisation results suggest that the functional infective unit may be just the 2.0 and 1.8 kbp ds-RNAs (bands 18 and 19 respectively). These are of sufficient size to contain the coding potential required for independent existence. The lower molecular weight bands are likely to be satellite ds-RNAs which represent fragments of the 2.0 and 1.8 kpb molecules which are replicated with the 2.0 and 1.8 ds-RNAs but do not have any coding function [11]. Satellites can

be capable of modulating disease expression [12]. It is hypothesized that the 2.0 and 1.8 kbp molecules represent a single virus in the *Partitivirus* class and it is proposed that this should be named Brown Cap Mushroom Virus, BCMV, [13], Green *et al*, paper in preparation). Typically a *Partitivirus* has two ds-RNA components coding for an RNA-dependent RNA polymerase (RDRP) enzyme and the coat protein. Recently a virus which causes brown discoloration in *Flammulina velutipes* has been characterised by sequencing and named *F. velutipes* Browning Virus [14]. This virus consists of two ds-RNAs, 1.9 and 1.7 kbp encoding putative RDRP and coat proteins. Data-base comparisons of the limited sequences of the *A. bisporus* BCMV investigated in this study failed to find clear similarity to RDRP or coat protein. Full sequencing of all of the MVX bands is currently underway using Next Generation Sequencing technology.

Development of a PCR-based test to detect MVX infection in compost

All of the available sequences for *A .bisporus* and MVX were screened against non-infected and MVX infected samples to identify gene sequences which could be used in a PCR test to detect the presence of MVX in mycelia from a range of growth environments. Whole genome microarrays (version 4) were used for this experiment based on the 60,000 probe format. RNA was extracted from non-infected and MVX infected fruitbodies, compost and casing taken from farms and experimental crops and also from axenic (laboratory) culture. These were hybridised to microarrays and the results compared between non-infected and infected samples.

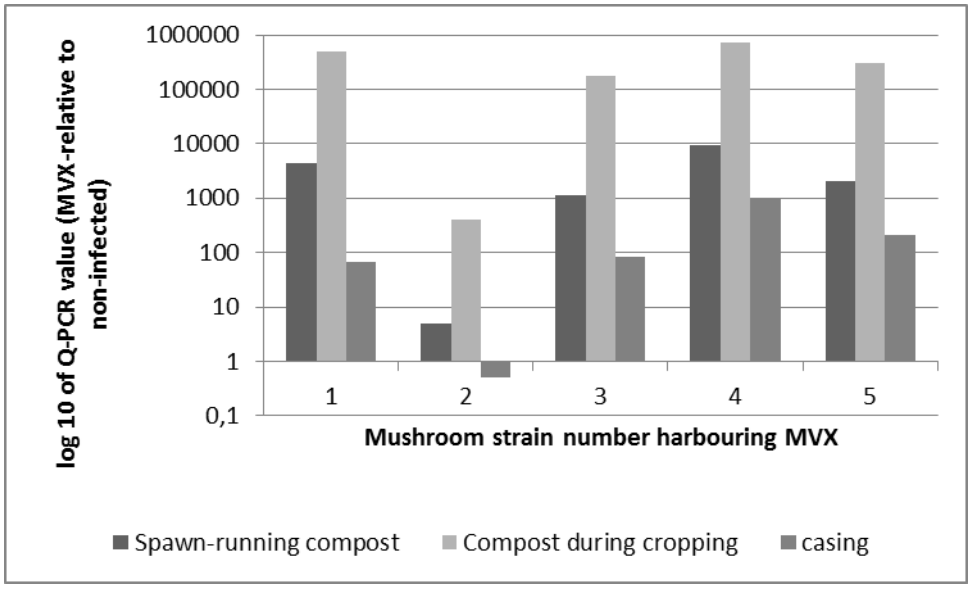

Figure 2: Bar chart showing transcript levels (Q-PCR data) of MVX-infected mycelia sampled from different growth environments: spawn-running compost, compost during cropping and casing. The data shown are the increase in Q-PCR signal relative to non-infected controls on a $\log_{10}$ scale. The five strains were originally collected from commercial farms; strains 1-3 cause the 'patch' symptom and strains 4 & 5 produce the browning symptom.

A number of probes showing at least 100-fold difference between control (non-infected) and MVX infected samples were identified: 97 probes in fruitbody samples; 102 probes in mycelium of colonised casing; 88 probes in mycelium of colonised compost; and 31 probes in mycelium grown in axenic culture. All but three of the identified probes related to non-*Agaricus* genome transcripts i.e. probably of MVX origin. The 3 genes of *Agaricus* origin were identified

from the compost samples. A selection was then made of 8 genes that showed the most consistent and significant up-regulation and the best 'spread' of detection across the range of MVX types examined.

PCR primers were designed to the 8 selected genes based on the full sequence known for each band or gene. Using these primer pairs Quantitative PCR reactions were carried out with the RNA extracted from samples of compost (during spawn-run and cropping) and casing of the 5 strains harbouring MVX and the control non-infected mushroom strain. The five strains used harbour different types of MVX, three strains causing the MVX 'patch' symptom (a discrete area with no mushroom production) and two strains producing the brown symptom.

A combined test of two quantitative PCR reactions (based on different primer pairs) was found to be successful in detecting all of the MVX forms that were investigated. The quantitative results of this project also show that small amounts of early infection at the time of spawning leads to massive increases (100 fold) in infection in the compost during cropping (Figure 2). This strongly infers that any amount of infection, no matter how small, is likely to lead to serious disease development. The transcript levels of the virus in the casing were consistently low suggesting that MVX does not thrive in the mycelium of casing and that compost is the better substrate for testing for MVX (Figure 2).

CONCLUSIONS

The hypothesis is made that the browning symptom of MVX is caused by a distinct virus consisting as a minimum of a 2.0 and 1.8 kbp ds-RNAs in the *Partitivirus* class. These ds-RNAs are actively transcribed to very high levels and satellites of these molecules are also present in infected tissues but it is unknown what effect these have on disease progression or symptom development.

A new diagnostic technique based on PCR, has been developed that can detect both forms of MVX (browning symptom and pinning disruption symptom) at low levels in spawn-run or Phase III compost. This test can be *predicative* to detect the presence of MVX in compost providing advanced warning to growers (i.e. before cropping), it can be used to identify the sources of infection and it could be used to certify compost as MVX-free.

This research is now focussed on fully sequencing all of the bands which will allow the viruses to be characterised and classified, and diagnostic tests will be developed for all ds-RNAs and viruses.

ACKNOWLEDGEMENTS

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CAN VOLATILES EMITTED BY COMPOST DURING SPAWN RUN BE USED TO DETECT GREEN MOULD INFECTION EARLY?

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ABSTRACT

In recent years green mould (*Trichoderma aggressivum*) has presented big problems to the Dutch mushroom industry. *T. aggressivum* infects compost at a very early stage and in the Dutch situation infection most likely takes place at the compost yard. Even though compost producers in the Netherlands are very keen to prevent green mould problems, occasionally still a number of crops get infected. Therefore there is a need for a reliable method that allows early detection of *Trichoderma* green mould.

Although qPCR methods have been developed for quantitation of *T. aggressivum*, these cannot be used for detection in compost. In the Netherlands spawn run is performed in bulk at the compost yards and is referred to as phase 3 composting. During this process, spawned compost is incubated in tunnels and ventilated with large volumes of air to control compost temperature. During this process the compost is inaccessible for sampling.

Literature data showed that *Agaricus bisporus* and *T. aggressivum* use volatiles to affect each other's growth rate. We tested the possibility to detect *Trichoderma* green mould using the volatiles that are emitted during spawn run. This eventually could lead to a sophisticated non-invasive detection method of *T. aggressivum* in the process air of the tunnels, without the need to sample inside the tunnel during spawn run.

For this we compared volatiles that are produced in non-infected compost with volatiles that are produced in infected compost. In our experimental model, 300 g of phase 2 compost, is spawned and inserted in aerated glass vessels. Compost is colonised at an air temperature of 24°C. After 7, 10 and 14 days of spawn run, process air is sampled both in infected and non-infected cultures and analysed by coupled gas chromatography mass spectrometry (GC-MS). During this 14-day period white mushroom mycelium develops in the non-infected compost. In the infected compost the compost turns black with occasional tufts of white mycelium and green spores.

Volatile blends that are produced during normal compost colonisation (when *Agaricus bisporus* interacts with *Scytalidium thermophilum* and other micro flora present in compost) differ from those produced during colonisation of *T. aggressivum* infected compost. Some of the volatiles appear to be specific for *T. aggressivum* infected compost. Next to this also consistent differences in the overall pattern of volatile production are seen. Infections with *T. harzianum*, *T. atroviride*, an *Aspergillus* species, or Smokey mould (*Penicillium citreonigrum*) produce different volatile patterns. Significant differences between the volatile blends of infected and non-infected compost are visible after 7 days of compost colonisation. In commercial practice of phase 3 composting, tunnels are likely to be partially infected. On-going research is directed at studying larger amounts of compost that is only partially infected.

Keywords: *Trichoderma aggressivum*; button mushroom; *Agaricus bisporus*; volatiles; phase 3 compost; metabolomics.

INTRODUCTION

Many "green moulds" that occur in mushroom cultivation, are considered to be "weed-moulds". *Trichoderma aggressivum*, however, causes problems that are much worse, as it can totally wipe out crop production. The first reports of devastating green mould infection in the U.K. and Ireland date from around 1986 [1, 2, 3]. A few years later, severe green mould infections were experienced in Canada [4], the Eastern part of the U.S. [5], California [6], Spain [7] and France [8]. Despite being one of the main button mushroom producers, the Dutch industry remained spared of this disease for a long time. Nevertheless, in 2006 the first incidents were reported [9]. In about the same period, also the Hungarian industry experienced green mould problems [10].

Identifying the aggressive pathogen among the non-aggressive *Trichoderma* strains and species proved to be challenging. As morphological identification alone was not able to easily discriminate aggressive and non-aggressive strains [11], research focused on molecular techniques [12, 13, 14]. Eventually, Samuels et al. combined morphological characteristics and molecular data to reach a species description [15]. The aggressive *Trichoderma* strains, formerly known as *Trichoderma harzianum* biotypes 2 (European origin) and 4 (American origin) were renamed as *Trichoderma aggressivum* f. *europeanum* and *Trichoderma aggressivum* f. *aggressivum*, respectively. For diagnostic purposes several researchers published PCR based methods [16, 17].

T. aggressivum infects compost at a very early stage and in the Dutch situation infection most likely takes place at the compost yard. Unfortunately, diseased compost cannot be recognized at the compost yards. Even though compost producers in the Netherlands are very keen to prevent green mould problems, occasionally still a number of crops get infected. Therefore, there is a need for a reliable method that allows early detection of *Trichoderma* green mould.

In the Netherlands spawn run is performed in bulk at the compost yards and is referred to as phase 3 composting. During this process, spawned compost is incubated in tunnels and ventilated with large volumes of air to control compost temperature. During this process the compost is inaccessible for sampling of the compost. In addition, it is anticipated that small samples such as normally taken for PCR, may not be representative for the large volumes of compost that are produced. Also the short span of time between emptying of phase 3 tunnels and delivery of the compost at the growers renders PCR based methods less useful.

Mumpuni et al. [18] showed that *A. bisporus* and *T. aggressivum* use volatiles to affect each other's growth rate *in vitro*. This suggests that it may be possible to detect infected compost by analysing the volatiles present in the process air during phase 3 composting. Here we show results of experiments where we used chemical analysis of volatiles produced during phase 3 compost combined with an untargeted metabolomics approach to detect an infection with *T. aggressivum* at early stages. We demonstrate that this eventually could lead to a sophisticated non-invasive detection method of *T. aggressivum* in the process air of the tunnels, without the need to sample inside the tunnel during spawn run.

MATERIALS AND METHODS

Organisms and strains used. For all experiments commercial spawn of *A. bisporus* strain A15 (Sylvan Inc.) was used. Strains of *Trichoderma aggressivum* (MES 13067), *Trichoderma*

harzianum (MES 12998) and *Trichoderma atroviride* (MES 13083) were all isolated from diseased commercial crops in the Netherlands within the period 2007-2009. Species identity was determined by sequence analysis of the internal transcribed spacer of the ribosomal genes of part of the translation elongation factor 1α gene using the database of the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (http://www.isth.info/index.php). The strains of *Aspergillus fumigatus* (MES 11163) and smokey mould (*Penicillium citreonigrum*) were isolated from diseased crops in the Netherlands in 1994 and 2009, respectively, and species name was determined by microscopy.

Infection experiments. Portions of 300 g of spawned compost were placed in glass vessels on top of a nylon gauze sheet which in turn rests on a piece of oasis foam (Fig. 1). Infection was established by inserting 15 grains of sorghum that were overgrown with *T. aggressivum* mycelium into the compost. To establish infections of *T. harzianum*, *T. atroviride* and *P. citreonigrum*, 15 g of spawn grains overgrown with the pathogens was mixed with 5 g of mushroom spawn and used to inoculate 900 g of phase 2 compost. The spawned compost was divided in three equal portions and inserted into the experimental vessels. Controls were left uninfected. Subsequently, the vessels were closed and incubated for 3 weeks at 24 °C under continuous aeration by blowing purified air underneath the compost layer using a copper tube. At the outlet volatiles from process air were collected at different time points. Three vessels infected with *T. aggressivum* and three non-infected controls were incubated simultaneously.

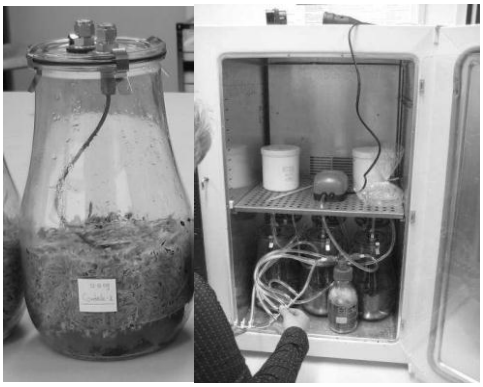

Figure 1: Experimental setup of compost experiments.
Portions of 300 g of compost were placed on top of a nylon gauze which in turn rests on a piece of oasis foam. Air is blown underneath the compost layer using a copper tube. At the outlet process air can be sampled.

Headspace sampling of volatiles. Volatiles produced by the compost were collected on stainless steel cartridges filled with 200 mg of Tenax TA (20/35 mesh, Grace Alltech) at the start of spawn run (T=0), after 7d (T=7), 10d (T=10), and 14d (T=14) for 1 hour at an air flow rate of 200 $ml.min^{-1}$. The cartridges were directly connected to the outlet if the vessel. During this period a volume of 12 litres of air was blown through the vessel. A vessel holds a volume of 2.5 litres, so during a sampling time of 1 hour, the volume of the vessels was refreshed about 5 times. After sampling the cartridges were capped on both sides and stored until analysis.

Chemical analysis of headspace volatiles. Headspace samples were analysed by coupled gas chromatography - mass spectrometry (GC-MS) with a Thermo TraceGC Ultra connected to a Thermo TraceDSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA). Before thermodesorption, the cartridges filled with Tenax TA were flushed with helium at 30 ml min^{-1} for 15 min to remove moisture and oxygen. After flushing the collected volatiles were

desorbed from the Tenax traps at 250 °C (Ultra; Markes, Llantrisant, UK) for 4 min with a helium flow of 30 ml min^{-1}. The released compounds were focused on an electrically cooled sorbent trap (Unity; Markes, Llantrisant, UK) at a temperature of 4 C. Volatiles were injected on the analytical column (ZB-5Msi, 30 m x 0.25 mm ID, 1.0 µm–film thickness, Phenomenex, USA) in splitless mode by ballistic heating of the cold trap to 260 C for 3 min. A constant column flow was set to 1ml/min^{-1}. The temperature program started at 40 C (3.5-min hold) and rose 10 C min^{-1} to 280 C (7-min hold). The column effluent was ionised by electron impact (EI) at 70 eV. Mass scanning was done from 45 to 450 *m*/*z* with a scan time of 5.13 scans s^{-1}.

Data processing. We used an untargeted metabolomics approach to analyse the GC-MS raw data [19]. GC-MS raw data were processed by using MetAlign software [20] to extract and align the mass signals (s/n≥ 3). Mass signals that were below an s/n of 3 were awarded randomized values between 2.4 and 3 times the calculated noise value. Only mass signals that were present in at least 2 samples were retained for analysis, all others were discarded. Signal redundancy per metabolite was removed by means of clustering and mass spectra were reconstructed [21]. The eluted compounds were identified using Xcalibur software (Thermo, Waltham, USA) by comparing the mass spectra with those of authentic reference standards or with NIST 08 and Wageningen natural compounds library spectra. Linear retention indices were calculated for each compound according to van den Dool & Kratz [22] and were compared with those published in the literature. The quantitative composition of the volatile blends was evaluated by principal components analysis (PCA) and orthogonal projection of latent structure discriminant analysis (OPLS-DA) using the software program SIMCA-P 12.0.1. (Umetrics AB, Umea, Sweden) [23]. Data were log-transformed and then variables were mean-centred, and pareto scaled.

RESULTS

Result of infections. An infection of compost with *T. aggressivum* was easy to establish when 15 spawn grains covered with *T. aggressivum* mycelium were inserted into 300 g of freshly spawned compost. Uninfected compost has a brownish colour with clearly visible white mycelium of *A. bisporus*. *T. aggressivum* infected compost is black with occasionally green tufts of spores. There is no visible sign of the white *A. bisporus* mycelium.

Lowering the number of infected spawn grains to 5 /300 g of freshly spawned compost still resulted in well infected compost after 10 days of spawn run. When using only one infected spawn grain, results were more variable, showing lots of *A. bisporus* mycelium by day 10 of spawn run. However, even from compost that did not show visible green mould symptoms by day 18 of spawn run, *T. aggressivum* could still be isolated. Infection with *T. harzianum*, *T. atroviride*, and *P. citreonigrum* was much more difficult to establish. Replacing 75% of the *A. bisporus* spawn used to inoculate phase 2 compost with *T. harzianum* colonized spawn resulted in some spots with green mould in the vicinity of spawn grains. However, most of the compost did not show visible signs of infection after 18 days of spawn run. In contrast, infection with *T. atroviride*, *Aspergillus* spp. and *P. citreonigrum* showed clearly visible signs of infection at the end of the experiment. Especially *T. atroviride* infected compost showed massive signs of infection with lots of pustules with green spores. After infection with an *Aspergillus* species the compost showed a less abundant presence of *A. bisporus* mycelium. After infection with *P. citreonigrum* signs of infection (lack of the white mycelium of *A. bisporus*) were somewhat slow to develop. Infection with *P. citreonigrum* was apparent after 18 days of spawn run. However, after a prolonged incubation (24 days) brownish spots of infection were seen throughout the whole compost layer.

Volatile production after infection with *T. aggressivum*. After data processing 578 potential volatile compounds were retained and relative intensities were subjected to PCA. PCA analysis resulted in a model with 5 significant PCs according to cross validation explaining 78% (R^2X) of the total variation (Q^2X=54%). There are no major differences in volatile patterns of infected and non-infected compost immediately after spawning (Fig. 2).

However, after 7, 10, and 14 days of spawn run the volatiles blends of *T. aggressivum* infected compost differ from those of non-infected compost. In uninfected compost, the largest differences in volatile pattern occur during the first 10 days of spawn run (Fig. 3).

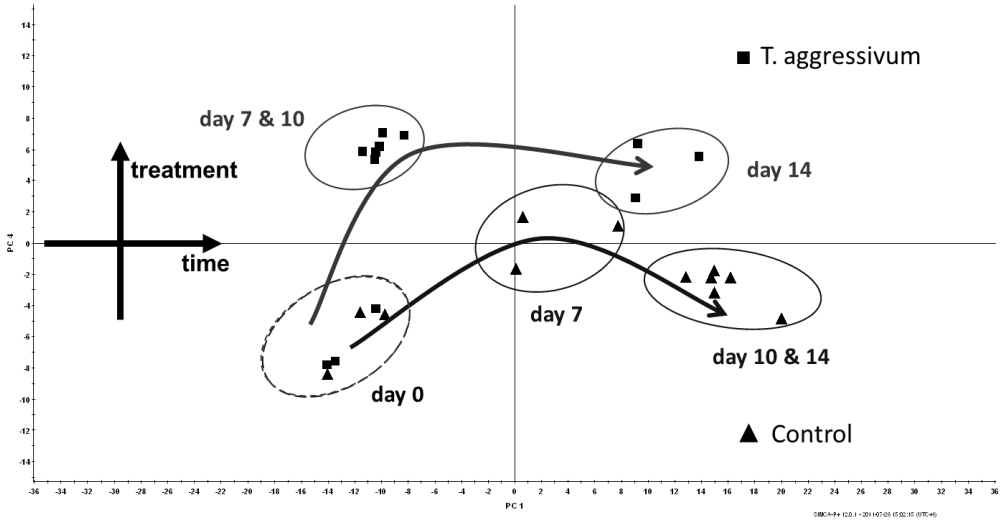

Figure 2: Principal Component Analysis the volatiles blends sampled during spawn run. PC 1 and PC 4 explaining 52% of the total variation are given. There are no major differences in volatile patterns in infected and non-infected compost immediately after spawning. However, after 7, 10 and 14 days of spawn run the volatiles in *T. aggressivum* infected compost differ from the volatiles in uninfected compost.

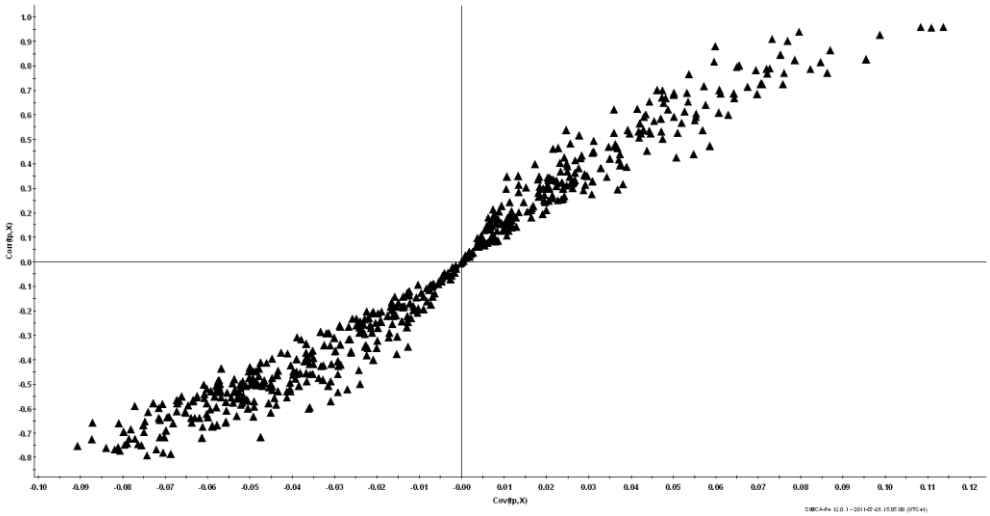

Figure 3: S-plot of an OPLS-DA of volatiles produced by *T. aggressivum* infected compost and non-infected compost.

The S-plot visualizes both the covariance and correlation loading profiles of the volatiles. Each triangle represents a volatile compound. Volatiles located at the upper right end of the plot are significantly more abundant in *T. aggressivum* infected compost on a 95% confidence level.

Volatile patterns in non-infected compost show no major differences after 10 and 14 days of spawn run. In *T. aggressivum* infected compost, the time course of volatile patterns is different. There are clear differences in volatile pattern between spawning and after 7 days of spawn run. Volatile patterns obtained after 7 and 10 days of spawn run do not differ much from each other. However, during the last 4 days of incubation, there are again differences in volatile pattern.
To determine which volatile compounds differ between *T. aggressivum* infected and non-infected compost, the samples of day 7, 10, and day 14 were pooled and the pooled samples were subjected to OPLS-DA. OPLS-DA is a supervised classification technique that aims to find a set of latent variables that discriminate the data according to the predefined treatments (infected *vs* non-infected) in the best possible way. Volatiles that were significantly different between infected and non-infected compost were selected from the so-called S-plot and jack-knifed confidence intervals (Figure 3). The S-plot visualizes the influence of volatiles in an OPLS-DA model. It is a scatter plot that combines the covariance and correlation loading profiles resulting from the predictive discriminant component [24]. This corresponds to combining the contribution or magnitude (covariance) with the effect and reliability (correlation) for the volatile compounds [24].The S-plot visualizes both the covariance and correlation between the volatiles and the modelled class designation [24]. Volatile compounds that are located at the upper right end of the S-shaped plot are significantly more abundant in *T. aggressivum* infected compost compared to non-infected compost (Figure 3). One of the volatiles that most reliably differs between *T. aggressivum* infected compost and non-infected compost is an unknown compound that from its mass spectral signature appears to be a sesquiterpene ($C_{15}H_{24}$). Next to this two monoterpenes (monoterpene 1 and monoterpene 2) are important to discriminate *T. aggressivum* infected compost and non-infected compost.

Specificity of potential marker volatiles of infected compost. Reliable volatile indicators for *T. aggressivum* infected compost should be specific for *T. aggressivum*. Infections of compost with *T. harzianum, T. atroviride, Aspergillus* species, or *P. citreonigrum* are considered to pose a lower risk than infection with *T. aggressivum*. Figures 5a-c show presence of the indicator sesquiterpene, monoterpene 1 and monoterpene 2 in the GC-chromatograms produced by the different pathogens. The sesquiterpene that is produced in *T. aggressivum* infected compost also appears to be produced in compost that is infected by *T. harzianum*, but appears absent in compost that is infected by *T. atroviride*, an *Aspergillus* species, and *P. citreonigrum*.

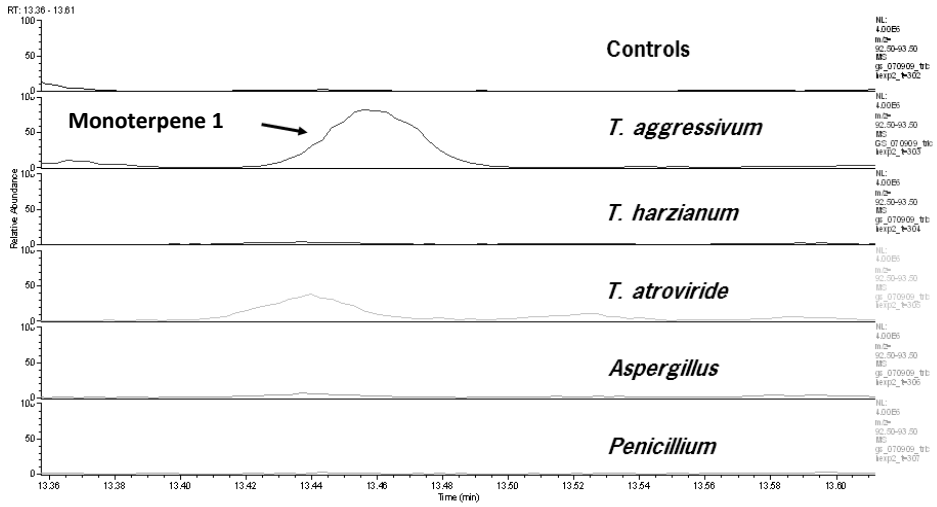

Figure 5a: Specificity of sesquiterpene as indicator of infection with *Trichoderma aggressivum*.

Figure 5b: Specificity of monoterpene 1 as indicator of infection with *Trichoderma aggressivum*.

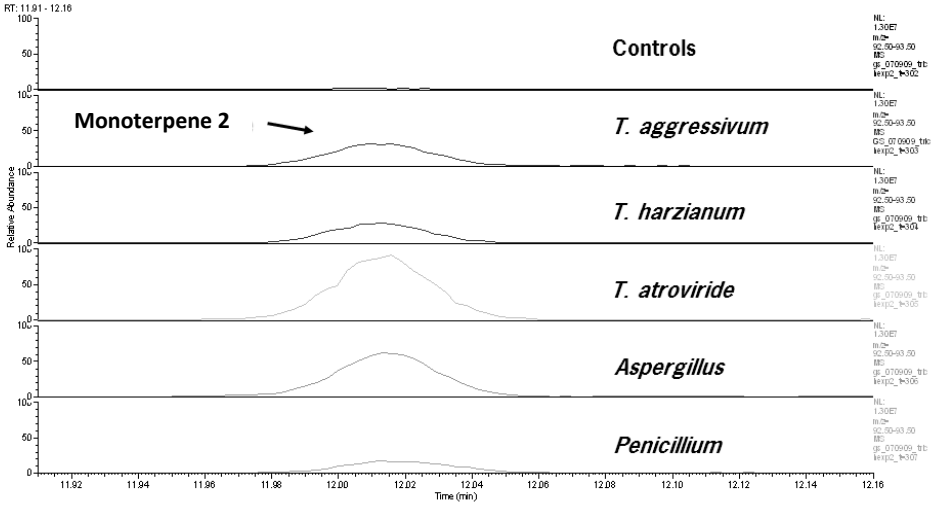

Figure 5c: Specificity of monoterpene 2 as indicator of infection with *Trichoderma aggressivum*.

The emission of monoterpene 1 appears to be truly specific for infection of compost by *T. aggressivum*. The emission of monoterpene 2 occurs in all infected composts and is therefore not useful to discriminate the pathogens.

DISCUSSION

The results of the experiments described here, were performed to study the possibility to identify *T. aggressivum* infected compost already during spawn run by the emitted volatiles. Mumpuni et al. [18] showed that volatiles (other than CO_2) produced by *T. aggressivum* were able to significantly reduce radial growth of *A. bisporus* colonies. The chemical nature of these volatiles was, however, not determined. For *T. harzianum* [25] and *T. atroviride* [26] some information on the chemical nature of the volatiles they produce is available. Also *A. bisporus* is known to produce a large number of volatiles. Some of these volatiles, for instance the C_8-compounds, are

involved in repelling pests and pathogens and controlling sporulation [27-31]. For instance, 1-octanol shows fungitoxic activity [32]. Especially *Trichoderma* species are known for producing antifungal volatiles [33]. The antifungal volatile lactone, 6-pentyl-pyrone (6-PAP), is well described as an antifungal compound produced by *T. harzianum* as secondary metabolite [34, 35, 36]. The production of volatiles is influenced by the substrate on which the mycelium is growing [25] and by the age of the mycelium [26].

In our experiments there is a change in the volatile pattern as spawn run is proceeding (both in *T. aggressivum* infected and non-infected compost). Differences become apparent already after 7 days of spawn run. Specific volatiles that are present in the process air of *T. aggressivum* infected compost but not in the uninfected compost are an as yet unidentified sesquiterpene, and the two monoterpenes. The unidentified sesquiterpene is present in process air of both *T. aggressivum* - and *T. harzianum* infected compost. The monoterpene 2 is present in the process air of all composts that were infected with pathogens. It is likely that this monoterpene is produced by *A. bisporus*. The monoterpene 1 appears to be specific for *T. aggressivum* infected compost.

Krupke et al. [37] studied growth inhibiting substances produced by *T. harzianum* and *T. aggressivum*. For this they extracted substances from infected compost and malt extract broth. When grown in malt extract broth, 3,4-dihydro-8-hydroxy-3-methylisocoumarin (mellein) was the major antifungal compounds produced by *T. aggressivum*. However, we could not detect this compound in the volatile blends of *T. aggressivum* infected compost. Nor were we able to identify 6-PAP in the volatile blends of *T. aggressivum* infected compost.

CONCLUSIONS

Compost infected with *T. aggressivum* can be distinguished from uninfected compost during spawn run, based on its volatile blend. Volatile blends are distinguishable already a few days (7-10d) after infection with *T. aggressivum.* Some terpenoids within the volatile blend might serve as indicators due to their specificity for *T. aggressivum*.

ACKNOWLEDGEMENTS

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DETECTION OF SOURCES OF *LECANICILLIUM* (*VERTICILLIUM*) *FUNGICOLA* ON MUSHROOM FARMS.

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ABSTRACT

The objective of this work was to identify sources of *L. fungicola* on commercial mushroom farms. For detection of *L. fungicola* two methods were used: selective media and Real Time PCR (RT PCR). Two selective media were compared using 438 samples and an RT PCR method was used with 375 samples. There was no difference in the success rate of the two selective media but *L. fungicola* grew faster and was more easily detected on the novel PDA media than on MRSM. RT PCR detected *L. fungicola* more frequently than the selective media. This might indicate a high level of dead *L. fungicola* on farms. Living *L. fungicola* was detected at most locations on farms but was much higher in growing rooms where 14, 26 and 47 % of samples from 1st, 2nd and 3rd flush, respectively, tested positive. Live *L. fungicola* was detected in 19 % of samples taken from other locations (outdoors and canteen area). When data were organised by type of sample *L. fungicola* was not detected on ruffling, filling or empting machines by either method, but in other places (inside, floor of growing room, crates, flies, door handle) the pathogen was detected by both methods.

Keywords: *Lecanicillium fungicola*, mushroom farms, selective medium, Real Time PCR

INTRODUCTION

Today *Agaricus bisporus* is cultivated in more than 70 countries in the world [1]. This monoculture is affected by many pathogens and pests. The most important pathogen of *A. bisporus* is *L. fungicola* which causes the disease called "dry bubble". The symptoms of dry bubble disease are un-differentiated masses of mushroom tissue, split stipes and cap spotting.

Lecanicillium fungicola produces large numbers of conidia which are held in sticky mucilage and these conidia can be very easily spread around the mushroom farm. Conidia are spread and dispersed in many ways via water, flies, humans and machinery [2, 3]. The conidia can also survive for a long time (7-12 month) in dry or moist casing soil mixture [4, 5]. All these factors make *L. fungicola* a very serious pathogen of the mushroom industry that is difficult to eliminate.

The first comprehensive study to search for sources of *L. fungicola* on mushroom farms was presented by Wong and Preece [6]. They used two different microbiological media for the detection of *L. fungicola* in samples from a large mushroom farm in the UK which was seriously affected by dry bubble disease.

Nair and Macauley [7] reported that the most common source of *L. fungicola* var. *fungicola* was soil from around mushroom farms. The peat moss and water which were tested were not a source of the pathogen. They detected *L. fungicola* by preparing a serial dilution of a sample and plating it out on potato dextrose agar with antibiotics.

Information about sources of *L. fungicola* on mushroom farms was also reported by Rinker *et al.* [8], who designed a selective medium for *L. fungicola* to test samples from mushroom farms.

Grogan [9] confirmed Gandy's [10] information about the spread of *L. fungicola* by the debris-dust fraction which is present on a mushroom farm. Debris samples collected from inside and outside mushroom houses that were added to casing soil, caused dry bubble symptoms to occur.

The objectives of this work were to detect *L. fungicola* on commercial mushroom farms in Ireland using two methods – one microbiological and one molecular. Identification of the possible sources of *L. fungicola* on mushroom farms could provide useful information for managing dry bubble disease.

MATERIALS AND METHODS

Mushroom Farms Visits. Between 2008 and 2010 samples were collected during 18 visits to 9 mushroom farms with different levels of dry bubble disease. In total 438 samples were collected from different stages of the crop cycle from spawn running to 3rd flush, as well as from other locations. Samples were categorised (1) by location or (2) by crop stage. Samples were examined using microbiological (selective medium) and molecular (Real Time PCR) methods.

Sample Collection and Preparation. Most samples from mushroom farms were collected by passing a sterile wet swab over the selected surface. The swab was then put into a 50 ml centrifuge tube. Other samples were collected directly (e.g. 5-10 flies, few gram soil, discarded gloves, hair net etc.) into either a 50 ml centrifuge tube or a clean plastic bag. The samples were stored at 4°C overnight and sample preparation started on the following day. Centrifuge tubes with samples were filled with sterile water up to 50 ml and shaken vigorously for 30 min. at 120 rpm. After that the sample was filtered through a square of UV sterilised Miracloth. Sample filtrate was concentrated by centrifugation (GS-6 Centrifuge, Beckman) for 10 min at 3,000 × g. After centrifugation samples were left overnight to sediment. The following day the upper layer was removed using a disposable transfer pipette and the debris pellet plus a small amount of water was left in the bottom of the tube to give a final sample volume of around 3 ml. Of this, 600 µl was used for selective media tests and the remainder of the sample was transferred into a 2 ml Eppendorf for molecular tests.

Selective Media. Two media were used for the detection of viable *L. fungicola* in mushroom farm samples: a "Modified Rinkers Selective Medium" (MRSM) and a "Novel PDA based Selective Medium" (NPDASM). The MRSM is based on a selective medium for *L. fungicola* described by Rinkers *et al.* [8]. Both media are described in Piasecka [11]. A 100 µl aliquot of sample was spread onto 3 replicate Petri dishes for each medium, which were then incubated for 6-7 days at 20°C.

DNA Extraction. DNA extraction was performed using Wizard Magnetic DNA Purification System for Food (Promega) and QuickGene Mini 80 device with QuickGene DNA tissue DT-S DNA isolation kits (Fujifilm) following the extraction protocol.

Real Time PCR. Real Time PCR was done using the primers and probe designed by Zijlstra *et al.* [12, 13, and 14]. A result of Real Time PCR was recorded as positive when 6-FAM signal was present after 45 cycles or less. If 6-FAM signal was present after more than 45 cycles and/or was negative the sample result was recorded as negative.

Data Analysis. Results from the microbiological and molecular detection methods were compared using McNemar's test for comparison of proportions from paired binary outcomes. This is a nonparametric test for a 2 × 2 contingency table with matched subjects where the outcomes are not independent. McNemar's Test statistic was calculated using SAS Software (SAS Institute Inc. 2004. SAS/STAT® 9.1, Cary, NC: SAS Institute Inc.).

RESULTS AND DISCUSSION

When the effectiveness of detection of *L. fungicola* on NPDASM and on MRSM selective medium was compared there was no significant difference between the media, but *L. fungicola* grew better on NPDASM compared to MRSM (see Fig. 1) making detection easier.

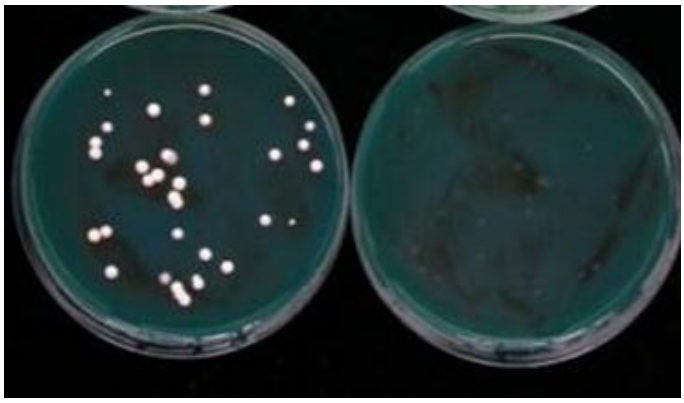

Figure 1: Growth of *L. fungicola* on NPDASM (left) and on MRSM (right) selective medium.

The Real Time PCR method gave significantly more positive results (63 %) for presence of *L. fungicola* compared with the selective media (18 %) at $P \leq 0.05$ except for three sample categories (machinery, watering equipment and worker clothes) where there were too few samples for an accurate comparison (see Fig. 2). This high detection rate by Real Time PCR may reflect the detection of dead *L.fungicola* by the Real Time PCR method as 50-90% of samples in all categories tested positive, while the corresponding selective media results had fewer positives.

When data were analysed and organised by location of samples *L. fungicola* was not detected on machinery by either method, but in in samples from other locations the pathogen was detected by both the selective media and Real Time PCR. Samples containing casing soil (e.g. growing room floors, pickers trolleys and platform) often gave more positive results on selective media compared to other samples. Samples which did not contain polymerase inhibitors (flies, door handle, water equipment, crates, etc.) gave more positive results with Real Time PCR compared with selective media (see Fig. 2).

When data were analysed and organised according to crop stage, viable *L. fungicola* was detected by selective media at all crop stages except during the spawn running stage (see Fig. 3). The number of samples testing positive for viable *L. fungicola* increased from 14 to 47% between the 1st and 3rd flush while 19% of samples from non-crop locations (e.g. outdoors and canteen areas) were positive for viable *L. fungicola*. Real Time PCR testing detected *L. fungicola* in 50-80% of samples for all crop stages which is a cause for concern. However it is unlikely that all Real Time PCR positive results reflect live *L. fungicola* therefore its use as a diagnostic tool on mushroom farms might be limited. There is likely to be a high level of dead

L. fungicola on farms that use steam to kill off diseased crops increasing the likelihood of false positives with Real Time PCR.

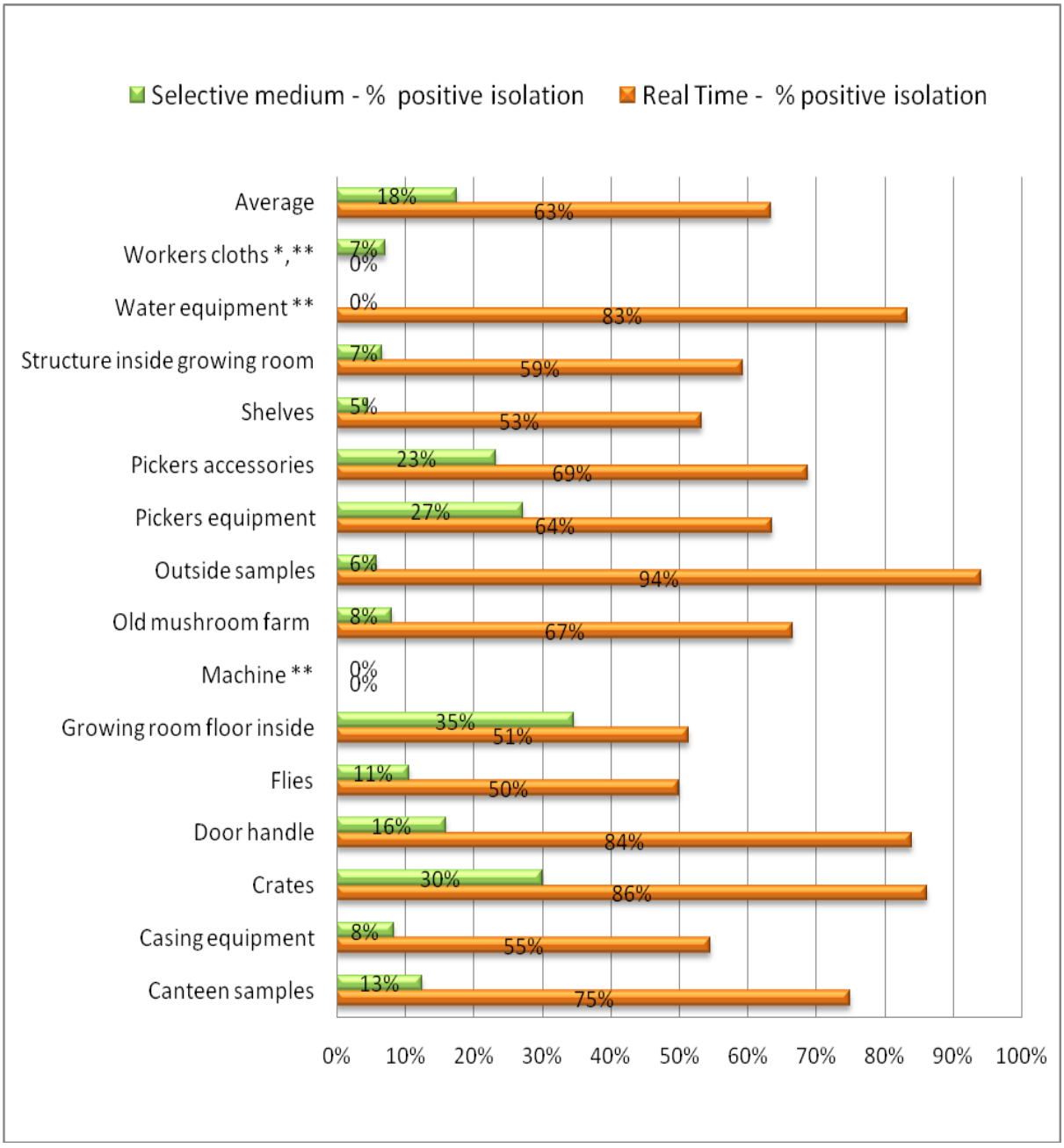

Figure 2: Detection of *L. fungicola* in samples from different locations. * Only selective medium tested, ** not enough samples for comparison.

The results suggest that selective medium may be a more reliable method for routine detection of *L. fungicola* on mushroom farms. It is not an expensive method for detection, but it requires a few days to give results. It detects only live material such as spores and mycelium so that a positive result identifies a very real disease risk for a farm. Real Time PCR is a fast but

expensive method for detection of *L. fungicola* from samples from mushroom farms however in these experiments detection levels were very high suggesting that non-viable background levels of *L. fungicola* were being detected. In order for Real Time PCR to be more useful in *L. fungicola* diagnostics it needs to be more specific for living material.

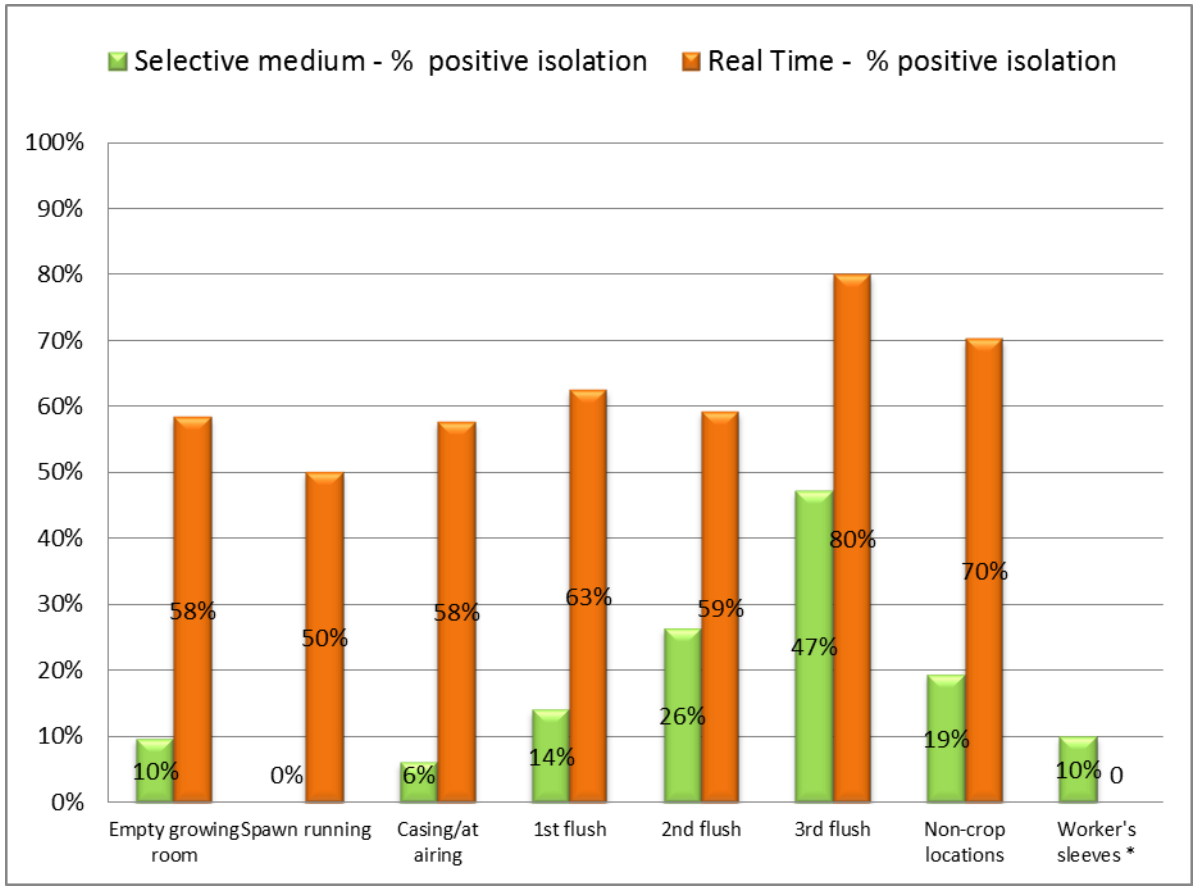

Figure 3: The percentage detection of *L. fungicola* using selective media and Real Time PCR after 45 cycle. * Only selective media tested.

CONCLUSION

Live *L. fungicola* was detected at most locations on mushroom farms but was most abundant in 2nd and 3rd flush growing rooms and outdoor samples. Real Time PCR detected four times more *L. fungicola* than selective media but it is likely that Real Time PCR is also detecting non-viable *L. fungicola*.

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CASING LAYER DISINFECTION BY COLLOIDAL SILVER AND ACTIVE OXYGEN, EFFECTS ON YIELD OF *AGARICUS BISPORUS* AND CONTROL OF COBWEB DISEASE

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ABSTRACT

Cladobotryum dendroides is the most frequently found soil-borne mushroom pathogen in Serbia [1]. Although many investigations have been carried out to find an adequate disinfectant of mushroom casing, so far, none has been found to be completely effective thus non-toxic to humans. The aim of this study was to explore the potential of Ecocute, based on colloidal silver [2-3] and Peral-S (active oxygen) [4-5], as environmentally friendly casing disinfectants. Efficacies of the disinfectants and incidence of cobweb disease were evaluated in bags of compost cased with a black peat/lime casing soil, previously disinfected with Ecocute at 30 mg L^{-1} per m^2 and Peral-S at 1.7 ml L^{-1} per m^2. Plots were inoculated with suspension set on 1000 conidia per m^2 of *C. dendroides*. Both disinfectants were applied alone and in combination with prochloraz-manganese. Biological efficiency of colloidal silver applied alone was higher than in treatments with active oxygen in all trials. The colloidal silver did not control cobweb disease satisfactorily, and resulted in diseased mushrooms in all three trials (1.1%, 5.7% and 5.9%). Application of colloidal silver did not decrease the total productivity of *Agaricus bisporus*, and resulted in comparatively higher cumulative biological efficiency than that of active oxygen. On the other hand, active oxygen provided better disease control compared to colloidal silver. Treatment of active oxygen with prochloraz-manganese had the highest effectivenes in disease control and also satisfactorily mushroom productivity.

Keywords: Ecocute; Peral-S; *Cladobotryum dendroides*.

INTRODUCTION

Soil-borne pathogens, such as *Lecanicillium fungicola*, *Mycogone perniciosa*, *Cladobotryum* spp. and *Pseudomonas tolaasii*, cause the most serious growing mushroom diseases, respectively, dry and wet bubble, cobweb disease and bacterial brown blotch [6-7]. *Cladobotryum dendroides* is the most frequently found soil-inhabiting mushroom pathogen in Serbia [1]. The disease symptoms were: cottony fluffy white or yellowish to pink colonies on mushroom casing, rapid colonization of casing surface, covering of host basidiomata by mycelia, and their decay. Infection intensity depended on the development stage of fruiting bodies. The use of both peat casing and indoor disinfectants, such as formalin, sodium hypochlorite, potassium permanganate, sulphur, calcium chloride and chlorinated compounds, is a general practice in mushroom cultivation process. The selective fungicide prochloraz-manganese is officially recommended in mushroom production facilities in EU countries [6]. However, the use

of chemicals induces a problem with residues in mushroom fruiting bodies. So far, many investigations have been carried out to find an adequate disease control in edible mushroom industry, but none has been found to be completely effective thus non-toxic to humans. Many alternative compounds, e.g. biopesticides, disinfectants, plant extracts, essential oils and their components, have been tested as control agents against mushroom pathogens in general [1, 8-10]. Environmental awareness has grown, with the result that the enforcement of pollution control laws has become more and more effective. This situation forced the mushroom industry to develop technologies that ensure production with the least possible harmful effects on the environment [11]. Evaluations of the commercially available disinfectants effects on mushroom pathogenic bacterium *Pseudomonas tolaasii* and fungi *Trichoderma harzianum* and *C. dendroides*, have already been reported [12-13]. Reducing environmental pollutants and microorganisms by colloidal silver has been developed in hospitals, diaries, food processing and even drinking water disinfection [14-15]. Environmentally friendly solution based on silver combined with hydrogen peroxide showed synergism having strong bactericidal and antiviral effects [2-3]. Other ecological disinfectant is active oxygen, arising as a result of break-down of peracetic acid with its high oxidizing potential. It is broadly effective against microorganisms for indoor use on hard surfaces. Its use sites include agricultural premises, food establishments, medical facilities etc. [4-5]. The aim of this study was to explore the potential of colloidal silver and active oxygen, as ecological casing disinfectants, against Serbian *C. dendroides* isolate.

MATERIALS AND METHODS

Commercial fungicide formulation prochloraz-manganse (Octave WP, Bayer Crop Science, Germany, prochloraz-manganese complex 50%, kaolin 35%, other ingredients 15%) was used in this study. The ecologically friendly disinfectants Peral-S (Vetprom, Belgrade, Serbia, active oxigen 0.9%) and Ecocute (IHIS Techno Experts, Belgrade, Serbia, colloidal silver 30 mg, hydrogen peroxide 1 L) were tested as potential antifungal agents against Serbian *C. dendroides* isolate in a mushroom growing room.

An isolate of *C. dendroides*, strain Vegr2C7, was grown on potato dextrose agar (PDA) at 20^{o}C for four days. Conidia were harvested by flooding the plates with 10 ml of sterile distilled water and Tween 20 (v/v 0.01 %) (REANAL Finomvegyszergyar Rt., Hungary, No.: 805383) followed by filtration through double layers of cheesecloth. Each plot of the infested casing was treated with a total volume of 10 ml of conidial suspension at a rate of 1000 conidia per m^2.

Plastic bags, 0.60 x 0.40 x 0.25 m (*l* x *w* x *h*), filled with 18 kg of compost spawned with *A. bisporus* strain 737, (Sylvan, Hungária zRt), were incubated (spawn-run) for 18 days at 24^{o}C. Compost surface was divided by wooden barriers into two sections so that each experimental compartment, measuring 0.30 x 0.40 x 0.25 m, had a total area of 0.12 m^2, and contained 9 kg of spawned substrate. Each plot was cased with a 40-50 mm layer of black peat/lime casing soil (Ramski rit – Treset, Veliko Gradište, Serbia) and incubated at 21^{o}C for 8 days (case-run), and then air temperature was reduced to 16^{o}C. Drench applications of prochloraz-manganese were applied on day 5 and 20, and relevant plots inoculated with conidial suspension of *C. dendroides* Vegr2C7 isolate 7 days after casing. The treatments were as follows: *(1)* uninoculated and untreated control; *(2)* inoculated and untreated control; *(3)* inoculated, treated with prochloraz-manganese at standard product application rate (0.6 g a.i. in 1.8 l H_2O m^{-2} of mushroom bed area); *(4)* inoculated, treated with prochloraz-manganese at standard product application rate and Peral-S ((active oxygen 0.9%) 1.7 ml in 1 L H_2O m^{-2} of mushroom bed area); *(5)* inoculated, treated with prochloraz-manganese at standard product application rate and Ecocute ((colloidal silver 30 mg in 1 L hydrogene peroxide) 250 ml in 1 L H_2O m^{-2} of mushroom bed area); *(6)* inoculated, treated with Peral-S (1.7 ml in 1 L H_2O m^{-2} of mushroom bed area); *(7)* inoculated, treated with Ecocute (250 ml in 1 L H_2O m^{-2} of mushroom bed area). The plots were arranged in

a randomized block design with three replicates per treatment. All experiments were repeated three times.

The mushrooms were hand-picked in three successive production flushes. The harvested mushrooms were weighed, counted, and divided in two groups based on visual observation: fruiting bodies without symptoms and those covered with *C. dendroides* mycelium. Disease incidence was recorded as a percentage value, based on the number of diseased sporophores visually recorded. The effect of fungicides on mushroom productivity was evaluated by the biological efficiency (BE) calculated as the ratio of the fresh weight of total yield of harvested mushrooms (healthy and diseased) to the weight of dry substrate at spawning and expressing the fraction as kg/100 kg compost [16]. The incidence of cobweb disease in inoculated plots was calculated as percentages of diseased mushrooms of all mushrooms harvested.

Fungicide effectiveness was calculated by using Abbott's formula [17]:

% effectivness= [(Ic -It)/Ic] x 100

(where Ic = disease incidence of the control; It = disease incidence of the treatment)[18].

Analysis of variance was performed in order to determine treatment effects. Data were analysed using ANOVA and the means separated by Duncan's multiple range test.

RESULTS AND DISCUSSION

The incidence of cobweb disease in untreated inoculated plots in three trials was 4.6%, 7.6% and 4.5%, calculated as percentages of all mushrooms harvested. Prochloraz-manganese prevented disease development in one trial, while disease symptoms were recorded in the two other trials (3.1% and 2.3%, respectively). The disinfection with colloidal silver did not control cobweb disease satisfactorily, and resulted in diseased mushrooms in all three trials (1.1%, 5.7% and 5.9%). Active oxygen gave better control than colloidal silver, and disease incidence was 0%, 5.9% and 3.0%.

Table 1. Biological efficiency (BE %) of the different treatments on *Agaricus bisporus* artificially inoculated with *Cladobotryum dendroides*

Treatments ($g\ m^{-2}$)	BE (%)		
	Trial I	Trial II	Trial III
Uninoculated and untreated	74.28 c[1]	90.14 b	79.63 e
Inoculated Untreated	64.70 f	85.47 d	108.94 c
Inoculated Untreated Peral S 1.7 ml L^{-1}	57.08 g	66.09 e	72.78 f
Inoculated Untreated Ecocute 250 ml L^{-1}	67.46 e	88.38 c	101.79 d
Inoculated Octave WP 1.2 mg L^{-1} Peral S 1.7 ml L^{-1}	97.48 a	91.62 b	110.42 b
Inoculated Octave WP 1.2 mg L^{-1} Ecocute 250 ml L^{-1}	70.98 d	94.78 a	127.47 a
Inoculated Octave WP 1.2 mg L^{-1}	91.16 b	84.89 d	50.08 g

[1]Means within the same column followed by the same letter are not significantly different (P=0.005).

Regarding the effect of treatments on mushroom productivity evaluated by biological efficiency, the greatest productivity was obtained by the combination of prochloraz-manganese and active oxygen in the first trial, and with both disinfectants in the second and the third trial (Table 1). The fungicide also showed high productivity when applied alone in the first trial. The poorest results were obtained with active oxygen. Biological efficiency of colloidal silver applied alone was higher than in treatments with active oxygen in all trials. Additionally, yields in the treatment with colloidal silver, both applied alone and in combination with prochloraz-manganese, exceeded the yields in uninoculated control in the third trial. When both

disinfectants were combined with fungicide, efficiency of colloidal silver was higher than of active oxygen in two trials.

In the first trial, a 100% effectiveness was shown in treatment when active oxygen was involved, alone or with prochloraz-manganese (Fig. 1). Prochloraz-manganese applied alone exhibited the least effectiveness in the first trial (32.6%). Colloidal silver was effective both applied alone and with fungicide (76.1 and 45.7%, respectively). In the second trial, the 100% effectiveness was attained in all treatments with prochloraz-manganese. Active oxygen and colloidal silver had much lower effectiveness, 22.4 and 25% respectively. In the third trial, fully effective was a combination of fungicide and active oxygen. Active oxygen applied alone also was highly effective (93.3%). Less effective was fungicide applied alone and with colloidal silver (48.9 and 48.7%) and the least effective was colloidal silver applied alone.

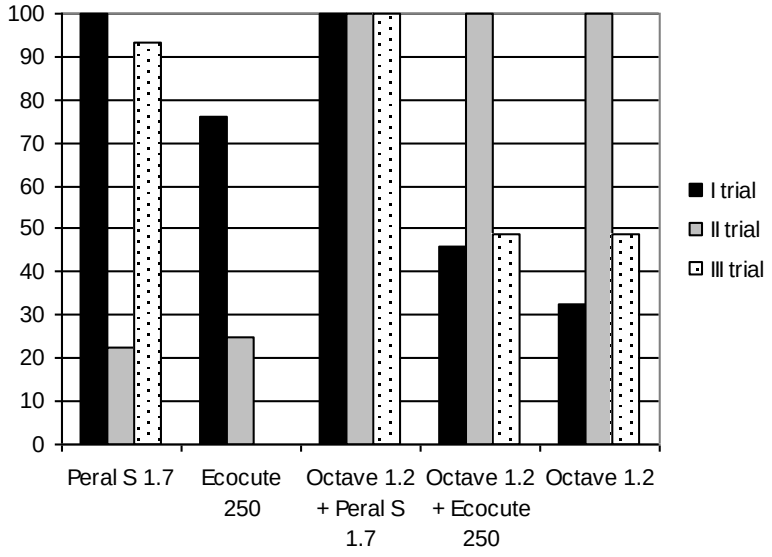

Figure 1. Effectiveness (%) of different treatments on control of *Cladobotryum dendroides* in artificially infected *Agaricus bisporus*; LSD_{005} =68.52.

Application of colloidal silver did not decrease the total productivity of *A. bisporus*, and it resulted in comparatively higher cumulative biological efficiency than that of active oxygen.

CONCLUSION

Colloidal silver caused a significant reduction in cobweb disease levels, but less than active oxygen. Results indicated that colloidal silver had no negative interference with *A. bisporus* physiology. Active oxygen provided better disease control compared to colloidal silver, but this disinfectant had negative influence on *A. bisporus* yield. On the other hand, treatment of active oxygen with prochloraz-manganese had the highest effectiveness in disease control and also satisfactorily mushroom productivity. Based on this finding colloidal silver and active oxygen should be tested further to examine effectiveness against other *A. bisporus* pathogens.

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NEW DEVELOPMENTS IN INTEGRATED PEST MANAGEMENT FOR MUSHROOM CULTURE, CHALLENGES AND OPPORTUNITIES IN QUALITY MUSHROOM PRODUCTION

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ABSTRACT

The continuous and increasing consumer demand for reduced pesticide use in the production of food products highlighted the need to develop novel pest and disease control programs in mushroom cultivation. Strict pesticide regulations in the E.U. and U.S. give farmers less opportunity to use highly effective pesticides against pathogens. The combined technologies in integrated mushroom production might be effective in a high quality, well-maintained shelf system house. The air-circulation systems, over-pressure cultivation tunnels and steam cook-out ability, are helping growers to reduce their use of pesticides. This gives the grower the chance to produce "pesticide-free" *Agaricus* mushrooms. The above-mentioned technological elements are available only on mushroom farms that were built recently. In the very new mushroom units it is possible to produce *Agaricus* mushrooms without using any synthetic pesticides or fungicides. The well-controlled environment provides the potential to integrate pest control with other practical elements (high quality compost and casing, good hygiene) to provide more effective and sustainable pest and disease control. Mushroom houses with less technological instrumentation face a higher risk of pathogens. At the moment, the bulk of mushroom production in the world comes from less effective farms and this highlights the need for new approaches to solving the many problems that they face.

DIFFERENT SYSTEMS - SIMILAR SOLUTIONS

There are no mushroom farms in the world that are exactly the same therefore pest and disease management technologies can vary between growers. Integrated pest and disease technology should be adapted to the local circumstances as the types and ages of mushroom units can vary from farm to farm and problems may arise due to those variable structures. Additionally the growing systems (tray, shelf, bag, block etc.) may have an influence on the pathogen status of a farm. Farms built on the Dutch design usually have highly efficient air circulation and filtration system and the dominant fly species (Sciarids, Phorids and Cecids) must face over-pressured growing rooms, therefore reducing their numbers. The reduced number of mushroom flies reduces the risk of cross-contamination with fungal diseases. The cleaning process of such buildings often includes washing with high-pressure cleaners but this cannot be used on older farms where the floor condition is poor and high-pressure washing with water could spread pathogens hidden in crevices in the floor.

Despite the variability in types of mushroom farms, the main components of an integrated pest and disease management program are similar. The components are summarized in Table 1 followed by Fletcher & Gaze [1].

Table 1. The main components of an integrated pest and disease control programme [1]

Principles of pest and pathogen management	Practices and operations
Entry	Identification and records Filtration, ventilation, air movement Disease removal
Containment	Harvesting hygiene Disinfectant pads Crop termination Farm design
Elimination	Chemical control Environmental control Genetic control

Pathogen pressure can vary depending on continent and climatic conditions but the main mushroom pest and disease species are found wherever mushroom or compost production is significant.

ENTRY

It is essential to understand the importance of some very simple practices to avoid the spread of diseases. A fault in a daily routine, like hand washing, at the mushroom farm may have a high risk of spreading fungal spores. Use of a glowing gel that shows how well the pickers have washed their hands - by illustrating bacteria they missed while washing - may significantly improve hand hygiene. A study that was conducted on children, but which is also relevant for mushroom farms, – showed that it is necessary to improve hand washing techniques. To demonstrate proper hand washing, simply rub a specific lotion on the picker's hands (eg. Glo Germ), and the lotion simulates the spread of tiny plastic fluorescent 'germs' on their hands. Under a black light, the gel creates a yellow glow in areas where dirt and 'germs' are present. Then ask pickers to wash their hands as they normally would, following the prescribed farm process. Finally, a cheap UV lamp can be used to highlight any remaining 'germs'. Under the lamp the 'germs' fluoresce or glow brightly (Figure 1) so they can be easily seen by the pickers [2].

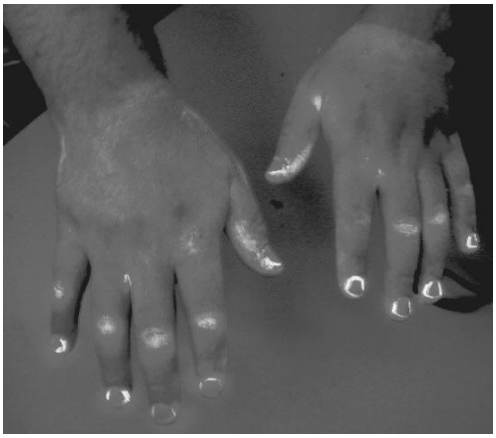

Figure 1. 'Dirty' hands after hand washing to simulate effectiveness of hand washing (from: http://www.teachersource.com)

CONTAINMENT

An effective way to isolate cobweb on the casing is still to cover with paper towel and salt [1]. It is often difficult to find the origin, where the infection comes from. A good preventative standard would be to identify and isolate the origin of the fungal diseases at the farm. Regular sample collection and analysis by molecular and classical methods from mushroom farms helps to find for example the sources of *Verticillium (Lecanicillium) fungicola* [18]. The major principles are to get as many samples as possible from different areas of the farm (entry, room, storage, social rooms, etc) and collect samples at different stages of the crop; before filling, after casing, after emptying, etc. A simple draw or graphical map about the farm, where the sources of pathogen are marked may help the grower to treat the infected areas with disinfectants.

ELIMINATION

The introduction of a new synthetic pesticide-fungicide for use in mushroom cultivation is not to be expected due to high costs of developing a new substance and the relatively low value of the mushroom sector, compared to other areas of agriculture. There are several ways to deal with pathogens but, if possible, they should be cheap and effective methods and techniques are necessary for growers, especially who have only less well-equipped unit.

The presence of *Trichoderma* species, and their negative effects on the yield and quality of mushrooms, is one of the high risks of mushroom production. Many species of *Trichoderma* have been described, producing a range of different symptoms. Many publications have helped to understand the biology of green mould [3, 4]. Farmers who buy Phase III compost usually have fewer problems with green mould, compared to Phase II composts. As Rinker & Castle found in 2005, '*Trichoderma* is a disease that must be managed preventatively' [5]. Recent research showed strains of *T. aggressivum* var. *aggressivum* were resistant to the fungicides thiophanate-methly and benomyl [6]. This underlines the importance of the prevention of green mould. The grains in spawn can serve as food source for *Trichoderma*, non-grain based and reduced grain spawn formulations have been developed and marketed [7]. As in nature *Trichoderma* species have antagonists; a biofungicide containing *Bacillus subtilis* was introduced to button mushroom cultivation in 2008 and its efficacy against green mould in oyster production was good [8]. The complex structure and high-range types of bacterial colonies in the compost may have some, as-yet unknown beneficial effects on the crop.

Other major fungal diseases like *Cladobotryum dendroides, Mycogone perniciosa* or *Lecanicillium fungicola* var. *fungicola* (formerly known as *Verticillium* [9]) are almost always present in the old farm structures. The well-maintained and disinfected Dutch-type houses are less susceptible to high losses due to of these pathogens but if they are present, then hygiene protocols must be reviewed. Fungal diseases cause more serious problems in older structures. The limited number of permitted pesticides in mushroom culture against fungal pathogens can make disease control more difficult. However, more tolerant and resistant pathogens can also be 'selected' for because of automatic and routine usage the fungicides. Recent researches also indicate that more aggressive strains of dry bubble disease can occur [10] and may cause serious losses to growers in the future. On the other hand, an earlier article suggested that the lack of variation in the recent isolates as compared to the older isolates (more than 45 year) indicates that the *L. fungicola* population may be becoming more homogeneous [11]. To prepare for the appearance of more aggressive pathogens it is essential to understand their biology. The host-pathogen interaction and its model is widely described; it is always under revision, particularly from with regard to humans [12]. More focused research is needed in relation to the edible mushrooms group. Mushroom breeders and geneticists are interested in what happens at gene level when a pathogen appears in cultivation. Improving the genetic resistance in mushroom

cultivars is very costly and it is usually done by large spawn manufacturers, who could charge premium prices for new resistant cultivars.

The biological control of *Sciarids* has been used for years with good experiences; no tolerance has neen detected so far. *Steinernema feltiae,* an entomopathogenic nematode is effective against immature dipterous insects, including fungus gnats. Its life cycle is very short, completed in a few weeks and hundreds of thousands of new infective juveniles emerge in search of fresh hosts [13]. Casing can be treated by *Bacillus thuringiensis* var. *israeliensis* as well. It produces a toxin, which is poisonous to sciarid larvae [1]. The usage of these biopesticides is not so frequent with Hungarian growers because of the high costs of the products and limitations in storage conditions and spraying techniques.

Since antiquity, essential oils have been widely used for bactericidal, virucidal, fungicidal and insecticidal applications [14]. Most of them are extracted by distillation from aromatic plants and they contain a variety of volatile and non-volatile molecules. *In vitro* physicochemical assays characterized most of them as antioxidants [14]. In eukaryotic cells – such as fungi or animals - essential oils can act as pro-oxidants affecting inner cell membranes and organelles. Depending on type and concentration, they exhibit cytotoxic effects on living cells but are usually non-genotoxic [14]. Many essential oils were tested against many pathogens especially in plant production *in vitro* with variable results. The oils from mint and thyme were shown to have antifungal activity against *Mycogone in vitro* [15]. From the results it is a reasonable assumption that many essential oils may have a negative effect on the growth of fungal pathogens. The main objective is to find those oils (and concentration), that do not harm the mushroom mycelium and yet are still effective against the pathogen. Essential oils are natural products and are more easily approved for use, in addition they can be used for organic production. The importance of essential oils may increase in the future, but many questions remain to be addressed concerning when and how to use them in a crop.

The sterilizing ability of UV radiation is well known in laboratory practice and the food industry. Nowadays it is used in mushroom culture to increase Vitamin-D level [16] and might be effective in disease-prevention. A recent study showed the effect of UV light *in vitro* on *Verticillium fungicola* and *Mycogone perniciosa* tissue culture. The aim of the study was to determine what UV light range and irradiation time was most effective against the two pathogens. The results suggested that with proper application, UV irradiation could function as an additional technique in mushroom protection [17]. Certainly more tests are needed to evaluate the results in mushroom production conditions.

The toxic disinfectant formalin is very effective and relatively cheap but is no longer permitted for use in mushroom production in many countries. Therefore, other substitutes will need to be found which are less toxic to human health.

As a conclusion, new simple techniques in mushroom cultivation are needed in the future to reduce costs and increase efficiency of integrated pest management.

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THE ECTOMYCORRHIZAL FUNGUS *TRICHOLOMA MATSUTAKE* IS CAPABLE OF FACULTATIVE SAPROTROPHY

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ABSTRACT

We studied carbon acquisition in *Tricholoma matsutake* by combining morphological, chemical and enzymatic experiments conducted both in the laboratory and natural setting. Associations between host plants and isolates of *T. matsutake* from Finland (2) and Japan (1) were confirmed via *in vitro* formation of ectomycorrhizae (ECM). Chemical properties and enzyme-activity rates were determined for samples of mycelia-soil aggregation (shiro) collected from sites of sporocarp formation and nearby control spots. Annual growth and seasonal changes in tissue and ECM health were monitored in a natural population of matsutake. Finally, several organic substrates were evaluated as the sole carbon source for *T. matsutake* growing *in vitro* and according to the most active enzymes in the shiro.

Matsutake formed typical ECM with the conifers *Pinus sylvestris* and *Picea abies* but did not form associations with Silver Birch (*Betula pendula*). Finnish isolates formed ECM on both conifers but the Japanese strain was less compatible, with only a partial Hartig net being observed in *P. sylvestris*. Saprotrophic feeding of the Japanese isolate was observed in culture with *P. abies.* Preferred organic carbon sources and enzyme activities *in vitro* corresponded to those observed in the shiro. Enzyme assays confirmed the presence and increased production of organic carbon degradation related enzymes during sporocarp formation, when ECM root tips were necrotic. Mycelial growth on culture media consisting of complex polysaccharides was similar to that composed of simple sugars (e.g., glucose). In addition to its typical life strategy as an ECM symbiont, results suggest that *T. matsutake* can exist as a saprotroph.

Key words: Matsutake; Mycorrhization; Saprophytic potential; Fungal ecology

INTRODUCTION

Tricholoma matsutake (S. Ito *et Imai)* is an ectomycorrhizal (ECM) fungus found in pine and spruce forests in the Northern hemisphere [11, 20, 18]. The fungus produces commercially valuable mushrooms that have been revered in Japan for their flavour, medicinal properties and iconic significance for centuries. Over the past 50–60 years, these edible fungi have become increasingly rare in Japan where the annual yield of matsutake has decreased from 12,000 tons in the 1940s to a few hundred tons today. One of the reasons for this reduction may be the introduction and spread of the pine nematode (*Bursaphelencus lignicolus*) in Japanese forests. Nearly 3000 tons of *T. matsutake* or closely related species are exported to Japan annually, with a retail value of approximately one billion US dollars [13]. This mushroom was known as *T.*

nauseosum in Nordic countries until recently when molecular techniques revealed its conspecificity with *T. matsutake* [2]. Matsutake mushrooms are distributed patchily throughout Finland [8], where they became a commercially harvested mushroom in 2007. The new and growing value of this non-woody product has received increasing attention in Nordic countries.

Many studies have been focusing on improving sporocarp formation in nature, e.g., by outplanting the mycorrhizal seedlings, and cultivating matsutake under controlled conditions. However, efforts to cultivate this species have not been successful, and knowledge of sporocarp formation in the shiro remains in its infancy. The shiro is a unique and massive aggregate of mycorrhizae, mycelium, host plant roots and soil particles [6, 12].

The objectives of this study are to confirm the relationships between *T. matsutake* and the major forest tree species in Finland, and to evaluate the saprotrophic potential of *T. matsutake* both *in vitro* and *in vivo*. We combined morphological observations, enzyme activity measurements and physico-chemical profiling of soil dominated by *T. matsutake*. We tested the following hypotheses: (1) *T. matsutake* is a typical ectomycorrhizal fungus but can exist as a facultative saprotroph, and (2) sporocarp formation is related to the amount of available organic carbon and other degradation products in the shiro.

MATERIALS AND METHODS

Three isolates of *T. matsutake* (S. Ito et Imai) Sing. were screened in this study: Japanese isolate (JA) (Tm 0945, [10]), Finnish eastern isolate (EF) (GQ904716, [18]) and Finnish southern isolate (SF) (JF346748). The *in vivo* study was conducted in Nuuksio national park (60°18'16"N, 24°31'10" E) in southern Finland, which supports a mixed forest of Scots Pine (*Pinus sylvestris* L.), Norway Spruce (*Picea abies* [L.] H. Karst.) and Silver Birch (*Betula pendula* Roth). At this site, sporocarps of *T. matsutakae* were found continuously during the study period (2008–2010).

RESULT AND DISCUSSION

Formation of ectomycorrhizae between *Tricholoma matsutake* and the main forest tree species in Finland. We tested the extent to which a local Finnish isolate (GQ904716, [18]) and a Japanese isolate (Tm945, [10]) formed ectomycorrhizae with the three most common tree species in Finland; *P. sylvestris*, *P. abies* and *B. pendula*. Under laboratory conditions, *T. matsutake* formed typical ectomycorrhizae with *P. sylvestris* (Fig. 1a & 1b from [18]) and *P. abies* but not *B. pendula*. Germinated seedlings of *P. sylvestris* and *P. abies* were inoculated with either isolate, and after eight months the Finnish isolates had formed a sheath and Hartig net on both host species. Inoculation with the Japanese isolate resulted in an initial Hartig net-like structure in *P. sylvestris* but not in *P. Abies*, but a fully formed Hartig net was not observed in either. Ectomycorrhizal *P. sylvestris* seedlings inoculated with the Finnish isolates showed the same shoot height and dry mass as controls, whereas those of *P. abies* had similar shoot height but slightly less dry mass than control seedlings. For both tree species, inoculation with the Finnish isolate resulted in reduced total nitrogen content per seedling but carbon content was unaffected. Seedlings of both species inoculated with the Japanese isolate showed significantly reduced growth, dry mass, nitrogen and carbon content per seedling and shoot height (in spruce) compared to the controls. These results document and describe the *in vitro* ectomycorrhization between *T. matsutake* and *P. sylvestris* and *P. abies* and the variable mycorrhizal structures that strains of matsutake can form [18].

Interestingly, in addition to the apparent preference shown by the Japanese isolate for *P. sylvestris*, we observed its saprotrophic behaviour in co-culture with *P. abies*. It should also be

mentioned that although we did not find any Hartig net or similar structure in inoculated seedlings of *Betula pentula*, root tips exhibited signs of necrosis (Fig. 1c & 1d, unpublished data). Host specificity or host preference of *T. matsutake* remains an ongoing study in our group.

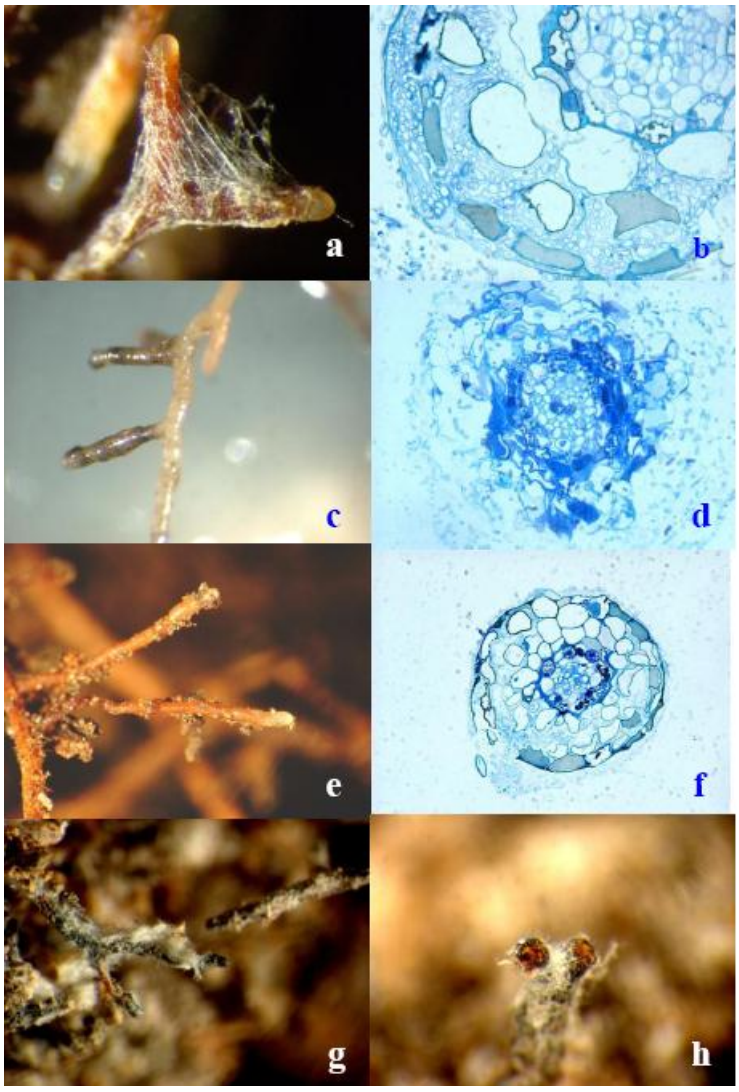

Figure 1: a-b, External morphology and light micrographs of fungus-inoculated root system of *Pinus sylvestris* eight months post-inoculation by the Finnish isolate (GQ904716): (a) the dichotomous lateral root is colonized by dense fungal mycelium; (b) transverse section of ectomycorrhizal root showing multiseriate Hartig net development within the cortex, between cortical cells, tannin cells present in cortex.
c-d, External morphology and light micrographs of fungus-inoculated root system of *Betula pentula* two months post-inoculation by the Finnish isolate (GQ904716): (c) the monopodial lateral root with light necroses; (d) transverse section of root tip showing no Hartig net formation within the cortex.
e-f, External morphology and light micrographs of root tips from *T. matsutake* shiro spot in early summer of 2009: (e) the monopodial lateral root with a loose external mycelium; (f) transverse section of root tip showing the well-developed Hartig net structure within the cortex.
g-h, External morphology of root tips from *T. matsutake* shiro spot in autumn of 2009: (g) many mycorrhizal root tips were necrotic; (h) or clearly suffering.

Mycorrhizal root tips showed signs of necrosis in the shiro. In our study site, live mycorrhizal root tips and Hartig net (or similar) structures were observed in the early summer (Fig. 1e & 1f, unpublished data). However, we found that many mycorrhizal root tips were necrotic (Fig. 1g) or clearly suffering (Fig. 1h) during the fruiting season. This phenomenon has also been reported elsewhere [5]. A breakdown in the association between matsutake and host plant during the reproductive season may be connected to its facultative saprotrophy.

Saprotrophic potential of *Tricholoma matsutake*. In this study, eight enzymes were assayed in soil samples dominated by *T. matsutake* (shiro+) or nearby control (PCR-negative: shiro-) spots. We assayed the same enzymes in an *in vitro* culture system in which mycelium of *T. matsutake* was used to inoculate bark chips of *P. sylvestris*. Results indicated higher activities of cellulose and hemicellulose degradation related enzymes in shiro+ than in shiro- soil immediately following sporocarp harvesting (unpublished data). It should be emphasized that many root tips in shiro+ samples were necrotic [11, 5].

In a *T. matsutake*–sawdust culture system, Vaario et al. [17] observed mycelial growth in xylem tissue and reported earlier that *T. matsutake* produced mainly β-glucosidase when pine bark was used as the substrate *in vitro* [16]. Subsequently, Kusuda et al. [7] purified and characterized β-glucosidase in wild matsutake. These findings suggest that *T. matsutake* can secrete cellulolytic and hemicellulolytic enzymes to acquire carbon from its environment, and results from the bark culture experiment confirmed this (unpublished data). Furthermore, enzyme activity between day 20 and day 40 were similar, which suggests the capacity to utilize this form of organic carbon (i.e., bark chips) is stable. Hyphae continued growing throughout the two-month study period.

We provided polysaccharides as the sole carbon source in a liquid medium to measure the growth capacity of *T. matsutake*. After a 60-day incubation period, a similar net increase of mycelial biomass was observed in culture media containing either simple sugar (e.g., glucose) or polysaccharides. The *in vitro* culture data suggest that *T. matsutake* can secrete a cocktail of enzymes suitable for bark as well as a medium containing simple sugars. This finding suggests that *T. matsutake* has, at least, the chemical means to feed saprotrophically.

Because *T. matsutake* can use organic carbon and secrete related cellulolytic and hemicellulolytic enzymes, we suggest three possible mechanisms to explain how *T. matsutake* gains extra energy for shiro and fruitbody formation. Firstly, different kinds of organic carbon compounds exist in the litter layer that could be leached to the mineral layer where most of the mycelium is found. Such nutrient leaching would be especially important if most of the mycorrhizal root tips are necrotic and the symbiotic association is no longer in place. Secondly, *in vitro* enzyme profiles suggest that *T. matsutake* can produce certain cellulose degradation related enzymes, which could facilitate the degradation of cellulose and make its products available, e.g., cellobiose [9]. Finally, our earlier work showed that several saprotrophic and mycorrhizal/litter-decay fungi (e.g., *Trichoderma viride*) in the litter layer above the shiro were positively correlated with the presence of matsutake [19]. It seems possible that the degradation carried out by these fungal associates could provide the available carbon source leached to the mineral layer for *T. matsutake* uptake.

Deacon and Fleming [4] reviewed the succession of ECM fungal guilds and suggested that species may be classified as early (low sugar requirements, small and ephemeral sporacarps with easily germinated spores) or late (high sugar requirements, large and persistent sporocaps with spores that are difficult to germinate) stage. According to this classification, *T. matsutake* conforms to a late stage species. Whether high sugar requirement is the limiting factor in shiro and sporocarp formation needs to be examined *in vivo*. Recent studies on the functional and ecological significance of ECM symbiosis have emphasized its importance in the ecosystem [1, 3]. Increasing attention is being given to the functional diversity of ECM fungi [14], and how

they may occupy a position along the biotrophy-saprotrophy continuum [15]. A flexible trophic ecology would be a considerable advantage for *T. matsutake* in shiros where disconnection from host plants occurs, and may be a necessary stage of the life cycle.

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SYMBIOTIC VERSUS SAPROTROPHIC STRATEGY DURING *TUBER MELANOSPORUM* ASCOCARP DEVELOPMENT. PRELIMINARY RESULTS BASED ON ^{13}C AND ^{15}N NATURAL ABUNDANCE AND ON *IN SITU* $^{13}CO_2$ PULSE-LABELLING.

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ABSTRACT

Despite their renown, the life cycle of the true truffles belonging to the genus *Tuber* is not well known. The growth of the ascocarp is poorly understood. It is not known if a direct transfer of carbohydrates takes place between the host tree and the developing ascocarps through the ascogonial filament or whether ascocarps become independent from their hosts after several weeks or months and are able to use dead host tissues or soil organic matter as carbon and nitrogen sources. From a first work based on ^{13}C and ^{15}N natural abundance, we found that *Tuber* ascocarps do not exhibit a saprotrophic strategy during their development [1]. However, *in situ* ^{13}C and ^{15}N labelling experiments are the only way to solve the question of carbon and nitrogen allocation during *Tuber* ascocarp differentiation. A first *in situ* $^{13}CO_2$ pulse-labelling experiment was carried out in 2010 on a 20-year-old hazel tree mycorrhized with *Tuber melanosporum*. The preliminary results showed that the transfer of carbon from the leaves to the fine roots is slow but continuous during several months even during winter at low temperature. The fine roots act as a pipe to transfer the carbon to the mycorrhizas. From the mycorrhizas, ^{13}C accumulates into the ascocarps, which constitutes a carbon sink. These results contradict the statements of recognized truffle handbooks and could be of some importance for the improvement of truffle cultivation methods.

Keywords: *Tuber*; Ascocarp; Development; Carbon; Nitrogen.

INTRODUCTION

The development of *Tuber* ascocarps is poorly understood. It is not known if a direct transfer of carbohydrates takes place between the host tree and the developing ascocarps through the ascogonial filament or whether ascocarps become independent from their hosts after several weeks or months and are able to use dead host tissues or soil organic matter as carbon and nitrogen sources. According to several studies, the ascocarp becomes independent from the host very early [2, 3, 4]. It would be able to use soil carbon sources through external mycelium independently from the host. During its development, the truffle ascocarp develops from its

peridium external mycelium which could colonize dead cells from living roots, dead roots, other dead organic tissues or mineral structures [5]. These external hyphae could absorb, water, orthophosphates and simple sugars, which would be then transferred inside the ascocarp [6]. Similarly, in pure culture, the mycelium of *T. melanosporum* could use cellulose, cellobiose, lignin, chitin and tannins as sources of carbon [7, 5]. Nevertheless, the sequencing of *T. melanosporum* genome has shown that this fungus has much fewer GH-encoding genes than saprotrophs [8]. The cellulases from families GH6 and GH7 are absent, whereas family GH45 cellulase and hemicellulases from families GH10 and GH43 are present. *T. melanosprum* has an invertase gene allowing the hydrolysis of the plant-derived sucrose.

The aim of our work was to determine the way of carbon allocation to the ascocarps: saprotrophic pathways from surrounding soil organic matter or dead host tissues or symbiotic pathways through carbon allocated by the host.

The first part of our work was based on the determination of ^{13}C and ^{15}N natural abundance in different compartments of a truffle orchard of holm oaks in the South East of France. The second part of this work was based on an *in situ* $^{13}CO_2$ pulse-labelling experiment carried out on a 20-year-old hazel nut tree mycorrhized with *Tuber melanosporum* in a truffle orchard of hazel nut trees in East of France.

MATERIAL AND METHODS

^{13}C and ^{15}N natural abundance. Samples were collected at Châteauvert, Visan, Vaucluse, France, in a holm oak truffle orchard (Longitude: 4.916° - Latitude: 44.366°; altitude: 200 m, annual winter-dominant rainfall 759 mm; mean annual temperature 13°C. The bedrock consists of calcareous alluvial deposits. The soil is a deep brown calcarisol with a sandy-silty texture, high alkaline $pH_{(H2O)}$ (8.5) and a limestone content of 41%. It is very poor in available phosphorus and moderate in available K and Mg. It is a highly granular and aerated free-draining soil. Each year, the site is superficially ploughed and irrigated (100 mm in summer).

The holm oak (*Quercus ilex* L.) stand (10000 m^2) was planted in 1976 on a vineyard site. The seedlings were mycorrhized with undetermined naturally occurring fungi and were naturally mycorrhized with *T. melanosporum* and other ectomycorrhizal fungi.
In 2002, five trees, having produced *T. melanosporum* ascocarps over many years, were selected for sampling. Leaves, fine roots, branches, mycorrhizas, soil samples and *Tuber* ascocarps were collected in August 2002 and January 2003 underneath these five trees. In January 2003, sporophores of epigeous fungi were also collected throughout the site.

Percentages of C and N plus isotopic composition were determined at the INRA centre of Nancy-Champenoux using an online continuous flow CN analyser (Carlo Erba NA 1500) coupled with an isotope ratio mass spectrometer (Finnigan delta S). Values were reported using the standard notation ($\delta^{13}C‰$ and $\delta^{15}N‰$) relative to Pee-Dee Belemnite for C, using PEF (IAEA-CH-7) as a standard, and relative to atmospheric N_2 for N, using $(NH_4)_2SO_4$ (IAEA-N-1) as a standard. $\delta X=(R_{sample}/R_{standard}) -1) \times 1000$, where R is the molar ratio $^{heavy}X/^{light}X$.

$^{13}CO_2$ pulse-labelling experiment. The experiment was carried out in Rollainville situated in the West part of the Vosges, France, on a limestone plateau of the Jurassic era (latitude 48° 18' 42'', longitude 5° 44' 13''; elevation 360 m ; annual rainfall 941 mm with a maximum in July ; mean annual temperature 9.5°C. The soil is a brown calcarisol with a silty clay texture, a high alkaline pH (water pH 7.97), a moderate content of organic matter (9.4%) and a limestone content of 8.8%. It is poor in available phosphorus and moderate in available K and Mg. It is a free draining soil highly granular and aerated.

Established in 1990 and 1991 on a previously cultivated site with *Quercus robur* (L.) and

hazel nuts trees previously inoculated with *Tuber aestivum* (Vittad.) (summer truffle) and *T. melanosporum* (Vittad.) (black Perigord truffle). The first truffle crop has started in November 1995 for *T. melanosporum* and in October 1996 for *T. aestivum*.

In 2010, we have started a labelling experiment with $^{13}CO_2$ on a hazel nut tree selected for having previously produced *T. melanosporum* ascocarps. On the day of labelling, the whole tree was enclosed in a 28 m^3 cylindrical polyethane chamber into which $^{13}CO_2$ gas was injected. The content of $^{13}CO_2/^{12}CO_2$ was followed by a Mahiac (SICK 710). A first introduction of $^{13}CO_2$ was done the 6th of July 2010 and a second one the 1st of September 2010. During the two labelling periods, the tree has assimilated 16.7 g de ^{13}C.

The isotopic composition of leaves, fine roots, mycorrhizas and ascocarps was determined as previously described for natural abundance.

RESULTS AND DISCUSSION

^{13}C and ^{15}N natural abundance. As reported by several authors [9, 10, 11, 12, 13, 14, 15, 16, 17], in the Châteauvert site $\delta^{13}C$ values differed between sporophores of saprotrophic and ectomycorrhizal fungi (Figure 1b), while total C did not differed among the two groups (Figure 1 a).

Sporophores of the two saprotrophic fungi from the Châteauvert site showed $\delta^{13}C$ enrichment compared to their substrates. *T. hiemalis* displayed a $\delta^{13}C$ of -24.2‰ against -26.1‰ for soil organic matter (Figure 2). Similarly, *S. hirsutum* displayed a $\delta^{13}C$ of -20.3‰ against -22.7‰ for dead wood (Figure 2). Cellulose and lignin degradation could be involved in ^{13}C enrichment of sporophores of saprotrophic fungi, although few fungal culture studies on known ^{13}C complex substrates have yet been conducted.

Sporophores of the two saprotrophic fungi displayed no or little ^{15}N fractionation compared to their substrate (Figure 2). *T. hiemalis* displayed a $\delta^{15}N$ of -4.4‰, very close to the $\delta^{15}N$ of soil organic matter (-4.6‰). Similarly, *S. hirsutum* displayed a low $\delta^{15}N$ fractionation (-1.1‰ against 0.6‰ for dead wood).

^{13}C natural abundance of ectomycorrhizal fungi sporophores varied from -24.6‰ to -27.2‰ (-25.7 to -25.8‰ for *T. melanosporum*) (Figure 1 b).

$\delta^{15}N$ natural abundance of ectomycorrhizal sporophores was much more variable than $\delta^{13}C$ natural abundance. For example, *I. fastigiata* displayed a weak nitrogen natural abundance ($\delta^{15}N$ of 2‰), while *A. strobiliformis* displayed a huge natural abundance ($\delta^{15}N$ of 13.5‰). These results are congruent with those of several research teams [10, 12, 13, 18, 15, 17, 19, 20] who all observed high ^{15}N abundance in ectomycorrhizal fungi sporophores. The three *Tuber* species displayed a high $\delta^{15}N$ value (9.3 to 10.4‰). Hobbie et al. [21] also found for *T. gibbosum*, a North American truffle, high $\delta^{15}N$ values (12 to 16.6‰). From the Châteauvert results, congruent with those of Hobbie et al. [21] for *T. gibbosum* in Oregon, it seems improbable that *Tuber* ascocarps could use, through protease excretion, nitrogen incorporated in soil organic matter which display on average a $\delta^{15}N$ of -4.6‰, while *T. melanosporum* ascocarps display $\delta^{15}N$ values ranging on average from 9.3‰ in winter to 10.4‰ in summer. A transfer of nitrogen *via* mycorrhizas, which displayed a positive $\delta^{15}N$ (4.6‰ in nursery conditions), seems much more probable. From the isotopic index $\Delta_{CN} = \delta\ ^{13}C - \delta\ ^{15}N$, which allows assignment of a mycorrhizal or a saprotrophic strategy for sporophore differentiation, it is obvious that *Tuber* ascocarps do not display a saprotrophic strategy (Table 1). If we consider the $\delta\ ^{13}C$ values alone, the conclusions are the same as Hobbie et al. [21] when considering that the limit between saprotrophic and symbiotic strategies is 24‰. From the Châteauvert results, it seems that, during ascocarp differentiation, *T. melanosporum, T. brumale and T. rufum* behave like ectomycorrhizal

fungi and not like saprotrophic fungi, despite the fact that sporophore initiation and development are rapid (some days) in ectomycorrhizal Basidiomycetes and very slow in *Tuber* species (six months ore more). Moreover, there were no statistically significant differences between newly formed *T. melanosporum* ascocarps collected in summer (August) and mature ascocarps collected in winter (January of the following year) (Figure 1c). This implies that the processes of carbon allocation remained identical during the entire period of *T. melanosporum* ascocarp development.

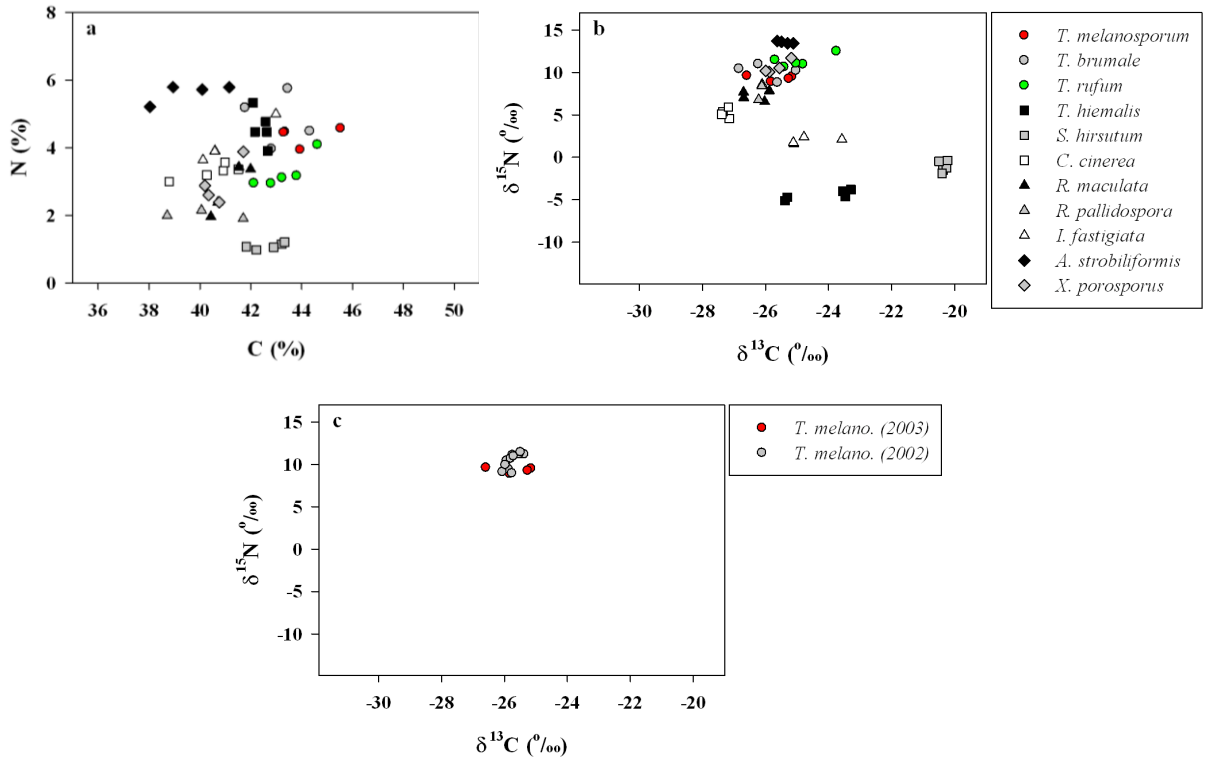

Figure 1: Discrimination among *Tuber* ascocarps (*Tuber melanosporum* Vittad., *Tuber brumale* Vittad. and *Tuber rufum* Pico) and sporophores of epigeous fungi (two saprotrophic species, *Stereum hirsutum* (Willd.) Pers. and *Tubaria hiemalis*, var. *hiemalis* Romagn. ex. Bon, six ectomycorrhizal species, *Clavulina cristata* (Bull.) J. Schröt., *Russula maculata* Quél. & Roze, *Russula pallidopsora* J. Blum ex Romagn., *Inocybe fastigiata* (Schaeff.) Quél., *Amanita strobiliformis* Paulet ex Vittad. and *Xerocomus porosporus* Imler) collected in the Châteauvert stand in 2003 according to:
a - Total C and total N (all sporophores, average and standard deviation for each species). Two species differed significantly ($P < 0.001$) from the other species for total N, *A strobiliformis* (high values) and *S. hirsutum* (low values). No significant differences were observed between saprotrophic and ectomycorrhizal fungi.
b - $\delta^{13}C$ and $\delta^{15}N$ (all sporophores, average and standard deviation for each species). Saprotrophic and ectomycorrhizal fungi differed significantly for $\delta^{15}N$ ($P < 0.001$). *I. fastigiata* displayed an intermediary position. *S. hirsutum* differed significantly for $\delta^{13}C$ from all of the other species ($P < 0.001$).
c - Discrimination among sporophores of *T. melanosporum* collected in the Châteauvert stand in 2002 (summer) and 2003 (winter) according to $\delta^{13}C$ and $\delta^{15}N$ (all sporophores, average and standard deviation for the two dates). There were no significant differences between the two dates.

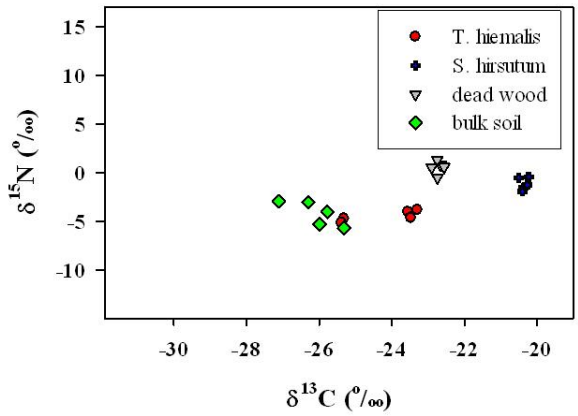

Figure 2: Discrimination among the two saprotrophic sporophores collected in the Châteauvert stand in 2003 according to $\delta^{13}C$ and $\delta^{15}N$ (all sporophores, average and standard deviation). Comparison with soil and wood $\delta^{13}C$ and $\delta^{15}N$. *Tubaria hiemalis* lives on soil and *Stereum hirsutum* on wood. There is a statistically significant $\delta^{13}C$ shift between soil and *Tubaria hiemalis* and between wood and *Stereum hirsutum*. There is no statistically significant $\delta^{15}N$ shift between the two fungi and the two corresponding substrates.

Table 1: Total C, total N, $\delta^{13}C$, $\delta^{15}N$ and isotopic index Δ_{CN} ($\delta^{13}C$ - $\delta^{15}N$) of fungal sporophores collected in the Châteauvert stand (average and standard deviation, n = 5 or 4)

Genus	***Species***	**Authors**	**Date**	**C (%)**	**N (%)**	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**Δ_{CN} = $\delta^{13}C$ - $\delta^{15}N$**
Tuber	*melanosporum*	Vittad.	08-2003	43.0 (3.81)	4.0 (0.47)	-25.76 (0.21)	10.40 (0.87)	-36.16 (0.73)
Tuber	*melanosporum*	Vittad.	01-2003	44.0 (1.05)	4.4 (0.28)	-25.71 (0.66)	9.32 (0.32)	-35.02 (0.78)
Tuber	*brumale*	Vittad.	01-2003	43.1 (1.07)	4.9 (0.78)	-25.93 (0.78)	10.10 (0.92)	-36.03 (1.42)
Tuber	*rufum*	Pico.	01-2003	43.3 (0.96)	3.3 (0.48)	-24.94 (0.75)	11.32 (0.71)	-36.26 (0.54)
Tubaria	*hiemalis var. hie.*	Romagn. ex Bon	01-2003	42.4 (0.27)	4.6 (0.52)	-24.20 (1.05)	-4.45 (0.53)	-19.75 (0.69)
Stereum	*hirsutum*	(Willd.) Pers.	01-2003	42.7 (0.65)	1.1 (0.09)	-20.35 (0.11)	-1.09 (0.66)	-19.26 (0.66)
Clavulina	*cinerea*	(Bull.) J. Schröt.	01-2003	40.5 (1.05)	3.3 (0.21)	-27.28 (0.11)	5.25 (0.49)	-32.54 (0.51)
Russula	*maculata*	Quél. & Roze	01-2003	41.2 (0.72)	2.8 (0.73)	-26.33 (0.43)	7.27 (0.58)	-33.60 (0.73)
Russula	*pallidospora*	J. Blum ex Romagn.	01-2003	40.2 (1.50)	2.0 (0.12)	-26.15 (0.06)	8.21 (0.98)	-34.08 (0.93)
Inocybe	*fastigiata*	(Schaeff.) Quél.	01-2003	41.1 (1.29)	4.1 (0.61)	-24.65 (0.73)	1.96 (0.37)	-26.60 (0.63)
Amanita	*strobiliformis*	(Paulet ex Vittad.) Bertill.	01-2003	39.6 (1.35)	5.6 (0.28)	-25.39 (0.23)	13.54 (0.15)	-38.93 (0.37)
Xerocomus	*porosporus*	Imler	01-2003	40.8 (0.68)	2.9 (0.66)	-25.66 (0.37)	10.62 (0.74)	-36.27 (0.41)

$^{13}CO_2$ pulse-labelling experiment. The flux of pulse-derived ^{13}C from the tree to the fine roots, the mycorrhizas, and the ascocarps was traced and quantified over a seven-month post-labelling period. The preliminary results of this $^{13}CO_2$ pulse-labelling experiment showed that a significant transfer of ^{13}C towards the roots was detectable 5 days after labelling. However, the $\delta^{13}C$ of fine roots always remained low during all the post-labelling period, indicating that fine roots acted mainly as a pipe (Table 2). The transfer of ^{13}C from the fine roots to the *T. melanosporum* mycorrhizas occurred between 5 and 20 days after labelling (Table 2). The mycorrhiza $\delta\ ^{13}C$ reached a maximum of +22.75 ‰ 22 days after the first labelling. Then it decreased when the young truffles started to grow. It increased again after the second labelling to reach a maximum of 55.35 ‰ before decreasing again. The mycorrhizas formed a carbon sink and accumulated ^{13}C for all the tree growing period and for all the period of the ascocarp development. Then the mycorrhizas transferred their ^{13}C to the truffles, which accumulated carbon from the host until their complete maturity, 200 days after the first labelling. At the end of September, the gleba of the ascocarps, which were in full growth, was six times more enriched in ^{13}C than the mycorrhizas and ten times than the fine roots (Table 3). This role of carbon sink of the *Tuber* ascocarps occurred several months after the end of carbon assimilation by the host and at low temperature.

Table 2: Rollainville: Evolution of the $\delta^{13}C$ of leaves, fine roots and *T. melanosporum* mycorrhizas in ‰ according to the time (average of 4 samples at each date)

Days after labelling	1	4	5	22	55	83	133	264
Leaves	-27.66	290.71	35.26	29.65	469.07	11.45	-4.45	76.70
Fine roots	-27.62	-26.62	- 9.73	-13.15	-19.34	- 6.73	9.87	3.82
Tuber mycorrhizas	-27.50	-24.09	-24.60	22.75	-5.31	26.3	52.35	18.85

Table 3: Rollainville: Evolution of the $\delta^{13}C$ of *T. melanosporum* ascocarps (peridium and gleba) in ‰ according to the time (average of 3 to 7 samples at each date; the $\delta^{13}C$ of *T. melanosporum* ascocarps cropped under non labelled trees were in average -25.7‰ for the gleba and -26.25‰ for the peridium).

Days after labelling	83	101	133	168	204
Peridium	87.01	60.36	69.56	59.43	77.36
Gleba	125.38	78.94	79.13	67.17	82.12

CONCLUSIONS

^{13}C and ^{15}N natural abundance measurements and a $^{13}CO_2$ pulse-labelling experiment gave results going in the same direction. *Tuber* ascocarps do not exhibit a saprotrophic strategy during their development. The results demonstrate for the first time under field conditions that *Tuber* mycorrhizas provide a slow but important pathway of carbon flux from tree to the ascocarps. Almost the whole of carbon used by the truffle ascocarps seems to be allocated by the host. It becomes now evident that *Tuber* ascocarps are dependent from their hosts during their whole development. These preliminary results contradict the statements of well-recognized truffle handbooks and could be of some importance for the improvement of truffle cultivation methods.

A replication of the $^{13}CO_2$ labelling experiment is in course. We have also carried out two new experiments of soil labelling (^{15}N and ^{13}C) in order to determine if truffle ascocarps could partly use dead host tissues or soil organic matter as carbon and nitrogen sources.

Nevertheless some questions are remaining. It is not known how the transfer of carbohydrates takes place between the host tree and the developing ascocarps. The most probable is a transfer through the ascogonial filament, which could provide a direct connection between mycorrhizas and ascocarps.

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EFFECT OF NUTRIENT SOURCES AND PLANT HORMONES ON MYCELIAL MORPHOLOGY OF THE BLACK PERIGORD TRUFFLE *TUBER MELANOSPORUM*

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ABSTRACT

Physiological studies on strain Mel-28 of *Tuber melanosporum*, a highly prized edible ectomycorrhizal truffle species having a pungent and earthy fragrance, were undertaken to standardize its growth under *in vitro* conditions as it grows very slowly under cultural conditions. A total of ten media were evaluated, of which, minimal medium with slight modifications supported the best growth, followed by malt extract PVP medium. The media with pH 6.5 and 7.0 were optimal for the fungal growth. Preferred carbon and nitrogen sources were sucrose and organic nitrogen, followed by nitrates. Effect of the strigolactone analogue Gr-24 and auxin (Indole-3-acetic acid) was also studied on the growth, mycelial morphology and branching pattern of the fungus using confocal microscopy. Supplementation of the medium with Gr-24 resulted in highly branched mycelium, whereas elongation of hyphal tips was noticed in auxin-supplemented medium along with increased number of nuclei per cell. On the other hand, the radial growth of the fungus was lower in Gr-24, whereas it was higher in auxin-supplemented medium.

Keywords: Truffles; *Tuber melanosporum*; Growth conditions; Strigolactones; Auxin

INTRODUCTION

The true truffles are a group of several valuable and highly sought-after edible species of underground ascomycetes belonging to the genus Tuber [1]. All are ectomycorrhizal and are found in close association with tree roots. There are hundreds of truffles, and while none are known to be poisonous only a few of them are considered to be delicacies by humans. Among them, there are four commonly known edible taxa: the white autumn truffles (*Tuber magnatum*), the black winter truffles (*Tuber melanosporum*), the white spring truffles, and the black summer truffles (*Tuber aestivum/uncinatum*). The black winter truffles are also known as Perigord truffles and are the most expensive and sought together with *T.magnatum* among the truffle species. They can be described as "gourmet mushroom" and have a pungent, intense, earthy fragrance, which offers a unique flavor to food. *T. melanosporum* grows naturally as mycorrhized fungus on roots of oak (*Quercus* spp) and other trees in certain parts of France, Italy and Spain, and to a lesser extent in other countries [2, 3]. The annual harvest of black Perigord truffles from France, Italy and Spain combined is currently 50-80 tons [1].

The worldwide demand for this truffle has fuelled intense efforts for improving cultivation and production. Identification of processes that condition and trigger fruit body and symbiosis formation, ultimately leading to a more efficient production, will be facilitated by the knowledge of genomic traits and by a thorough analysis of the fungal physiology. While the first step has been reached with the sequencing of the truffle genome [4] data on the nutrient requirements in cultural conditions are still limited [5, 6, 7]. In the present study, the physiological requirements of *T. melanosporum* (strain Mel-28) were studied so as to standardize a medium for *in vitro* growth of the fungus for further studies. The effects of two growth promoters like auxins and strigolactone analogue Gr-24 on the growth and the branching pattern of the fungus were investigated by using confocal microscopy.

MATERIAL AND METHODS

Germplasm: Mel-28 strain of *Tuber melanosporum was* provided by INRA, Nancy [4] and the other strain Rey-t from Dipartimento di Biologia Vegetale dell'Università, Sezione di Torino, Torino.

Growth studies: For physiological studies, a basal medium is a prerequisite. For the identification of the black truffle basal medium, a total of ten media were tested (Table 1). Out of the ten media used a minimal medium with some modifications was selected for further studies (Tables 2, 3).

Table 1: Cultivation media used

Medium	Composition	Medium	Composition
HM medium	Standard composition	Malt extract PVP agar	Malt extract 10g; dextrose- 5g; PVP-1g;KH_2PO_4-1g; $MgSO_4.7H_2O$-0.5g
Hill & Kafer medium	Standard composition	MMN medium with mannose	Standard composition
Potato Dextrose Agar	Standard composition	MMN medium with Glucose	Glucose instead of mannose
Malt extract Agar	Malt extract 20g; dextrose-20g	MMN with malt extract	Malt extract instead of mannose
Potato dextrose malt agar	100g Potato extract; 10g malt extract	Modified Minimal medium	$Ca(NO_3)_2.4H_2O$ in place of $NaNO_3$; Gellan gum in place of agar

For identification of optimal pH the fungus was grown in a range of five pH values i.e. 6.0, 6.5, 7.0, 7.5 and 8.0. Radial growth of the fungal strains was recorded at a weekly interval up to four weeks to assess the growth rates and optimum pH for the fungus.

To identify the best carbon source used by *T. melanosporum*, four types of carbon sources i.e. three monosaccharides (Mannose, Glucose and Galactose) and the disaccharide maltose were used while sucrose was used as a control as the minimal medium contained sucrose.

Table 2: Minimal Medium composition (100 ml)

Sucrose	1.0g	Stock-4	0.5 ml
Stock-1	10 ml	Stock-5	0.5 ml
Stock-2	0.1 ml	Stock-6	0.1 ml
Stock-3	10 ml	pH	6.5
Gellan gum	0.4 g		

Table 3: Compostion of the Stock Solutions used in Minimal Medium

Stock-1 (100 ml)		**Stock-2 (25ml)**		**Stock-3 (100 ml)**	
$MgSO_4.7H_2O$	0.731 g	$MnCl_2.4H_2O$	0.15 g	$Ca(NO_3)_2.4H_2O$	0.288 g
KNO_3	0.080 g	$ZnSO_4.7H2O$	0.118 g	**Stock-4 (150 ml)**	
KCl	0.065 g	H_3BO_3	0.0375 g	Na.Fe.EDTA	0.24 g
KH_2PO_4	0.0048 g	$CuSO_4.5H_2O$	0.033 g	**Stock-5 (150 ml)**	
		$(NH_4)_6Mo_7O_{27}.4H_2O$	0.0058 g	Glycine	0.09 g
Stock-6 (20 ml)				Thiamine HCl	0.003 g
Potassium Iodide	0.015 g			Pyridoxal HCl	0.003 g
				Nicotinic acid	0.015 g
				Myo-Inositol	1.5 g

To determine the preferred nitrogen source five nitrogen sources were used i.e. two ammonium sources [$(NH_4)_2SO_4$, $(NH_4)_2H.PO_4$], two amino acid sources (Alanine, Asparagine) and one amide source (Urea) along with one nitrate control. The amount of carbon and nitrogen were equalized according to their weight present in the control medium. A total of 3.4 mg of nitrogen was supplemented through these sources replacing stock-3.

Effect of Indole-3-acetic acid and strigolactone analogue Gr-24: For the study, four concentrations of Gr-24 viz. 10^{-5}, 10^{-7}, 10^{-9} and 10^{-11} Molar were used along with a negative control. The radial growth of *T. melanosporum* was measured placing the growing fungal hyphae in the centre and Gr-24 in a well at a distance of 1.7 cm from the growing mycelium. To see the effect of auxins on the truffle growth, Indole-3-acetic acid was used in three concentrations (0.136μM, 1.36 μM and 2.72 μM) along with one negative control. The medium used was the Minimal Medium. Solution of various concentrations of Indole-3-acetic acid was supplemented in the medium by sterilizing it through syringe filter of 0.22μ porosity. The growth of the fungus was recorded along two predefined axis on weekly basis up to 6 weeks. Each treatment was replicated five times.

Microscopic studies: The effect of the Gr-24 and Indole-3-acetic acid on branching pattern of the fungus was studied using confocal microscopy. For microscopic studies, the fungus was grown on a dialysis membrane treated with EDTA. The membrane was boiled with 1mM solution of EDTA for 30 minutes and then washed with distilled water several times. The membrane was sterilized and placed over the solid medium before fungal inoculation. After two weeks of growth the membrane was lifted and cut in small pieces using surgical blade under a stereo microscope and was left overnight for recovery from injury. Propidium iodide was used as a stain. The cut membrane containing the fungus was stained and viewed under a Leica confocal microscope (Laboratory of Advanced Microscopy, Department of Plant Biology, Torino).

Statistical Analysis: Statistical analysis was done using one-way analysis of variance (ANOVA) and critical difference (CD) was calculated by multiplying standard error with the value of two-tailed t-distribution on n-1 degree of freedom at 5%.

RESULTS AND DISCUSSION

A total of 10 media were tested for the growth of *T. melanosporum*. Only two media, Malt extract PVP agar and a slightly modified Minimal medium supported the fungal growth. Malt extract PVP agar supported the maximum growth with a growth rate of 8 mm per week. The results obtained are shown in Table 4.

The optimal pH was investigated keeping malt extract with PVP as the basal medium. The growth of the two strains of *T. melanosporum* (Rey-t and Mel-28) was recorded for their respective pH requirements. The growth patterns of the fungal strains were recorded at a weekly interval up to four weeks to assess the growth rates and optimum pH for the fungus (Table 5). The results indicated that the two strains differed in their pH requirements. The strain Rey-t grows optimally at the pH 6 whereas the strain Mel-28 prefers pH 6.5.

Table 4: Radial growth of *Tuber melanosporum* on different growth medium

Medium	Radial growth (mm)				
	2nd week	3rd week	4th week	5th week	6th week
HM medium	--	--	--	--	--
Kafer medium	--	--	--	--	--
Potato Dextrose Agar	--	--	9.00	11.00	12.00
Malt extract Agar	--	11.00	14.00	18.00	20.00
Potato dextrose malt agar	--	--	9.00	10.00	12.00
MMN medium with mannose	--	--	--	--	--
MMN medium with Glucose	--	--	--	--	--
MMN with malt extract	13.00	19.00	24.00	29.00	33.00
Malt extract PVP agar	14.00	20.00	28.00	34.00	40.00
Modified Minimal medium	15.00	22.00	31.00	39.00	47.00
SE					**1.16**
CD (0.05)	**--**	**--**	**--**	**--**	**2.46**

Table 5: Radial growth of *Tuber melanosporum* on different pH

pH	Radial growth (mm)									
	2nd week		3rd week		4th week		5th week		6th week	
	Mel-28	Rey-t	Mel-28	Rey-t	Mel-28	Rey-t	Mel-28	Rey-t	Mel-28	Rey-t
6	9	10	14	17	18	23	21	27	23	32
6.5	14	8	19	13	26	17	32	20	39	23
7	11	7	15	12	21	15	28	18	34	23
7.5	----	----	----	----	12	10	15	13	19	17
8	----	----	----	----	----	----	----	----	----	----

Further study was carried out on Mel-28 strain since the strain was a sequenced one. The carbon sources utilization was studied using modified minimal medium without any carbon source. Four carbon sources viz. mannose, glucose, galactose and maltose were tested and sucrose was used as control. Fungal bit of 3 mm dia was cut using cork borer from actively growing fungal culture on potato dextrose agar medium and inoculated in the test media. Interestingly, none of the carbon source except the sucrose supported the growth of *T. melanosporum*. Till 3 weeks of time, the fungus even did not start to grow in the carbon sources except sucrose. In the medium containing sucrose as sole carbon source the fungus could show 23.4 mm radial growth in 3 weeks of time.

To determine the preferred nitrogen source by *T. melanosporum*, five nitrogen sources were tested along with calcium nitrate as control. The results showed the best growth of *T. melanosporum* on amino acid alanine followed by the amide source urea (Table 6). However, the fungus showed good growth on almost all the nitrogen source. Minimum growth of the fungus was recorded on Ammonium phosphate.

Table 6: Effect of various nitrogen sources on growth of *T. melanosporum*

Treatments	Growth (Radial in mm)		
	1st Week	2nd Week	3rd Week
Ammonium sulfate	10.4	18.6	23.7
Ammonium phosphate	9.6	14.2	20.2
Asparagine	10.2	19.6	24.5
Alanine	12.2	21.5	28.2
Urea	11.0	19.6	26.6
Control ($Ca(No_3)_2$)	11.6	20.4	26.9
SE	**0.06**	**0.07**	**0.11**
CD (5%)	**0.12**	**0.15**	**0.21**

The studies on the effect of the strigolactone analogue Gr-24 on the branching and growth pattern of the fungus *T. melanosporum* indicated a negative effect and growth inhibition of the mycelium even at 10^{-9} M concentration (Figure 1). By contrast, the Indole-3-acetic acid revealed a positive effect on the radial growth as well as the biomass of the fungus (Table 7 and 8).

Table 7: Biomass enhancement of *T. melanosporum* by IAA

Treatment	Growth of mel-28 on membrane (in mg) in 4 weeks
0.136mM	84.33
1.36mM	83.66
2.72mM	64.67
Control	42.66
SE	**7.10**
CD (5%)	**14.87**

Table 8: Effect of strigolactone and IAA on the growth of *T. melanosporum*

Treatment	Growth of *T. melanosporum* (in mm)			
	1st week	2nd week	3rd week	4th week
Gr-24 (10^{-9}M)	3.8	10.2	11.6	12.4
IAA (0.136mM)	3.6	10.8	15.4	18.2
Control	4.2	10.5	12.4	15.6
SE	**0.10**	**0.24**	**0.32**	**0.56**
CD (0.05)	**0.22**	**0.41**	**0.68**	**1.21**

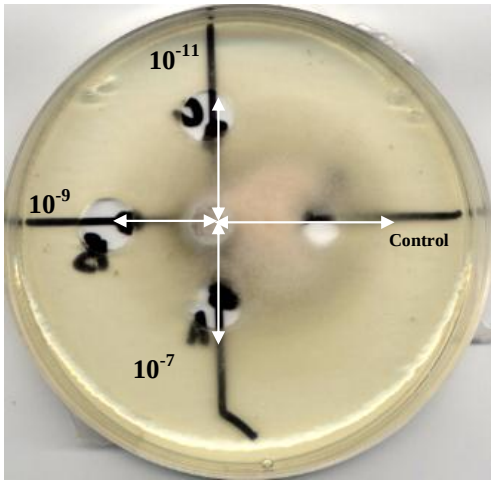

Figure 1: Growth of *T. melanosporum* as affected by Gr-24

To analyze the results obtained from Indole-3-acetic acid and strigolactone experiments, the fungal hyphae and their nuclear distribution were observed under confocal microscope. In the medium supplemented with the Indole-3-acetic acid, the length of the hyphal tips increased (Figure 2), while in Gr-24-treated mycelia, a more intense branching was observed, which may be the reason for the reduced radial growth (Figure 3).

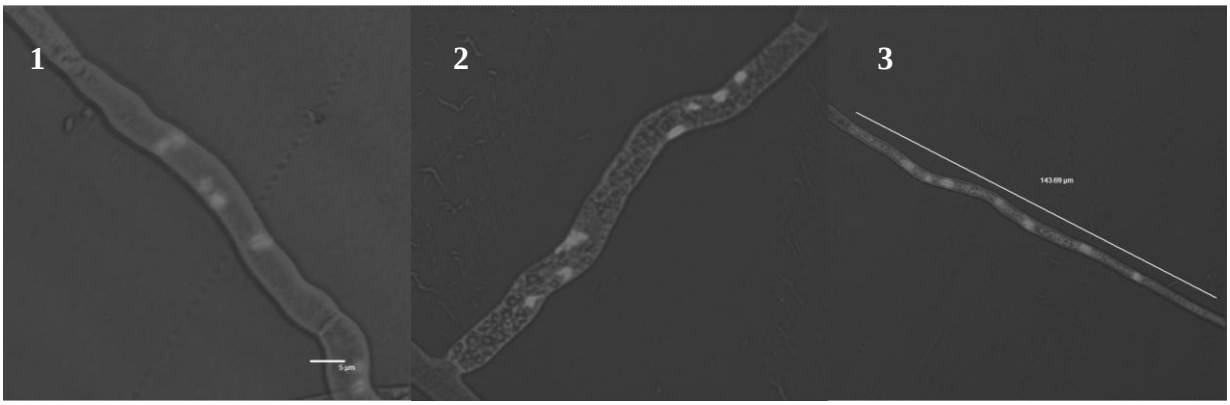

Figure 2: Elongation of hyphal tip in auxin treated mycelium (1 = Control; 2 & 3 = Auxin treated mycelium)

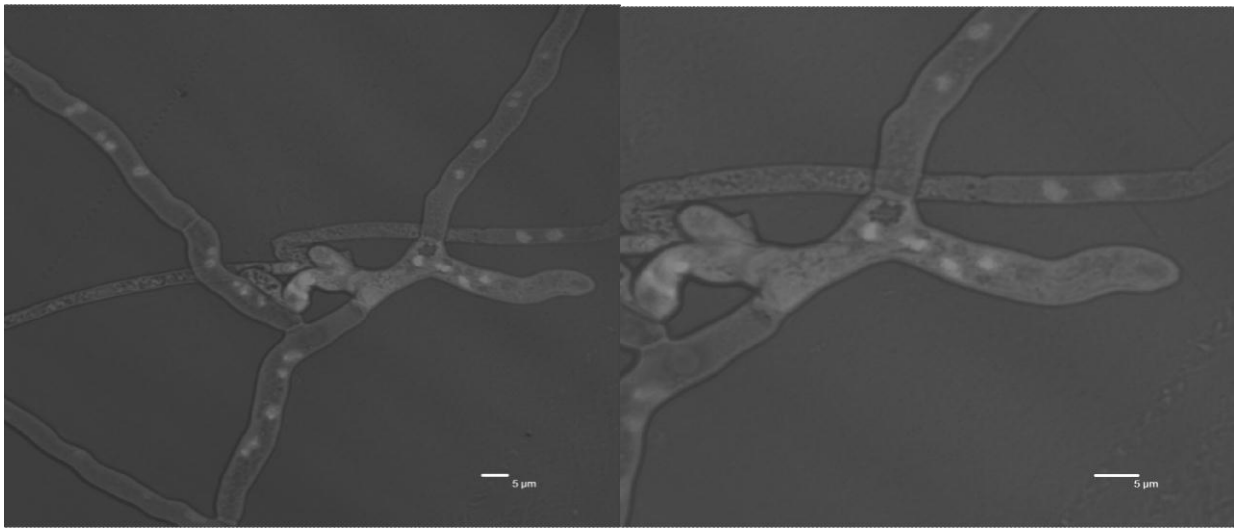

Figure 3: Change in branching pattern of Gr-24 treated mycelia of *T. melanosporum*

The genus Tuber groups ectomycorrhizal ascomycetes, which form mycorrhizas with a variety of hosts [8]. While the genome sequencing of the precious *T. melanosporum* has revealed the genomic traits, which are crucial for the establishment of the symbiosis [4, 9], a clear response to the slowness with which the fungus grows *in vitro* has not been found. This feature makes the study of the fungal physiology quite difficult. The results here reported largely confirm previous knowledge. Some studies regarding the use of different nutrient sources and culture media to optimize the growth of ectomycorrhizal fungi have been reported in the literature and, in particular, mycelium development has been assessed in relation to the carbon, nitrogen and phosphorus supply [10, 11, 12, 13]. Fontana agar medium [5] at either 20°C or 25°C (different growth chambers) was used for culturing *Tuber* mycelium. Use of modified Melin-Norkrans nutrient solution (MMN) (pH 6.6) by Molina [14] has been advocated for *in vitro* growth of truffles. MMN medium was also reported superior to Hagem + Modess medium [15] when growth was compared. As regards mycelial carbohydrate utilization, it was observed that ectomycorrhizal fungi utilize above all glucose and fructose [16], although sucrose and mannose each allowed substantial growth for *T. melanosporum* [7]. Mamoun and Olivier [7] suggested that the higher growth of *T. melanosporum* in mannose might be due to the fact that the fungus had adapted to environment, since mannose is an important constituent of plant photosynthetic sugars. In a previous study [17] using only *T. borchii* mycelium, utilization of glucose and fructose as carbohydrate sources was poor and stunted while good growth was recorded in sucrose [18]. Ceccaroli et al. [6] reported the utilization of mannose or mannitol as carbohydrate sources in culture by *T. borchii* strains. In contrast, during the present study the sugars other than sucrose did not support the growth of the fungus and we cannot propose any explanation for the same. Earlier all the studies on Mel-28 strain were conducted using potato dextrose medium, which also supported the growth of the fungus in this study. In fact, optimal production of mycelium is a fundamental step in the study of fungal metabolism, of the interaction between the truffle and its environment, of genetic variability and to obtain mycorrhization under controlled conditions. The hyphal morphology showed alterations in branching pattern when they were grown in mannose and mannitol [6, 19].

The above reports are in support of the present study that the morphology and branching pattern of the fungus *T. melanosporum* has shown changes with the change in the media and its composition. Interestingly, results obtained with the strigolactone (SLs) treatment suggest that *T. melanosporum* is sensitive to this novel class of plant hormones, similarly to arbuscular mycorrhizal fungi [19]. These fungi penetrate and colonize plant roots, where they develop highly branched structures called arbuscules, which are the sites of nutrient exchange. The natural SLs that have been identified so far have been examined for their activity on hyphal branching in the AM fungus *Gigaspora margarita*, and all the examined natural SLs were found to be active as branching factors. Although structural requirements for activity are very similar to those for germination stimulation of root parasites, some noticeable differences have been observed. For example, 3,6'-dihydro-GR24 was totally inactive as a germination stimulant but still showed distinct activity on hyphal branching (K. Akiyama, unpublished data).

The highly branched morphology in the *Tuber* mycelium was also observed during the present study and indicates that the synthetic stregolactone analogue GR-24 can also induce the mycorrhization by the fungus more efficiently and can be used for artificial inoculations of *T. melanosporum* for efficient colonization of the plants.

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BLACK TRUFFLE CULTIVATION AND COMPETING FUNGI

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ABSTRACT

Some of conditions required for *Tuber melanosporum* truffle production are known. The truffle fungus is introduced with controlled mycorrhized plants, in an appropriate soil (calcareous, aerated, free draining, with a good biodiversity), under a suitable climate which allows for a complete life cycle of the truffle. Culture techniques are usually concerned with the care of the soil, plantation irrigation, protection and pruning of the trees. Nevertheless, we observe the failure of some plantations because of the appearance of contaminating fungi.

Contaminating fungal species appear in truffle plantation according to the age of the trees and culture conditions. Many mycorrhizal or saprophytic species have been identified according to the age of different plantations in the South West of France, under mycorrhizal or sporocarp forms. A typology of this species was done to determine the level of danger it can cause in a success of truffle plantations.

These results show that certain species (*Hebeloma* sp.) can contaminate plants in the nursery. When *Tuber melanosporum* is cultivated in oak forest environment, *Tuber brumale* is the first contaminating fungus which takes advantage of the fall of biodiversity or some cultivation methods disturbing young truffle plantations. *Tuber aestivum* is a late contaminating fungus or it appears when the climate becomes drier. Many basidiomycetae are observed as plantations become old. When truffle cultivation is done in open landscape with cereals and vineyards, contaminations are rare. *Tuber melanosporum* can dominate the fungal train or succession for many years.

These observations require preserving the initial mycorhization of the planted trees with cultivation methods suitable to the environment with or without oaks forest. Bringing additional truffles spores can strengthen the initial inoculation with *Tuber melanosporum*. Thinning and pruning help *Tuber melanosporum* to be present and stronger in the truffle plantation as it is an early stage fungus.

Keywords: *Tuber melanosporum*, truffle, "brûlé" or burnt area, cultivation methods, mycorrhizal fungi.

INTRODUCTION

Truffle *Tuber melanosporum* cultivation has evolved since its beginning in the early 19th century when acorns were seed in a suitable environment already naturally producing black truffles. The process was codified on the 19th century and at the beginning of the 20th by Chatin [1], de Bosredon [2], de Ferry de la Bellone [3] and Pradel [4]. At this period of abundance, cultivation consisted mainly in growing oaks in limestone soils, that was to say to scuff the soil and prune oaks in order to keep the lasting of the production. After a period of declining production, following the two world wars, Rebiere [5] was the first to reformulate the truffle cultivation methods with the achievements of the modern agriculture intended to feed Europe. A new era began with the invention of controlled inoculated plants popularized since 1974 thanks to Gérard Chevalier. Grente and Delmas [6] specified the inoculation conditions of the environment with

trees mycorrhized with *T. melanosporum* and Delmas [7] analysed mostly the characteristic of suitable soils. First truffles, which were harvested under hazel trees only 4 years after planting, gave hope for a new rise of truffle production. French public institutions supported new truffle plantations with grants given until 1986. When it became obvious that truffle production would not really pick up again, all grants for planting mycorrhized trees, installing truffle irrigation and renewing old plantations were abolished. In 1994, the 11th National State Plan proposed that a step of experimentation in truffle cultivation should take place before giving out new grants for truffle plantations. In 2003, at the national truffle cultivation day organized by CTIFL in Cuzance (Lot, France), certain regions decided to encourage again truffle plantation with new financial aid.

Callot [8] underlined the importance of biological activity in truffle soil to explain the lack of good results. Sourzat [9] stressed the fact that soils had not changed in one century and biologic surrounding conditions had shifted as the farming methods had negative impacts. Fungal successions (fungi train) of wild truffle trees with those of plantation truffle trees where the soil was tilled were compared. In plantation, on shallow soil, no fungal succession but a disorder was observed. This disorder means generally that competing fungi species are favoured, particularly *Tuber brumale*. The principle of precaution in truffle cultivation, which was defined in "Truffe et Trufficulture" [9], recommends a technical itinerary with 3 stages:

- Stage One: in the first two years of planting, one must be sure to achieve the best possible success rate for the *T. melanosporum* infected seedling in the first two years of planting.
- Stage Two: it is important to focus on "a not too much" on the rapid growth of the mycorrhizal trees to avoid contamination by other types of mushrooms; the modification of the environment induced by the truffle itself (*i.e.* soil structure and biological activity) during the period of *brûlé* formation has to be considered.
- Stage Three: once fruiting has started, the aim is to improve the quality and quantity of the black truffle whilst ensuring a good perennial annual yield.

In 2008, the syntheses of truffle cultivation experimentations in France were published [10]. They enlighten the results provided since 1994 during the 11th and 12th National State Plans State-County with the subsidies from FranceAgriMer (Oniflhor, Viniflhor) and the Regions. These syntheses handle particularly of the truffle soils, host trees and seedling quality, technical methods (soil managing, truffle irrigation, pruning), *T. brumale* contaminations. Considering truffle soils have not really been altered for one century (in truffle traditional areas), cultural methods today are quite well known and seedlings are correctly mycorrhized, why is it so difficult to increase the level of the actual truffle production? The common answer is generally that climate has become warmer and dryer. Truffle growers who are irrigating their plantations credit regular production to this effort. Nevertheless, results are still heterogeneous. One observes that certain non-irrigated plantations can give quite regular results whereas irrigated or non irrigated other ones have bad results. Why?

The research programme SYSTRUF, funded by National Agency of Research (ANR), attempts to answer this question by studying particularly the biology of the truffle. The way of fungal competition by different species and observation of what happens in the fields are explored by the Station of experimentation on the truffle at Cahors-Le Montat. Why is the presence of such fungi more negative today than in the past? In other ways, why is the black truffle less dominant than in the past, why does it defend less against its potential aggressors?

MATERIAL AND METHODS

The three types of investigations involved surveys of the fungi species in the truffle environment, formation on the natural "truffières" on the edge of the plantation, contamination of truffle trees

in wooded environment or not wooded environment. The coherence of these three studies is motivated by the last question above and will be discussed in the conclusion.

Fungi surveys in truffle environment in Lot district. These surveys were done on four types of environments:

- Limestone grassland surrounded by *Quercus pubescens* woodland.
- Young truffle plantations (from introducing plants to the first harvested truffles).
- Actually producing truffle plantations.
- Old truffle plantations.

Study of the natural "truffières" on the edge of woodlands on natural pastures or Limestone grasslands. The conditions of formation of natural "truffières" are analyzed in traditional truffle areas. This type of truffières appears mostly in natural pastures, grassland or cleaned fallow land (moorland), linked to *Q. pubescens* woodland on the edge of the field in limestone areas. These situations are common in Lot district or other parts of Midi-Pyrénées region (Tarn, Tarn et Garonne, etc.). One example is selected on the commune of Le Montat to illustrate the phenomenom. This site is called grassland of Haute-Serre (pelouse de Haute-Serre) (Fig. 1).
This state is briefly discribed :

- Oaks on the edge, aged about fifty years, constitute a woodland relatively homogeneous, in average 6 to 8 meters high , with 2 to 8 meters between every trees.
- *Tuber melanosporum* « brûlés » appear on a strip located at 8 to 12 meters from the oak basis. This strip is 3 meters wide and is called the « melano strip » (plage à melano ».
- Some brûlés where *Russula delica* fruitbodies are observed at few meters from the basis of the oaks (2 to 4 meters).

Figure 1 : grassland of de Haute-Serre on **east side** with the 2 dotted lines delimiting the strip where *Tuber melanosporum* brûlés appear caused by the oaks on the edge. The 2 white and red stakes (1 m) at the right frame a producing brûlé. At the left, the tall red and white stake gives an idea of the size of the trees (one colour division = 0,5 m)

Beyond this report, we have investigated the fungal species repartition in the grassland of Haute-Serre by the way of the mycorrhizae indentification (microscope) plus classical surveys of fungi.

Conservation characteristics of initial mycorrhizal status by *Tuber melanosporum* or contamination by other fungi species in truffle plantations surrounded by woodlands or not. Examples chosen were explored in spring 2011.

Truffle plantations in wooded environments. These two explored plantations are located in Aujols (Lot district) and Daglan (Dordogne district). They are established on shallow and stony soils (rendosol), with mycorrhizal trees (*Q. pubescens, Quercus ilex, Corylus avellana*). They are well maintained with care and producing despite relatively dry summer. Investigations on these plantations concern the mycorrhizal status of the analyzed trees by the way of binocular loup and microscope. At Aujols, roots samples were taken mostly on producing trees whereas in Daglan, no producing trees were targeted.

Truffle plantation in cereals and vineyard plains landscapes. Explored plantations are located near Angoulême (Charente district) in vast plain of cereals cultivation and wineyard region for the Cognac production. There is neither woodland nor hedge of woods at least 300 meters from explored plantations. They had been done on "terres de Groie" (rendosols deeper than those at Aujols and Daglan) with mycorrhizal trees (*Q. pubescens, Q. ilex, C.avellana*), cultivated with care. Irrigation was by bringing water with big tanks (3000 liters) and watering only producing "brûlés". Growers brought spores from crushed truffles the 3rd year of plantation on the "brûlés" of young mycorrhizal trees which were not yet producing.
These plantations are producing better than those of the Lot and Dordogne districts. Harvest started earlier (4 years old) and percentage of producing trees was also higher (90 to 95 % instead of 30 to 60 %). Same type of investigations than above was done on these plantations.

RESULTS AND DISCUSSION

Results and their acquirement conditions are summarized without going into details in order to keep this article a reasonable size.

Mycological survey in truffle environment in Lot district. It was impossible to present the exhaustive mycological survey in the limits of this article. A synthesis was elaborated to understand the essential.

Limestone grassland, suitable for *T. melanosporum,* presented common mycological surveys. We observed that, both in grassland with *Bromus erectus* and *Festuca ovina* on edge of woodlands and in moorland with sparse *Qu. pubescens* and *Festuca ovina*, *T. melanosporum* could live with certain species (*Inocybe jurana*, *Hebeloma edurum*) but it excluded regularly the other fungi out of its production area (located in the "brûlé"). *Scleroderma verrucosum* (Fig. 2) was frequent at the limit of the *T. melanosporum* "brûlé" just starting or already producing. Its presence did not seem to penalize the truffle production. Its frequent observation in this condition means that *S. verrucosum* is preparing the field and helping to adapt and spread of the truffière, transforming organic matter and structuring the soil in front of the brûlé.

Tuber brumale presence in young plantations is generally a consequence of a disturbing technical itinerary which does not respect the fungi species dynamic and their repartition in space and time. The ecosystem is disturbed by some cultural practices such as frequent tilling with tractor-drawn tools. In these conditions, *T. brumale*, a well flexible species, occupies the ecological niche initially planned for *T. melanosporum*. A bad choice of establishment (insufficient drainage, soil with bad structure) or cultural methods unsuitable (soils compacted with mechanic tools, fresh organic matter ploughing in soil, and excessive irrigation before production) favours *T. brumale* at the expense of *T. melanosporum*.

Figure 2*: Scleroderma verrucosum* Pers. on a truffle « brûlé ».

Figure 3: *Tuber melanosporum* mycorrhizae (red ellipse) among infestation with *Hebeloma* sp. on root system of inoculated plant.

Figure 4: *Hebeloma mesophaeum* seen in a truffle plantation

Figure 5: *Scleroderma verrucosum* (first ellipse with metallic tool) closer to the trunk than truffles (2nd ellipse)

In producing plantation, "brûlés" which are producing *T. melanosporum* presented a very few species except some Incoybes like *I. jurana* and *I. splendens;* sometime, there are *Hymenogaster* (*H. luteus*) or *Hebeloma* sp. on the edge of the brûlé. On the other hand, trees without truffle production were associated with many other epigeous fungi (*Inocybe* sp. and *Tricholoma* sp. are frequent) and hypogeous (*Genea sp.*, *Hymenogaste*r sp., *Tuber sp.),* then *Russula* sp. and *Boletus* sp. when trees were becoming older. These fungi were not located

anywhere in the truffle plantation, particularly according to the "brûlé". These species are those of "old roots", located generally closer to the trunk of the tree, under the organic litter.

In old truffle plantations, according to the density of closure of the canopy, we observed thermophilic and limestone fungi usually found in *Q. pubescens* forest on calcareous soil. They are species which are very difficult to eliminate from renovated old plantations in order to produce again *Tuber melanosporum*. To succeed in renovating old plantations, it's necessary to open or clear and thin them, creating a variety of ecological niches. A good example exists with an old plantation which is producing again *T. melanosporum* at Laburgade (Lot district) [11].

These mycological surveys confirmed that each species or group of species have their specific ecological requirements corresponding with necessary conditions for the black truffle in the dynamic of its environment [12]. These groups or species have to be located at a particular place in the fungal succession (fungal train) observed with the environment change. *Tuber melanosporum* has its good place or stage as well as many other species.

Table 1 of main fungi which are not *Tuber* and living in the truffle environment.

Fungi quite negative	
Boletus luridus	Mycorrhizal fungus in open environment with grass. There is no truffle under trees producing this mushroom.
Russula lepida	Mycorrhizal fungus in closed environment, mainly in old truffle plantation. *Russula lepida* never lives with *Tuber melanosporum* under the same tree.
Russula delica	Mycorrhizal fungus in open environment. It creates « brûlés » similar with those of *Tuber melanosporum* .
Russula maculata	Mycorrhizal fungus in old truffle plantation and on the edge of woodland .
Amanita strobililiformis *Amanitopsis lividopallescens*	Mycorrhizal fungi living in open limestone environment. When they are colonizing « brûlés » producing *T. melanosporum*, truffle production disappears.
Hebeloma sinapizans *Hebeloma edurum*	Mycorrhizal fungi living mainly in old truffle plantation or limestone oak plantations. They persist in the soil when the forest is cleared. It was observed outside the "brûlé", doing a circle 0.50 to 0.80 m from the limit of the "brûlé".
Armillaria mellea	Dangerous parasite of the trees. It destroys truffle trees, mainly hazel. It's highly risky to plant truffle trees where this mushrooms is seen.
Fungi quite neutral	
Incocybe jurana	Mycorrhizal fungus quite frequent in truffle plantation. It is named « le truffier » in Provence where people eat it. Present on the edge of the producing « brûlé », it appears harmless for the truffle.
Scleroderma verrucosum	Myccorhizal fungus frequent on the outer fringe of the brûlé. It does not seem to interfere with *Tuber melanosporum.* It can coexist and fruit inside the brûlé.
Tricholoma scalpturatum *Tricholoma terreum*	Mycorrhizal fungi living in old truffle plantation. It follows the decline of the truffle production. It happens when there is an ecological disorder.
Helvella crispa	Non Mycorrhizal fungus frequent in truffle plantation when autumn is wet and hot.
Clavaria aurea	Non Mycorrhizal fungus living in limestone oak groves and rare in truffle plantation.
Morchella esculenta (and other morchella.)	Non Mycorrhizal fungus (?) rare in truffle plantation

Table 1 summarizes perception of species roles for the most frequent species living in truffle ecosystem in Lot district.

About the genus *Hebeloma*, *Hebeloma mesophaeum*, ubiquitous species all over the planet, is a big concern in greenhouses where nurserymen make mycorrhizal plants. They fight it with drastic sanitary precautions (Fig. 3-4).

Natural "truffières" on the edge of woodlands in natural pastures or Limestone grasslands. Fungi observed in the grassland of Haute-Serre belong to the species characteristic of the ecosystem of "natural pastures or Limestone grasslands on the edge of old *Q. pubescens* woodlands". A brief view of the main species observed with *T. melanosporum* is presented in relation to the fungal succession (Fig. 6). We defined where *Tuber melanosporum* was located in the limestone ecosystem previously described (Fig 7).

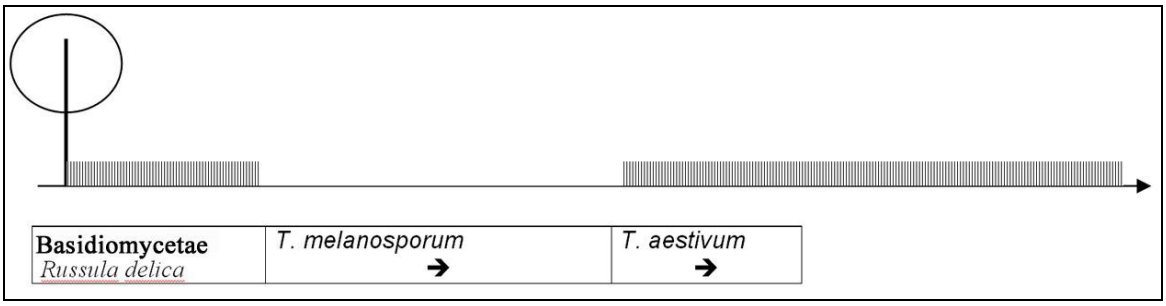


Figure 6: Fungal succession observed at Haute-Serre.

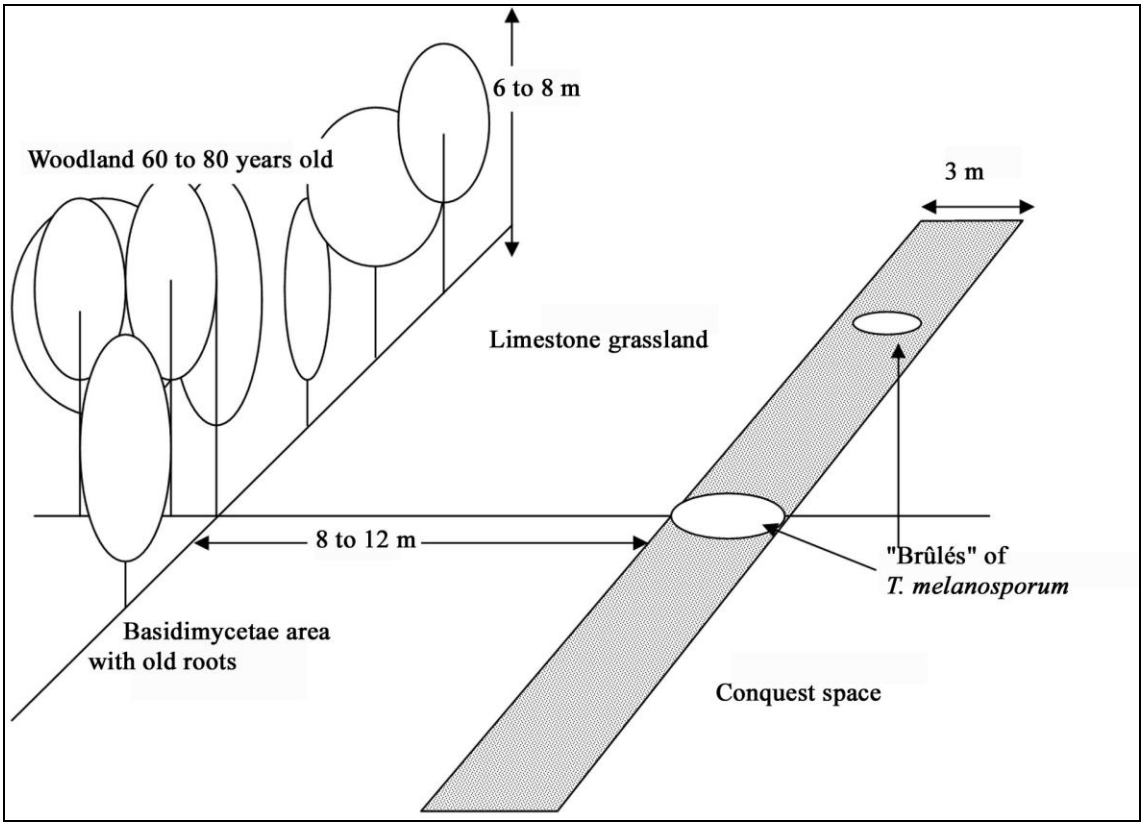


Figure 7 illustrating where *Tuber melanosporum* is expanding in the conquest space.

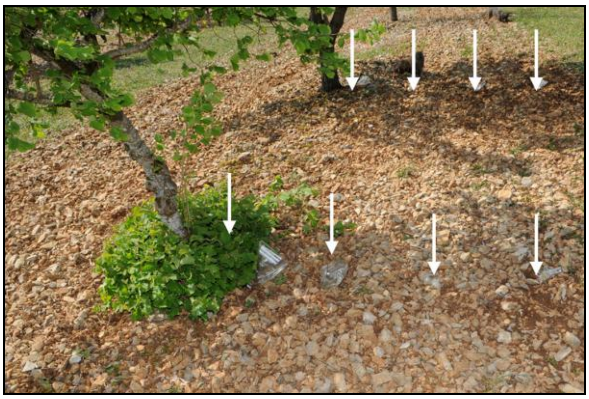
Tuber aestivum is a pioneer fungi in Limestone grassland and moorland. Nevertheless, if *Tuber melanosporum* fruits in this fungi succession, *Tuber aestivum* has a tendency to be quiescent ("sleeping"), that is to say that *Tuber* aestivum waits for the closure of the environment or woodland before it begins to fruit. In the meantime, *Tuber aestivum* is observed only on the form of mycorrhizae. Its mycorrhizae do not tolerate other kind of mycorrhizae like those of *Tuber melanosporum.*

The fungal succession observed at Haute-Serre is common in many areas in Lot district and French south-west.

Pioneer species like *T. melanosporum* are established in the young root system whereas basidiomycetae are on old roots. *Tuber aestivum* is located in front of or around the "brûlé" of *T. melanosporum*.

Evolution of the mycorrhizal status in relation to the type of environment. At Aujols, roots of producing trees were contaminated by basidiomycetae (*Hebeloma* sp.) on a radius of several tens centimetres from the trunk. Beyond, *T. melanosporum* was mostly present under the form of clusters of mycorrhizae (Fig. 8-11).

Figure 8: plantation at Aujols.



Figue 9: sampling method under the tree and every 60 cm at Aujols

Figure 10: view (with a measurement scale) of typical cluster of mycorrhizae of *T. melanosporum*.

Figure 11: backlighting of a mycorrhizae cluster of *Tuber melanosporum* which underlines young mycorrhizae

At Daglan, roots from non producing trees were contaminated with many undetermined fungal species. Inoculated trees with *T. melanosporum* on the edge of the woodland grew slower

than the other ones inside the plantation (feeding competition?). They were strongly contaminated from the oaks of the close woodlands.

We observed very dense root hairs on contaminated root system. On the other hand, roots inoculated with *T. melanosporum* revealed a sparse root hair. Phenomenon of the "brûlé" from *T. melanosporum* affected plants which can grow in the soil near the trees and the root system. Both were attacked directly or indirectly by *Tuber melanosporum.*

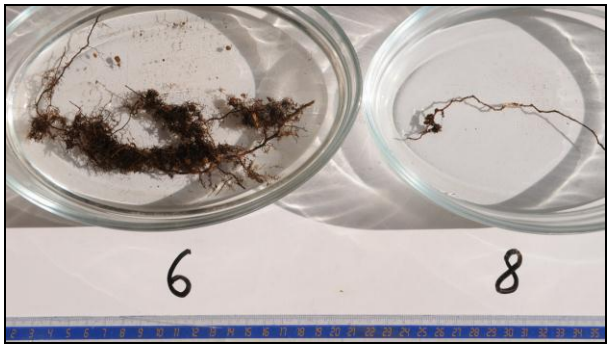


Figure 12: comparison of 2 root samples respectively from a contaminated area and a producing part of a *Tuber melanosporum* "brûlé".

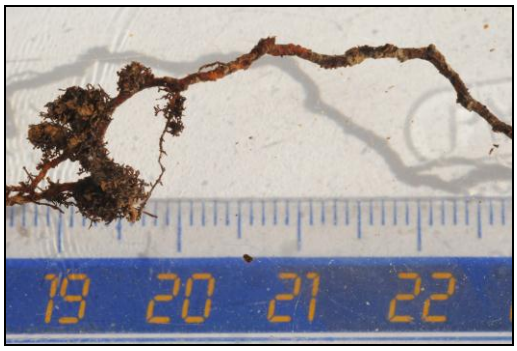


Figure 13: close-up of the sample 8

Taken with an equal soil volume, the two samples above (Fig. 12) illustrate what we could observe in truffle plantation at the good age to produce. Sample 6, from a non productive area, taken on a *Q. pubescens* shows a dense root hair. Sample 8, taken at 50 cm from the trunk of a good producing *Quercus pubescens*, in the part of the "brûlé" where truffles were found out, shows how the root hairs become sparse when the "brûlé" start to produce *T. melanosporum*.

Close-up above of the sample 8 (Fig. 13) shows how, in the productive part of the « brûlé », mycorrhizae were mostly grouped together in a cluster which the maximum size is about 8 mm as against 28 mm at Aujols. Note the tortuous shape of the root at the right side which indicates many scares of abscission. These scares look like puffiness. Depletion of the root system or lost of the root hair was typical inside the producing "brûlé". It seems *T. melanosporum* is attacking the root hair with a lost of some "branches" or ramifications.

If this aggression on a part of its root system does not kill the host tree, it is not the same for many plants which disappear when the « brûlé » spread. *Cistus albidus* sometimes produces *T. melanosporum*. In Corbières (Pyrénées-Orientales district), when « brûlé » is formed around the shrub the year « n », truffle is harvested on year « n+1 », and *Cistus* dies the year "n+2". *Tuber melanosporum* « brûlé » accompanying production becomes fatal for the host shrub.

We have done analysis with molecular tools on the roots of plants usually living in truffle "brûlés" or on the edge (Fig. 14). These plants were mainly *Sedum sediforme, Festuca ovina, Bromus erectus, Carex halleriana, Juniperus communis, Vitis vinifera*. We had found out the presence of *T. melanosporum* in the root tissues. This discovery in 2009 [13] validates the hypothesis that the truffle is not only a mycorrhizal fungus. It could be a parasite of plants that do not accept ectomycorrhizal fungi. When these plants are attacked by *T. melanosporum*, they can die.

When black truffle is observed in a very clear or net « brûlé » (said virulent), which is regularly gaining ground on the conquest space, we can observe that mycorrhization with *T. melanosporum* is very exclusive in the production area. It is not the case of many other species of fungi which accept cohabitation, particularly *T. brumale*.

« Brûlé » expansion is an indication of « virulence » or aggressiveness of the black truffle on its environment. The growth of the « brûlé » (10 to 30 cm per year in average) is the same on the good truffle trees, regardless of the region or the kind of environment. Truffle virulence or aggressiveness, pointed out in other words in books from the 19th century (de Bosredon, 1887), is a permanent characteristic. We could observe these effects both on the root system and the surrounding plants. We think that, in the past time, agricultural practices and pastoralism (or shepherding) were stimulating this property of *T. melanosporum* to be aggressive.

Figure 14: plantation at Daglan with a good level of production. Contaminated trees on the right side have not grown.

Figure 15: truffle plantation in Charente district in a landscape with cereal and wine cultivation.

Figure 16: sampling of mycorrhizae near the trunk of very good truffle tree in Charente district

In Charente (Fig. 15, 16), *T. melanosporum* mycorrhized trees were poorly affected by fungal contaminations. Contaminations (*Hebeloma* sp.) were located in a radius of 10 cm around the trunk of the host trees (Fig. 17). We observed that the clusters of mycorrhizae formed by *T. melanosporum* had a small size. They had few mycorrhizae in comparison with those from Aujols and also from Daglan. It was difficult to find mycorrhizae on the roots inside « brûlé » of the good truffle trees (Fig. 16). Fungi succession is limited or smaller than those we have seen in traditional truffle areas.

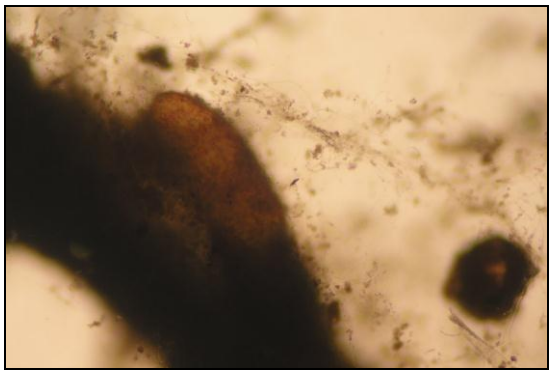


Figure 17: mycorrhizae of *Hebeloma* sp. With a characteristic mycelium reminding « cotton » which catches soil in its net.

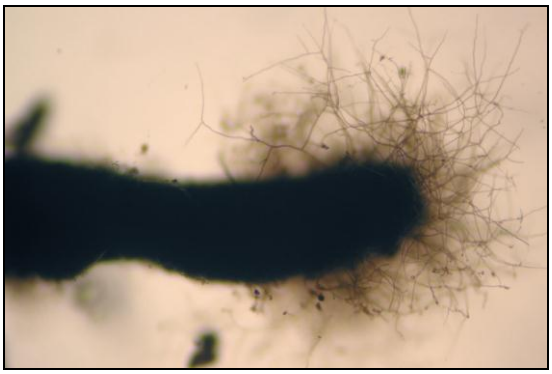


Figure 18: mycorrhizae said AD common in « brûlés » on edge of woodland

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We are trying to understand these differences in the fungal succession and the size of mycorrhizae clusters. When there is no woodland in the environment, it is logical that contaminating mycorrhizal fungi like AD (Fig. 18) are rarer. Forming big cluster of mycorrhizae could be a strategy to protect *T. melanosporum* against threat from other fungi. Maybe truffle can be a threat against itself, after an intense vegetative phase, when « brulé » appears. "Brûlé" appearance is concomitant with a depletion of the root system as if a truffle parasitic phase is beginning. Big clusters of mycorrhizae could also be a mean to have a big quantity of mycelium required for fruiting and feeding the fruit body when it is growing. These questions resulting from the evolution of the mycorrhizal status, regarding the environment, make so many hypotheses to study

CONCLUSION

Many species of fungi were observed in truffle environment. This fact emphasizes the difficulty to stimulate only one species cultivation, such as *T. melanosporum*, in space widely open to the contaminations with other fungi. *Tuber melanosporum* naturally finds its place in the dynamic of the environment as far as this one is weakly artificialized (no frequent tillage which reduces the biodiversity). In agricultural landscapes, without oaks hedge and woodland, truffle production is easier and earlier.

From these observations, we can recommend to the truffle growers certain precautions[14] summarized as following : 1) choose top-quality host trees (tree species suited to the environment, well mycorrhized) ; 2) limestone soil, aerated, well draining, good biological activity; 3) adapted cultivation techniques (tilling or plantation cleaning depending on the depth of the soil and physicochemical characteristics, truffle trees irrigation, pruning) ; 4) manage a favourable fauna and flora without excess to limit feeding competition but sufficient to maintain the necessary biodiversity ; 5) improve mycorrhizal potential by bringing truffle spores ; 6) take precautions with oak woodlands on the edge of plantations (cut oak roots from the edge with chisel, put mycorrhized trees at a good distance from the edge of the woodland creating a health perimeter) ; 7) thin the plantation in order to maintain a sufficient conquest space for the truffle (*T. melanosporum* is a pioneer fungi who needs space in its limestone environment).

A digression can be done about the truffle soil characteristics after the paragraph above. Discussion on this topic is still in progress and there are many advanced researches on it. In the traditional truffle areas, the physicochemical characteristics of truffle soils have not changed during one century. Of course, the consequence of a frequent tilling is a fast evolution of the organic matter. Soils with a high level of clay become more compact when they loose organic matter. Their cohesion and stability diminish. If the chemical fertilisation was vulgarized in the 20th century, it has not really affected the traditional truffle region with shallow soils. Moreover,

we observe very good results in truffle plantations established in large plains where there are cereals and vineyards cultivations without any oaks in hedge or woodlands near the plantations. Chemical fertilizers and soil fertility are not main factors to explain why some plantations cannot begin to trigger truffle production.

After at least one century of agricultural abandonment, we observe many oak woodland surrounding truffle plantations. They have grown naturally and progressively. Nowadays, they are penalizing the truffle production. Their impact depends of the « contaminating reach » of the oaks (10 meters in average) and of the « power of contamination » of the oaks on the edge. If the oaks on the edge are grouped in woodlands, their power of contamination is stronger than if they are in hedge [15]. This situation underlines the necessity to build « truffle bastion » in order to keep all *T. melanosporum* strength (power = strength x number of producing truffle trees). In the heart of big truffle plantations, there is a "truffle bastion" protected against fungi coming from outside.

The strength of the truffle is stated trough the concepts of « truffle virulence » or « truffle aggressiveness » which can be observed with "brûlés" spreading or gaining ground in good truffle plantations. In South-West of France, default of *T. melanosporum* "virulence" or "aggressiveness" is generally concomitant of 4 facts: 1) *T. brumale* is harvested instead of *T. melanosporum*, 2) *T. melanosporum* does not resist well during the drought, 3) truffle production has not durability in time (tree years in average in wild "truffières"), 4) harvest starts later in plantation after many years (as if there was an inertia to fruiting).

After the characterization of the lack of the truffle virulence, we can propose the opposite arguments to define positive truffle virulence. Many experiences or trials are done at the Station of experimentation on the truffle at Cahors-Le Montat and in some truffle areas in France and abroad. For these trials, we study another factor which could be the impact of the domestic animals like sheep and horses. We had observations which involve these animals in exceptional truffle production fields. We can suggest an analogy with other mushrooms production: *Agaricus campestris* L. and *Pleurotus eryngii* (De Cand.:Fr.) Quélet need respectively in their grassland habitat cows and sheeps.

We humbly recognize some explanations are still lacking. Why in 2006 (a good climatic year), the Lot district did produce 3 tons of truffles whereas its production was more that 300 tons in 1906? This 100 factor seems to summarize this lost of "virulence" or "strength" of the black truffle. This decline is usually explained by the deterioration of the *T. melanosporum* ecosystem, consequences of changes in human activities, mainly agriculture, animal farming and forestry. With less traditional activity, oaks woodlands are more and more present with their fungi of forest ecology.

Why is the black truffle less dominant than in the past, why does it defend less against its potential aggressors? This is the double question asked in the introduction. Does sick black truffle like other mushrooms suffer pathogen attacks like virus? This hypothesis is supported by some scientists [16] to explain the difficulty of truffle production. In this article, we favour that, during the abundance period, there were practices of mixed farming with organic matter restitution, biodiversity management. The rural world was entirely exploited and cultivated. Population density in the country was higher than today. Woodlands were rare and firewood was precious and collected. In limestone areas, landscapes were very open for the pioneer fungi like *T. melanosporum*: its "strength" or "virulence" was at the optimum. Furthermore, the harvesting pressure was lesser than nowadays, leaving in the soil a lot of truffle spores useful as natural inoculum. There were no inoculated controlled mycorrhized plants to sell and buy; nevertheless, there was an abundance of truffles.

ACKNOWLEDGMENTS

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MYCELIAL SLURRIES AS SPAWN FOR CULTIVATION OF THE EDIBLE ECTOECTOMYCORRHIZAL MUSHROOM, *RHIZOPOGON ROSEOLUS* (=*R. RUBESCENS*)

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ABSTRACT

Rhizopogon roseolus (Corda) Th. M. Fr. (=*R. rubescens* Tul. & Tul.), known as "shoro" in Japanese, is a hypogeous basidiomycete that is an important ectomycorrhizal symbiont of the Pinaceae. In order to cultivate this edible ectomycorrhizal mushroom, several researchers have tried to promote ectomycorrhization of this mushroom on roots of its host, *Pinus thunbergii*: Pine seedlings were inoculated with mycelium in vitro, or with crushed fruiting bodies in nature. However, this mushroom has not been successfully cultivated. We have developed useful mycelial slurries that enable production of abundant ectomycorrhizas and promote fruiting body formation under greenhouse nursery. We selected a superior isolate that rapidly colonized and produced a lot of ectomycorrhizas in roots of *P. thunbergii*. The mycelial slurries were composed of saline solution and a homogenate of a submerged culture of the mycelium. Addition of surfactant to the mycelial slurries resulted in stimulation of ectomycorrhizal formation in host roots. When the mycelial slurries were injected into a mother plant system in which the colonized seedling had been planted in the nursery, stimulatory effects were observed not only on ectomycorrhization of the seedlings but also on fruiting body formation. Genotype analysis of the fruiting bodies produced in the nursery showed that they had been produced by the inoculated isolate. These results suggest that the mycelial slurries could serve as mycelial spawn for cultivation of shoro.

Keywords: Ectomycorrhizal mushroom; Fruiting body formation; Mycelial slurry; *Pinus thunbergii*; *Rhizopogon roseolus*; Spawn

INTRODUCTION

Rhizopogon roseolus (Corda) Th.M. Fr. (=*R. rubescens* Tul. & C. Tul.), known as "shoro" in Japanese, is a hypogeous basidiomycete that is an important ectomycorrhizal symbiont of Pinaceae [1]. The fruiting bodies of this fungus are found in the sandy soils of *Pinus thunbergii* Parl. forest in seashore habitats. *R. roseolus* is a popular edible fungus in Japan, but factors such as deforestation, poor forest management and indiscriminate harvesting have led to recent decreases in production [2]. To cultivate this fungus, pine seedlings can be aseptically inoculated with mycelium or spores, then these ectomycorrhizal seedlings can be planted into a natural environment, and the fungus can be encouraged to grow [3-5]. However, cultivation techniques

have not been fully refined, because of the low efficiency with which ectomycorrhizal trees can be obtained, and the low levels of fruiting body production.

To successfully cultivate the fungus, inoculation and propagation methods must be developed, and isolates with superior traits must be selected. Such traits as vigorous mycelial growth, a tendency to form ectomycorrhizas and high levels of fruiting body production are desirable. Recently, we have discovered the superior isolate that rapidly colonized and produced a lot of ectomycorrhizas in root of *P. thunbergii*. Furthermore, we have developed a new inoculation method by using the isolate. In this paper, we demonstrate that the inoculation method enables to produce not only abundant ectomycorrhizas of host root, but also rapid fruiting body formation of this edible mushroom.

MATERIALS AND METHODS

Fungal isolates. Nineteen wild dikaryotic isolates of *R. roseolus* were used in this study (Table 1). The isolates have been deposited in the culture collection of the Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University, Japan. To prepare inoculum, the isolate was inoculated on malt extract agar (20 g malt extract (Oriental Yeast Co., Ltd., Tokyo, Japan), 20 g agar, and 1 L tap water) plates and incubated for 3 wk at 25 °C in the dark. After incubation, 5 mm square mycelial agar plugs were removed by a surgical knife and used for inoculation.

Table 1: Fungal isolates of *Rhizopogon roseolus* used in this study

TUFC number	Collection	
	Location	Year
10003	Tottori	2005
10004	Tottori	2005
10006	Ishikawa	2005
10007	Miyagi	1997
10008	Niigata	2005
10009	Niigata	2005
10010	Niigata	2005
10016	Miyagi	2003
10018	Shimane	2000
10019	Kochi	1998
10026	Tottori	2005
10031	Iwata	2003
10032	Miyagi	2003
10033	Miyagi	1997
10036	Kagoshima	1999
10042	Niigata	2005
10086	Saga	2001
10096	Ibaraki	1998
32018	Tottori	2005

In vitro infection. Roots of *P. thunbergii* were infected in vitro with *R. roseolus* using the Petri dish technique described previously [6]. Sterile *P. thunbergii* seedlings were germinated from seeds collected from a *P. thunbergii* forest at Tottori University in Tottori, Japan. The seeds were rinsed in running water for 24 h and surface-sterilized in 30% H_2O_2 for 30 min. Following three rinses with sterile distilled water, the seeds were aseptically transferred to a water agar medium and germinated at 23 °C under diffused light. At 2 wk following germination, the seedlings were transferred to filter paper overlying modified Melin and Norkrans medium [7] without added glucose and malt extract in Petri dishes (90 × 20 mm). The Petri dishes were sealed with lanolin and Parafilm (American Can Co., Detroit, MI, USA) and incubated in controlled environmental chambers at a constant 23 °C with 85% relative humidity under a 16-h day at 60 μmol m^{-2} s^{-1}. After 4 wk of incubation, 5 mm square *R. roseolus* mycelial agar plugs were transferred to *P. thunbergii* lateral roots and the seedlings were incubated as above for an additional 8 wk.

Preparation of mycelial slurries. Agar plugs of a dikarytotic isolate, *R. roseolus* TUFC10010 were inoculated into 50 ml of malt extract liquid medium (20 g/L malt extract and 0.2% Tween 80) in an Erlenmeyer flask and incubated at 25 °C. After 4 wk, the mycelia were harvested by filtration on 1 mm mesh Nitex cloth followed by washing with tap water. The harvested mycelia were suspended at 10 g wet weight in 100 mL saline solution (30 mM NaCl, 7 mM KCl and 4 mM $MgCl_2 \cdot 6H_2O$, pH 7.0) containing 0.2% Tween 80. About 500 mL of suspension was blended with an AHG-160A homogenizer (AS ONE Corp., Osaka, Japan) at 14,000 rpm for 1 min. The resulting mycelial slurries were used as inocula without dilution.

Inoculation in mother plant system in greenhouse nursery. For container-grown seedlings, seeds of *P. thunbergii* were planted in a plastic container (50 cm × 35 cm × 15 cm) containing horticultural grade vermiculite and bark-compost in equal proportions by volume. After 8 wk planting, an ectomycorrhizal mother seedling that had been inoculated in vitro with *R. roseolus* TUFC10010 was transferred into the center of the container. At 12, 16 and 20 wk after planting, seedlings were inoculated two or three times by injecting 500 mL of mycelial slurries into the root zone, approximately 2 cm underground, using a pipette. Ectomycorrhizal formation was evaluated 32 wk after planting by counting the total number of ectomycorrhizas.

RESULTS AND DISCUSSION

Pinus thunbergii seedlings grew well in plastic Petri dishes and produced root systems with first-order laterals suitable for ectomycorrhizal synthesis over a 5- to 6-wk period after germination. The total number of ectomycorrhizas formed by each isolate at 2, 4, 8 and 12 wk from inoculation is shown in Fig. 1. From 2 wk following inoculation, initial ectomycorrhizal formation could be observed in 4 isolates (TUFC10004, 10008, 10010 and 10042). After 8 wk, 2 isolates (TUFC10004 and 10010) formed abundant ectomycorrhizas. Figure 2 shows typical ectomycorrhizas of the seedling inoculated with TUFC10004.

Intraspecific variability of fungal isolates has to be taken into account in the selection processes aimed at obtaining superior isolates for controlled inoculation [8, 9]. The candidate fungi should exhibit the physiological capacity to form abundant ectomycorrhizas on the desired hosts [10]. Molina & Trappe [1] pointed out differences in colonization ability among *Rhizopogon* spp. fungal isolates. Habitat differences might be reflected in the colonization patterns, indicating some degree of specialization or host preference among isolates. Recent studies [11, 12] showed priority effects in inoculation experiments with *Rhizopogon* species,

demonstrating that more rapid root tip colonization of one species resulted in almost complete exclusion of the slower species. Our results obtained with in vitro experiments also showed intraspecific variation in colonization speed and ability to produce ectomycorrhiza among *R. roseolus* isolates. Rapid colonization ability may be a competitive advantage under natural conditions. Therefore, the fungal isolate used in natural conditions should have an excellent capability for rapid colonization as well as formation of a great number of ectomycorrhizas.

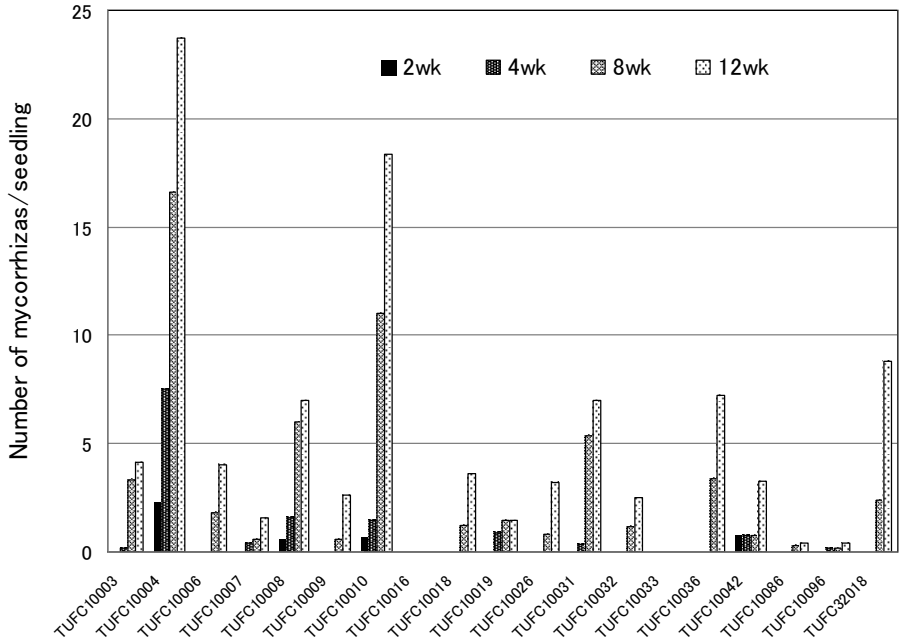

Figure 1: Variation in ectomycorrhizal forming ability among isolates of *Rhizopogon roseolus* on *Pinus thunbergii* seedlings using a Petri dish method.

Figure 2: Ectomycorrhizal root system of a *Pinus thunbergii* seedling inoculated with *Rhizopogon roseolus* TUFC10004.

Effects of injection of mycelial slurries on ectomycorrhizal formation in the mother plant system are shown in Fig. 3. Evaluation of ectomycorrhizal formation was carried out 32 wk after planting by counting the total number of ectomycorrhizas. A stimulatory effect of mycelial slurries on ectomycorrhizal formation by pine seedlings in the mother plant system was observed. At 32 wk after planting, 14 fruiting bodies were found in the nurseries: 1 fruiting body in the uninoculated treatment, 8 fruiting bodies in the treatment with two inoculations, and 5 fruiting bodies in the treatment with three inoculations (Table 2). Figure 4 shows the fruiting body produced in the nursery. Genotype analysis of the fruiting bodies produced in the nursery showed that they had been produced by the inoculated isolate.

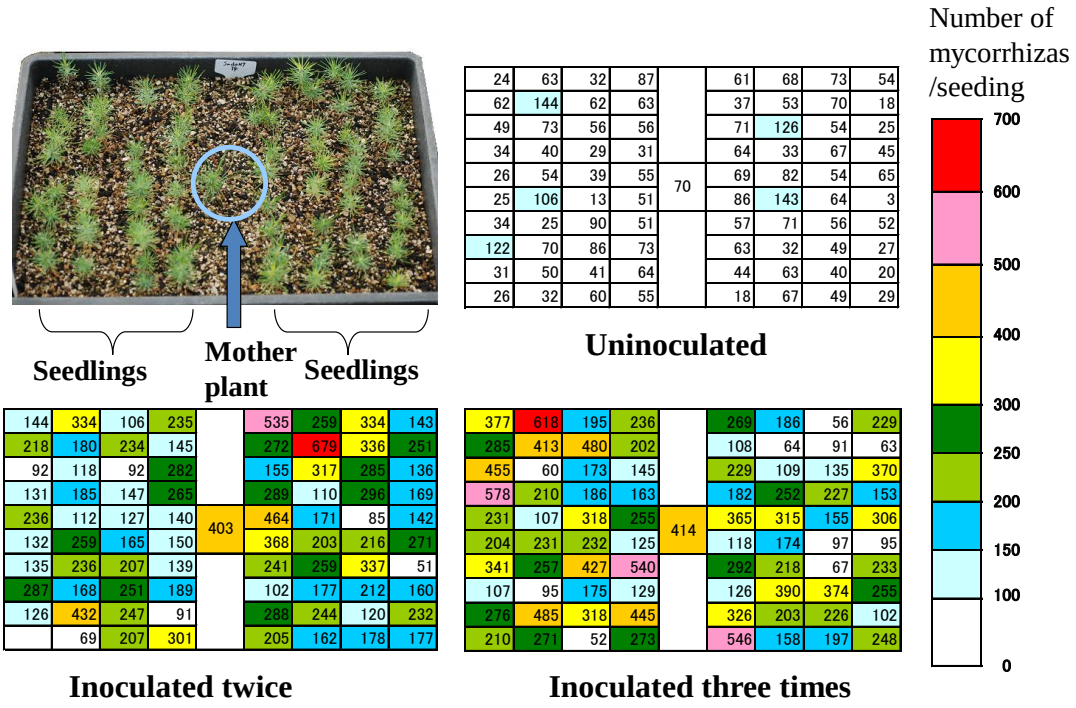

Figure 3: Effect of inoculation of mycelial slurries of *Rhizopogon roseolus* TUFC10010 on ectomycorrhizal formation on *Pinus thunbergii* seedlings in the mother plant system. Values in the figures indicate number of ectomycorrhizas/seedling.

Table 2: Effect of inoculation of mycelial slurries of *Rhizopogon roseolus* TUFC10010 on fruiting body formation in the mother plant system

Inoculation treatment	Number of fruiting bodies in four planters
Uninoculated	1
Inoculated twice	8
Inoculated three times	5

Figure 4: Fruiting body formation (arrow) of *Rhizopogon roseolus* in a *Pinus thunbergii* nursery.

In these experiments, we showed the usefulness of mycelial slurries as inocula. Although the use of solid carriers for mycelium is the standard method used to inoculate seedlings [13], it appears that mycelial slurries may have some advantages over solid for certain practical situations. Blended mycelial slurries of different ectomycorrhizal fungi were assessed for their suitability as inocula for the production of ectomycorrhizal trees, especially for plantations [14, 15]. On the other hand, difficulty obtaining ectomycorrhizal seedlings inoculated with mycelia of *Rhizopogon* spp. has been reported [1]. In the present experiments, we mixed a non-ionic surfactant with the inocula. Addition of surfactant promoted mycelial growth and ectomycorrhizal colonization (data not shown). Previous research [16, 17] showed that incorporation of Tween nonionic surfactants in soil-containing substrate strongly stimulated not only mycelial growth but also formation of shiro-like structure of the edible ectomycorrhizal mushroom *Tricholoma matsutake*. This stimulation is considered to be related to the higher hydrophilicity of treated hyphae, or to enhance lytic excretion and activity of hyphae [17]. Use of a surfactant might help overcome the difficulty in obtaining ectomycorrhizal seedlings.

The mother plant technique has been used extensively in the production of *Tuber magnatum* colonized plants, which are difficult to obtain using spore inoculation [18]. However, this method carries a high risk of spreading contaminating and possibly very competitive ectomycorrhizal fungi. Mycelial slurries developed in this study may reduce the risk of contamination.

CONCLUSIONS

In the present study, we showed that inoculation using mycelial slurries containing surfactant stimulates not only ectomycorrhizal colonization of host pine seedlings but also fruiting body formation, indicating that the slurries could be used as spawn for cultivation of the edible ectomycorrhizal mushroom, shoro.

ACKNOWLEDGEMENTS

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CULTIVATED EDIBLE SPECIALTY MUSHROOMS - SCOPE IN INDIA AND EU COUNTRIES

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ABSTRACT

In India and EU countries, the specialty edible mushrooms are not grown and available commonly. Any mushroom other than popularly grown white button mushroom *Agaricus bisporus* comes under the specialty category, and these mushrooms are generally not available in departmental stores/vegetable vendors. The mushrooms under this group are the different species of Oysters, Shiitake, Black Ear mushroom, Shimeji, Nameko, Enoki, Milky, Paddy Straw mushrooms and others. 11 varieties of specialty mushrooms were grown at the Centre on commercial scale for marketing in a niche market in India. This paper describes the cultivation technology of these mushrooms as grown at our Centre in controlled environment conditions. The mushrooms were grown on steam pasteurized/steam sterilized substrates without the use of chemicals/pesticides. The mushrooms were harvested fresh, chilled at 2-4ºC for 4-6 hrs before dispatch to the niche market. The mushrooms were sold at attractive prices as gourmet mushrooms. The raw materials used for cultivating these mushrooms were wheat straw, paddy straw, saw dust, wheat bran, rice bran and gypsum for pH adjustment. The environment was simulated in the cropping rooms with use of Air Handling Units with computer controls for temperature, RH and CO_2 concentration. One cropping cycle was completed under these conditions in 3-4 weeks, unless otherwise stated. Regular water sprayings were applied to keep the beds moist. Light was provided to the cropping beds wherever required.

Key words: *Pleurotus, Volvariella, Lentinula, Auricularia, Calocybe, Hypsizygus*, cultivation, controlled environment.

INTRODUCTION

The word specialty mushroom has different meaning for different countries. In India and EU countries, the specialty edible mushrooms are mushroom that are not grown commonly and available commonly in the market. Any mushroom other than popularly grown White Button mushroom *Agaricus bisporus* falls under the specialty category, and these mushrooms are generally not available in departmental stores/with vegetable vendors in India. In India and EU countries White Button mushroom *Agaricus bisporous* is cultivated commercially for local consumption and export to other countries. The specialty mushrooms for India and the EU countries are the different *Pleurotus* spp, *Lentinula edodes*, *Auricularia* spp, *Hypsizygus tessulatus*, *Pholiota nameko*, *Flammulina velutipes*, King Oyster mushroom *P.eryngii* and others. Similarly, specialty mushrooms in EU countries/USA and other developed countries in Europe/America are the mushrooms grown other than the White Button mushroom *A.bisporous.* But in China and many Far Eastern countries in Asia, most of our specialty mushrooms are commonly grown there as a home grown crop, and are easily available everywhere in these

countries over the counter, both in fresh and dehydrated form. In China, mushroom is the 6th important crop in the country as far as revenue generation for the nation is concerned. Button mushroom is exotic mushroom/specialty mushroom for these countries, and is chiefly grown for export/distant markets.

The Chinese mushroom growers cultivate these specialty mushrooms on a commercial scale, with substrate preparation/bottling/spawning done mechanically, semi-mechanically on a gigantic scale and mushrooms harvested are exported as such fresh or after dehydration. Today China is the largest producer of mushrooms in the world with 70% of world production happening in China. Chinese grow White Button mushroom for export, mostly seasonally. In China, mushroom is the base for most of the medicines available there for cure of wide spectrum of diseases. These mushrooms are listed under a separate group of mushrooms called medicinal mushrooms. Today China is trading medicinal mushrooms worth billions of dollars worldwide with increasing demand. Notable medicinal mushrooms/mushroom products are Reishi mushroom *Ganoderma lucidium* (immune system enhancer), Chinese caterpillar mushroom *Cordyceps sinensis* (hormone stimulator) and many others. These medicinal mushrooms together with nutritionally rich Shiitake mushroom are in great demand in USA, EU countries, India, Australia and other countries. While mushroom is the health food, medicinal mushrooms offer a big hope for cure of those human diseases where there is no known cure available in other systems of medicine in the world. Mushrooms are the health food and recommended by FAO-UN as a potent protein source along with soybean for all, especially in developing countries´

CULTIVATION OF SPECIALITY MUSHROOM

The commonly grown specialty mushrooms in India are listed in Table 1 and Cultivation technology as standardized suiting local conditions at Mushroom Research Development and Training Centre (MRDTC), Delhi under controlled environment is described below.

Table 1: Names of specialty mushrooms grown and their temperature range

Common name	Scientific name	Optimum Temperature Range
Grey Oyster	*Pleurotus sajor-caju*	20-28ºC
Black Oyster	*Pleurotus ostreatus*	18-22ºC
White Oyster	*Pleurotus florida*	20-28ºC
Pink Oyster	*Pleurotus djamor*	20-26ºC
King Oyster	*Pleurotus eryngii*	18-22ºC
Black Ear Mushrooms	*Auricularia polytricha*	22-26ºC
Shimeji Mushroom	*Hypsizygus tessulatus*	18-22ºC
Shiitake Mushroom	*Lentinula edodes*	18-22ºC
Milky Mushroom	*Calocybe indica*	28-32ºC
Paddy Straw Mushroom	*Volvariella volvacea*	30-35ºC

GREY OYSTER MUSHROOM

Pleurotus sajor-caju [1-5] the Grey Oyster mushroom, has its pileus color at pin head formation grey to dark grey in color. The color of the pileus changes to light grey on maturity, with fan shaped fruit body and thick texture. The fruit bodies are weighty when fully grown, and the pileus diameter may extend up to 4 inches. This Oyster mushroom is very commonly grown in India under seasonal growing conditions at temperature ranging between 20-28ºC, but growth stops at air temperature above 28ºC. This mushroom performs excellently when grown under controlled environment conditions.

Substrate Materials: *P. sajor-caju* grows best on wheat straw substrate which is easily available in India at affordable prices. Other alternative materials that are used for substrate preparation are maize stems, maize cob shells, pseudo banana stems and other agricultural recyclable wastes. These base materials are supplemented with N-rich supplements like wheat/rice bran at 10% dry weight of base material. These materials should be fresh and free from moulds and with maximum of 10-15% moisture content. The water used for wetting and watering should be at a pH of 7.

Substrate Preparation: The base material is wetted on composting yard with a water hose and left overnight for water absorption by straw cells. The heap is made rectangular shaped, about 2.5 to 3 feet in height and turned/mixed the next day after 24 hours before filling into the pasteurization chamber. The base material is alternatively soaked in a water tank in gunny bags overnight. The material is brought out of the tank and heaped on the floor of the composting yard to drain off excess water. The material after wetting is filled into the pasteurization chamber for steam pasteurization at 65ºC for 8 hours. The material is loaded directly onto the grated floor up to a height of 6 feet, blower fan switched on and steam injected to raise the temperature of the material to 65º**C**. The material is held at this temperature for 8 hours. Alternatively, the material is filled into the perforated plastic crates and loaded into the chamber for pasteurization of the material. The material after pasteurization is cooled to 24-25ºC overnight, and then spawned in aseptic spawning area located on the other side of bulk chamber. Rice bran/Wheat bran is filled into the perforated crates and pasteurized along with the substrate at same temperature, which is mixed with the substrate at the time of spawning.

Spawning and Spawn run: The spawning is done inside the glazed spawning area located on the cleaner end of the bulk chamber. The spawning is done at 2% spawn rate (2 kg per 100 kg wet substrate). The pasteurize rice/wheat bran is also mixed during spawning operation with the base material. The spawning is done by 'through spawning method', and seeded substrate filled into the perforated polythene bags, pressed hard to remove air. The bags are tied on the top and shifted to the spawn incubation room maintained at 24-25ºC and 90% RH. The spawn run takes about 12-14 days at 24 ±1ºC. The bags are allowed to become white on outside, and that is the stage the bags are shifted to cropping room.

Opening of the bags for Cropping: The polythene bags is cut and discarded, and spawn run substrate bundles are placed on the racks in the cropping room. The cropping room is maintained at a temperature of 17-19ºC, RH of 85-90% and CO_2 content of 800 ppm. The climate is automatically simulated to above mentioned levels, and the pin head/primordial formation takes place in another 4-5 days. The primordia/pin heads grow into harvestable mushrooms in another 3-5 days (Fig.1).

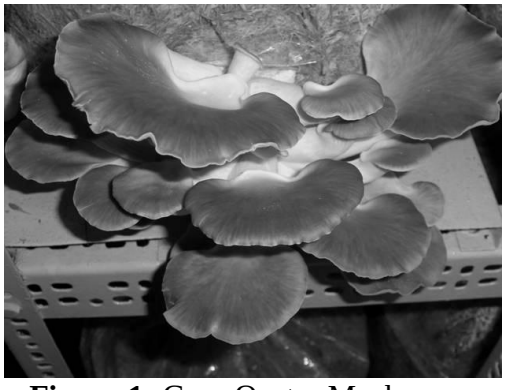

Figure 1: Grey Oyster Mushroom.

Cropping and crop Management: The color of the pileus is dark-grey to grey in color, which turns lighter on maturity. During the cropping period from pin head to maturation, light source of about 400 lux is made available to the developing mushrooms as per requirement of the mushroom. The mushrooms are allowed to grow to full biological maturity, say 3-4 inches of pileus diameter. The cropping room is maintained at above mentioned growing parameters throughout cropping phase. The filters installed on the Air Handling Unit required periodic weekly washing to remove the film of *Pleurotus* spores on the filters, which would hamper the air flow speed inside the cropping room. The cropping is done for maximum of 2-3weeks, and the spent substrate discarded at the end of cropping. On an average of 20-22 kg of excellent quality fresh mushrooms were harvested from 100 kg wet substrate. Grey Oyster mushroom, *P.sajor-caju* is large in size at harvesting time with hard/thick pileus, spreading out like a fan. Pileus diameter of 3-4 inches is considered right for harvesting, and is accepted by the consumer with a smile. During cropping regular water sprayings were given to the crop beds. No chemicals/pesticides were used during the entire process of cropping.

Post Harvest Handling and Marketing in India: The mushrooms are harvested at the right biological stage of the fruit body; that is when it is fully grown with pileus tight and curved outwards on the edge. The gills are decurrent and stipe is thin and short. The bulk of the fruit body is constituted by the pileus itself. This mushroom is very attractive to look at and fleshy in appearance. The mushrooms are plucked singly or in bunch from the substrate, collected in a basket, graded and packed in polythene bags/paper bags for the market. The polythene bags are provided with a small hole for air exchange to prevent the development of aflatoxins on the fruit body. The mushrooms are chilled inside a cold storage at 2-4ºC for 4-6 hours before dispatch to the market for enhancement of shelf life. These mushrooms are sold in niche market in big cosmopolitan cities in India.

BLACK OYSTER MUSHROOM

Pleurotus ostreatus, [2-3, 5-6] is the Black Oyster mushroom, as the pileus is black in color at the time of primordia/pin head formation. The entire process of its cultivation is similar to *P.sajor-caju,* except that this mushroom grows in vertically long bunches, with bunch growing in acropetal order (lower mushrooms younger/smaller, upper mushrooms larger). This mushroom is also called Hiratake mushroom in Japan, and this mushroom loves the lower temperature range of 18-22^{O}C. This mushroom is a prolific yielder, and yields 20-25% mushrooms of wet weight of substrate over a cropping period of 3-4 weeks. The mushroom shows distinct black color when young, which turns lighter on maturity. This mushroom is harvested in bunches, and packed/marketed like *P.sajor-caju.* This mushroom is in greater demand because of its velvety look/texture and excellent aroma. The diameter of the pileus of this mushroom is 2-3 inches, but mushroom length comprising of pileus and stipe is about 3-4 inches. The entire length of this mushroom is fleshy in texture, with decurrent gills on lower side. The fruit body looks like a horse shoe. This mushroom has preferred marketability value, and is readily accepted in the market by the consumers and the executive chefs of star hotels for its excellent taste (Fig.2).

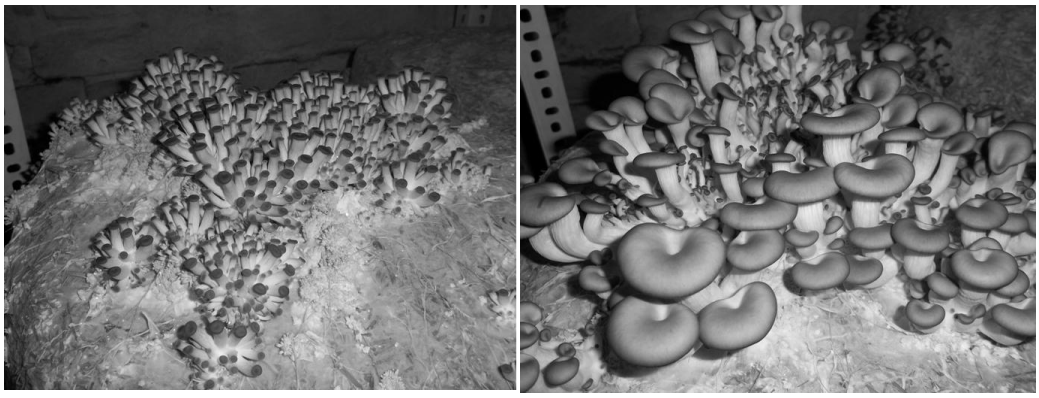

Figure 2: Black Oyster Mushroom.

WHITE OYSTER MUSHROOM

Pleurotus florida [1, 5, 7] the White Oyster mushroom, is white in color from primordia/pin head formation to maturity, and this mushroom also grows in bunches. The pileus of this mushroom is with thin margins, smooth and pileus thickness is lesser as compared to *P.ostreatus* and *P.sajor-caju.* The mushroom looks like a white disc, growing on a thick stipe with decurrent gills extending to the base of the stipe, unlike *P.ostreatus*/*P.sajor-caju*. This mushroom grew excellently at 18-22ºC temperature range but can grow up to 28ºC. White oyster looks graciously white, with delicate flesh which is turgid in texture. The marketability of this mushroom is quite high with increased demand in the niche market in India. These mushrooms are real gourmet mushroom, with high culinary value, and with intense mushroom aroma when used fresh (Fig.3).

Figure 3: White Oyster Mushroom.

PINK OYSTER MUSHROOM

Pleurotus djamor [7] the Pink oyster mushroom looks gracious on the bed and yields profusely. The cultivation process for this mushroom is similar as described for *P.sajor-caju*, excepting that this mushroom requires limited water spraying during its cropping. The mushroom pileus is thinner as compared to above species, leathery in texture and looks like a pink queen on the beds. The pileus is up to 3-4 inches in diameter, with little or no stipe and pileus thickness is 3-4 mm at the outer edges. Outer boarder on pileus top is pink, gills on the lower side are pinkish too. This mushroom is not fleshy as compared to above described 3 species. The marketability of this mushroom is excellent and sells at attractive prices in the niche market in India. It fruits profusely at lower air temperature of 18-20ºC in the cropping room (Fig.4).

Figure 4: Pink Oyster Mushroom.

KING OYSTER MUSHROOM

Pleurotus eryngii [8] is really the king amongst oyster mushrooms, not from size point of view alone but for its elegance in taste and aroma. This mushroom is also known by the name of Kabul Dhingri in India, and earlier it was harvested from natural habitats in Afghanistan and sold in India after dehydration at very attractive prices. This mushroom is now cultivated in cropping rooms all over the world, both under seasonal growing conditions and controlled environment. The cultivation technology is described in details as under.

Substrate materials. The commonly used substrate material for cultivating this mushroom is sawdust, supplemented with 10-30% wheat/rice bran. Wheat straw substrate supplemented with rice/wheat bran also supported the growth of this mushroom, but best quality mushrooms grew on sawdust substrate supplemented with rice/wheat bran.

Substrate preparation. Substrate sawdust is supplemented with 30% rice bran (w/w dry) and wetted on the composting platform, using water with pH 7. Gypsum/calcium carbonate is mixed with the substrate for pH adjustment only. The substrate materials after wetting were filled into the polypropylene bags of 1 kg capacity, material pressed hard into the bag with hands and a hole made with a wooden stick in the centre for inoculums. Plastic neck is fixed on the open end of the polypropylene bag and the bags are then cotton plugged and sterilized at 15 psi for 90 minutes. Wheat straw substrate was pasteurized at 65ºC with steam, but it did not support the spawn run. When wheat straw substrate was sterilized at 15 psi for 90 minutes as for sawdust, it supported good spawn run and fruited well, but yields were lower.

Spawning and spawn run: The bags were allowed to cool inside the autoclave and inoculated the next day on laminar flow deck using freshly prepared grain spawn of *P.eryngii*. About 40-50g of spawn was poured into the sterilized bag through the neck, and grain spawn pushed into the hole with a sterile glass rod, and cotton plug restored in the neck. The bags were then shifted to the incubation room maintained at a temperature of 22-24ºC. The spawn run takes 12-15 days for complete spawn impregnation of the substrate.

Figure 5: King Oyster Mushroom.

Opening of bags for fruiting. The bags were opened after complete spawn run, by cutting off the extra polypropylene near the upper edge of the substrate. The old spawn was scrapped off and discarded, and water sprinkled over the substrate to keep it moist. The bags were then shifted to the cropping room maintained at 17-19ºC temperature, 85-90% RH and 800 ppm CO_2 concentration. The pin heads started appearing on the top surface in 9-10 days, which grew into harvestable mushrooms in another 3-4 days. King Oyster loves cold temperature, and the production stops at temperature beyond 24ºC (Fig.5).

Cropping and crop management. The bags were given water spraying daily to maintain the substrate in moist condition. The mushrooms grow into large fruit bodies with brownish streaks on the pileus giving it an attractive look. The pileus stays curved inwards on the outer edge, the texture is tough and the fruit body is quite fleshy, with decurrent gills extended over the stipe. The stipe is quite thick and forms the bulk of the fruit body. The bags are maintained in the cropping room at 17-19ºC constantly with 85% RH and 800 ppm CO_2 concentration.

Post harvest handling. The fruit bodies are harvested when these are 5-6 inches tall, with average weight of about 100-250 g. These mushrooms grow large in size and require to be placed in larger containers/bags for marketing. King oysters have a superior shelf life and can withstand small distance transportation. These mushrooms are marketed in India to a niche market as a specialty mushroom of high value. The common consumer of mushrooms in India does not know much about this mushroom, and it does not sell fresh over the counter. This mushroom is grown in China/Korea/Japan and other Far-Eastern countries for export, and large quantities of King Oyster mushrooms are marketed fresh/in dried form in India, Europe, USA, Canada and other countries. In China, this mushroom is like a home grown crop and large numbers of mushroom growers are cultivating this mushroom on commercial scale for local consumption and export to neighboring countries.

BLACK EAR MUSHROOM

Auricularia polytricha [9], commonly known as Black Ear/Wood Ear mushroom is another specialty mushroom popularly used in Chinese food dishes all over the world. Its substrate preparation technology is similar as discussed for King Oyster mushroom. It grows on both wheat straw/sawdust substrates, but saw dust substrate showed superior results. The synthetic logs were prepared from saw dust supplemented with 10% wheat/rice bran, wetted on the composting floor and filled into polypropylene bags, cotton plugged and sterilized in steam autoclave at 15 psi for 90 minutes. The bags were spawned on cooling on laminar flow with grain spawn at 3% spawn rate. The spawn run was done at 23-25ºC, which was accomplished in

two weeks (Fig.6). The substrate bags were opened after complete spawn run and are being maintained for fruiting at two different temperatures in the cropping rooms at 20^{O} and 24^{O}C separately.

Figure 6: Spawn running bags of Black Ear Mushroom.

SHIMEJI MUSHROOM

Hypsizygus tesselatus, the Buna Shimeji mushroom was grown on sawdust substrate. Saw dust substrate was supplemented with 5% wheat/rice bran and wetted and filled into polypropylene bags and cotton plugged. The substrate was sterilized at 15psi for 90 minutes, and spawned on cooling on laminar flow with grain spawn at 3% spawn rate. Spawn run was done at 24-25C which took one month. The bags were opened after complete spawn run and maintained in the cropping room at 18C. The fruit bodies appeared 2-3 weeks after opening. This mushroom is slow grower and likes cooler temperatures. The fruit body is smaller in size, with light wood color on the pileus (Fig.7). The stipe is long and pileus about 1-1.5 inches in diameter, with pileus thickness of 2-3 mm. The large scale cultivation of this mushroom is in progress.

Figure 7: Buna Shimeji Mushroom.

SHIITAKE MUSHROOM

Lentinula edodes, [10] the prized Shiitake mushroom grows on wheat straw as well as on sawdust substrates. But the fruiting is superior on sawdust substrate. Sawdust supplemented with 6% rice/wheat bran and small quantity of gypsum (about 1% dry w/w) was mixed and wetted on the composting platform and filled into polypropylene bags, pressed hard to drive out air and a hole made with the wooden stick in the center. The substrate bags were cotton plugged (cotton plug fitting into the plastic neck on the open side) and the substrate bags were sterilized at 15 psi for 90 minutes. The moisture content of the substrate was maintained at 50-55%. The substrate

was spawned on cooling on laminar flow at 3% spawning rate, using grain spawn. This mushroom mycelium grows in its vegetative state in two phases, one is spawn run and the second is browning process. The vegetative growth/spawn run was done at 24±1ºC, which took 30-35 days. The bags after spawn run were opened and are being maintained in cropping room at 17-19ºC, 90% RH and 1000ppm CO_2 concentration for browning process (Fig.8). The bags after browning process for about 30 days will be shifted to the cropping room maintained at 22±1ºC. The complete cycle takes about four months on synthetic logs. The yield potential on synthetic logs is about 80-100% fresh mushrooms of the dry weight of the substrate.

Figure 8: Spawn run bags of Shiitake Mushroom.

MILKY MUSHROOM

Calocybe indica, [11] the Milky mushroom is snow white in color with a long stipe and a small pileus. The stipe is fleshy and constitutes the bulk of the mushroom. This mushroom is grown on wheat straw after steam pasteurization at 65ºC for 8 hours, in polythene bags. The spawning rate used was 5% of the wet weight of substrate. The bags after spawning were maintained in a cropping room at 28ºC. The spawn run takes about 12-14 days. The bags were opened after complete spawn run and beds cased with steam pasteurized casing material consisting of mixture of coirpith +FYM (Farm Yard Manure)+ ordinary soil (equal parts w/w). The casing thickness used was 2inches and after casing application water spraying was given regularly to maintain casing in wet state. Case run took 12-14days at 28ºC. After case run, the room temperature was lowered to 25-26ºC and the crop appeared in another 7-10 days after complete case run. The primordia developed into fully grown harvestable mushrooms in another 3-4 days (Fig.9). The mushrooms attained the height of 4-6 inches at harvest time. One kg of mushroom will have 10-12 fruiting bodies. This mushroom has a superior shelf life, and can be transported to short distances for marketing without damage to its quality.

Figure 9: Milky Mushroom

PADDY STRAW MUSHROOM

Volvariella volvacea, [12] the Chinese mushroom/the Tropical Paddy Straw mushroom is the tastiest mushroom amongst the edible group, with added aroma. But its shortcoming is its poor shelf life. This mushroom has to be consumed within hours of its harvest, or dehydrated.

Figure 10: Paddy Straw Mushroom.

This mushroom was grown on paddy straw bundles. Each bundle weighed about 500g (dry weight) with 2 feet length. The paddy straw bundles were wetted overnight in a water tank. The bundles were removed from the tank, left for a few hours at the composting platform to drain off extra water. The bundles were then steam pasteurized at 65ºC for 6 hours. On cooling the bundles were arranged into a bed over perforated shelf inside a cropping room. Each bed was made about 2 feet long and 2 feet wide, consisting of 5-6 layers. The bed was spawned in each layer on the periphery, sprinkling some gram powder - 200g per bed to provide nutrition. Spawning was done at the rate of 3% wet weight of substrate, using grain spawn. The bed was completely covered with a polythene sheet and maintained at a temperature of 30-35ºC with 100% RH. The spawn run was complete in 4-5 days, and on complete spawn run, the polythene cover was removed to allow entry of fresh air for primordial/pin head development. The pin heads developed in another 3-4 days, which grew into harvestable fruit bodies in another 3-4 days (Fig.10). The mushroom is harvested in egg stage, not allowing it to open like an umbrella. The mushrooms are harvested in egg stage when these are up to 2 inches in diameter. The mushrooms are packed in a polythene bag for the market. This mushroom is not recommended to be refrigerated, but can be stored at 15ºC for a few hours.

CONCLUSION

Cultivated specialty edible mushrooms like commonly grown Grey Oyster mushroom *P. sajor-caju* (20º-28ºC), Black Oyster mushroom *P. ostreatus* (18-22ºC), White Oyster mushroom *P. florida* (20-28ºC), Pink Oyster mushroom *P. djamor* (20-26 C), King Oyster mushroom *P. eryngii* (18-22ºC), Black Ear mushroom *A. polytricha* (22-26ºC), Shiitake mushroom *L. edodes* (18-22ºC), tropical Paddy Straw mushroom *V. volvaceae* (30-35ºc), Milky mushroom *C. indica* (28-32ºc) and Buna Shimeji mushroom *H. tessulatus* (18-22ºC) were grown under controlled environment conditions at MRDTC, Delhi, India on commercial scale. The substrates for cultivation of these mushrooms were steam pasteurized/sterilized, and no chemicals/pesticides were used during the cultivation of these mushrooms. Almost all the specialty mushrooms are lignicolous mushrooms, meaning lignin loving. These mushrooms grow on sawdust, wood, cereal straws as against White Button mushroom which grows on humus or fermented organic matter/compost.

The procedure for cultivation of White Button mushroom is lengthy, cost intensive and requires more space for building of infrastructure with additional costs on cooling of the

environment, as commonly grown White Button mushroom requires growing temperature of 15-17ºC. In comparison, most of our specialty mushrooms (for India/EU countries) grow directly on recyclable agro byproducts like cereal straw and plant residues/wastes after initial wetting and steam pasteurization. For button mushroom cultivation, environment controlled cropping rooms will be required for growing with special infrastructures for compost preparation. No such infrastructure is required for growing specialty mushrooms. These specialty mushrooms can be grown seasonally in your backyard in ordinary rooms, with no requirement of composting as these grow directly on unfermented cellulosic materials/sawdust.

For marketing White Button mushroom, we will require cool chain transport for delivery to the market place as button mushroom browns quickly and has short shelf life, but specialty mushrooms can be transported in ambient conditions with modified packaging, without any damage to the quality of the mushrooms.

Specialty mushrooms are rich in all the dietary components of 'mushroom fruit body', especially proteins, vitamins, minerals like potassium and high fiber content with no fats/sodium. It is the richest source of protein amongst the vegetable/fungal proteins and rich source of folic acid required for blood formation in anaemic patients. Specialty mushrooms like Shiitake and Oyster mushrooms are rich in mushroom aroma and considered delicacy in food world ever.

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EDIBLE MUSHROOMS: AN ALTERNATIVE FOOD ITEM

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ABSTRACT

Nutrition is the main living reason for all of the societies of the world. Food supply should be both an economic and ecological subject. Health and nutrition involve balanced and sufficient functional food components. Sufficient daily calorie intake is the main problem of developing countries. The people cannot supply an adequate intake of essential food compounds such as proteins containing essential amino acids, vitamins, minerals and essential fatty acids. The developing countries need to provide essential food components for nutrition. Edible mushrooms have these essential compounds and functional substances for human health. Mushrooms also contain bioactive components including β-glucans and chitin. The amount of edible mushrooms produced in modern plants for public nutrition that need balanced foods has increased. The production plant can bring economical benefits to unemployed people in these countries. The nutritional value of mushrooms is reviewed together with biochemical aspects, mushroom production and economical aspects.

Key words: Nutrition; Health; People; Economy; Nutrients and functional compounds

INTRODUCTION

Nutrition is the most important subject for humankind. A balanced nutrition is particularly important from the point of taking in essential elements such as minerals, vitamins and high quality proteins. Nutritional levels in societies depend on various factors such as economic conditions, ecology, nutritional habits, traditions and education. Successful development of countries is achieved by advancing economic subjects including levels of agriculture, industry and education.

Nutritional values of foods play an important role in human health. The people have to provide a balance diet containing essential food compounds; amino acids, fatty acids, minerals and vitamins. A sufficient and balanced diet should also include taking in enough carbohydrate and energy supplies. Mushrooms can provide balancing diet compounds in sufficient quantities for human nutrition, and contain medicinal compounds. They are in rich in crude fiber and protein. In fact, mushrooms also contain low fat, low calories and good vitamins and many mushrooms possess multi-functional medicinal properties [1].

The total number of edible and medicinal fungi is over 2300 species [2]. Cultivated mushrooms have become popular, and over 200 genera of macrofungi are useful for the people in the world. Most of them are cultivated on lignocelluloses waste materials and contribute to their re-cycling. The common mushroom species produced in suitable ecological conditions are: *Agaricus* spp., *Lentinula edodes* (shiitake), *Pleurotus* spp. (oyster), *Volvariella volvacea* (straw), Lion's head or pom pom (*Hericium*), ear (*Auricularia*), *Ganoderma* (Reishi), *Grifola frondosa* (maitake), Winter (*Flammulina*), white jelly (*Tremella*), *Pholiota* (nameko), and shaggy mane (*Coprinus*). The most common ones produced are *Agaricus bisporus* (button) mushroom, *Lentinula edodes*, and *Pleurotus* species [2]. The nutritional and chemical composition, and physical properties of edible mushrooms have been studied by different authors [3-7]. It is well know that mushrooms have a rich chemical composition and functional properties for health.

The aim of this article is to evaluate the importance of the economical contribution of cultivated and wild edible mushrooms, as well as their nutritional importance as an alternative food item.

RESULTS AND DISCUSSION

Evaluation of the economic aspects of agricultural production and collecting of edible mushrooms. Agriculture plays a vital role, especially in developing and underdeveloped countries all over the world. The development of societies implies similar or the same recipes that include fostering pro-poor economical growth and favoring poor people in access to all the services and other factors that support poverty eradication and define an acceptable standard of living [8]. Income growth of societies is essential in order to reduce undernourishment due to some factors such as better public services and sanitation that also are crucial. Growth and modern agricultural practices must become important and essential especially in developing countries like Turkey.

Cultivated mushroom production can apply in closed areas without depending on climatic conditions. Projection studies have shown that demand for agricultural products will continue to grow more slowly. Several factors influence this situation. World population will grow at an average of 1.1 percent a year up to 2030, compared with 1.7 percent a year over the past years [1]. Soylu et al. [9] studied the economic aspects of edible mushrooms grown in Turkey. There are many edible wild mushroom species growing in various ecological conditions such as the Black Sea, Marmara and Eagan Sea regions. Cultivated edible mushrooms can also grow in similar regions of Turkey including west of the Mediterranean Sea.

Commercial mushroom production began at the end of the 1970's. In 1973, 1983, 2008 and 2010, the quantity of cultivated mushroom produced in Turkey was 80 tonnes, 1,400 tonnes, 26,526 tonnes, and 45,000 tonnes, respectively [5, 9, 10]. Cultivated mushroom species have been produced for 34 years since 1970 [2, 11, 13] when mushroom production has contributed to the Turkish economy. The Mushroom Research Department investigates mushroom biology and science, and agriculture in the "Yalova Atatürk Horticulture Research Institute", which works by depending on the Agricultural Ministry in Turkey and reports on the amount of mushroom production, the export and import of mushrooms, and the marketing of mushroom [13].

As seen from Table 1, mushroom imports to Turkey fell during 1996 to 2001. In 1999, Turkey exported 17,920 tonnes of mushroom but this figure fell to 3,370 tonnes in 2001 [13]. Soylu et al. [9] reported mushrooms are wild edible mushrooms while 10% of them are *Morchella* sp. Turkey has at least 2,388 wild mushroom species [14]. The other mushroom species exported from Turkey are: *Terfezia bouderi*, (Chatin) & Tone, *Boletus edulis* and other *Boletus* species, *Leccinium scabrum*, *Lactarius* spp., *Tricholama calignotum*, *T. analtolicum*, *T. matsutake*, *Cantharellus* sp., *Craterellus cornocopioides* and *Amanita caesarea.* The total value of exported mushroom is US$28.5 million, while the total value of imported mushrooms is US$1.6 million [9].

Agriculture is the key factor for the development of the countries. Mushroom production is economically important since data have shown that mushrooms can contribute to the economy in terms of nutritional value.

Table 1: Mushroom import and export figures for Turkey (tonnes)

Year	1996	1997	1998	1999	2000	2001
Imports	350	1,020	10	10	0	60
Exports	5,450	6,290	5,580	17,920	3,370	3,730

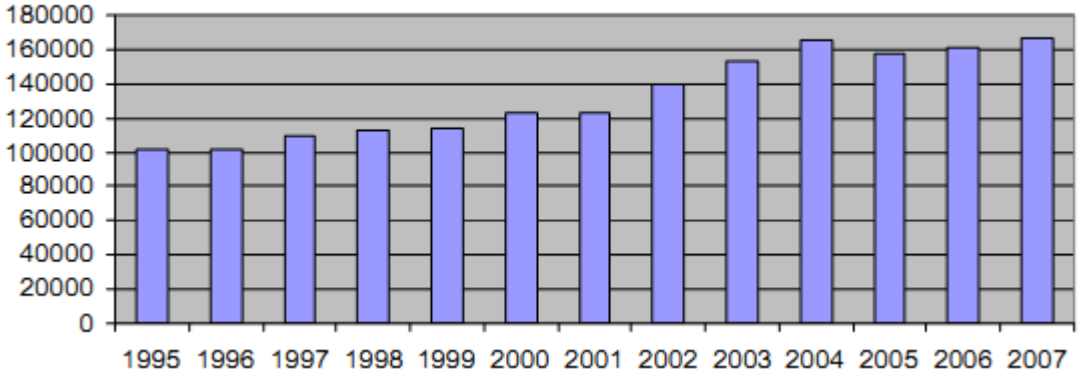

Figure 1: Total production of cultivated edible mushrooms (tons).
Data from Reference [15].

As seen from Figure 1, highest edible mushroom production occurred in 2004 and 2007 (over 160 000 tonnes) and similar amounts were produced between 2003 and 2007.

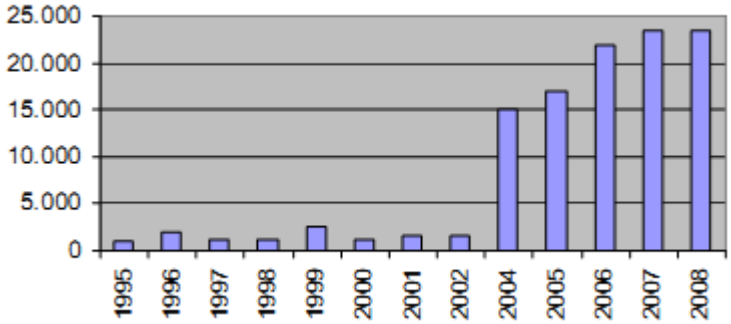

Figure 2: Production of cultivated edible mushroom in Turkey (tons)
Data from Reference [15].

Edible mushroom production in Turkey increased after 2002 and the increasing trend was strong between the 2004 and 2008 years (Figure 2).

The significance of mushroom nutrients for a balanced diet and health. Economic levels of societies affect their nutritional levels. The daily protein consumption for individuals is approximately 83 g per person and provided from sources of plant origin in Turkey. In the developed countries, 100 g protein intake per day is provided from animal sources. Nutrition develops parallel with public economical conditions [16]. Mushrooms should be a good alternative foodstuff with an especially balanced healthy nutrition for the person who has a low income.

The FAO reports mentioned that undernourishment is a characteristic feature of poverty and a direct violation of a universally recognized human right. Plenty of negative effects on human beings are seen in cases of undernourishment. This situation causes illness in people who are susceptible. Undernourished pregnant and nursing babies are born underweight, and so meet life with a nutritional handicap that may affect their health structure throughout their lives. Besides, undernourishment could also affect brain development and human productivity, which may be an obstacle to the learning performance. If energy and protein intakes are inadequate for the work requirements, muscle mass and labor productivity decline Deficiency of essential micronutrients cause some specific illnesses and reduce working capacity [17].

The main essential substances are shown in Table 2. Vitamins are considered to be micronutrients, as the human body requires only small amounts at any given time. The class of compounds called vitamins is a group of organic compounds. A good nutritious and well-balanced diet cannot replace by mere vitamin supplements (Table 2). While essential to life, the vitamins themselves do not provide any energy to the body. Vitamins are just one essential component of the human diet and the body requires many other substances besides vitamins for a full and adequate nutritional status. These include carbon-based compounds like carbohydrates, lipids, and essential fatty acids such as linoleic acid. The generally recommended daily protein intake for an average adult based on body size is 0.8 g per kilogram of body weight. High quality protein sources must contain essential amino acids (Table 2) and all essential trace minerals are necessary for a healthy diet. The health of the human body is not sustainable by vitamins alone and all other vital nutrients, such as minerals: Ca, P, Na, K, Cl, Mg, Mn that are produced N, S, Fe, Cu, I, Zn, F, Cr, Se, Mo, Si are acquired primarily from the food consumed by a person [17].

Mushrooms contain most of the essential elements (Table 2). Mushrooms can provide the balanced nutrition that comprises essential nutrients. There are data about nutritive values for both edible wild and cultivated mushrooms [18]. The data explained that studies dealing with the data related to the amount of vitamin C (L-ascorbic acid), B1 (thiamine), B2 (riboflavin), folic acid, B6 (pyridoxine), niacin, like *Cantharellus subalbidus*, (White chanterelle), *C. cibaruis*, *Craterellus cornucopiodes* (Black chanterelle), grown in Turkey (Table 3.). In this research, the species were fulfilling the required standards of recommended daily intake of folic acid, pyridoxine, and niacin. On the other hand, they fall short of the required amount of daily intake of protein and vitamin C. The folic acid, pyridoxine, and niacin contents of white, black and yellow chanterelle (mg/100g, wet basis), are as follows: (82.83; 1.83 and 96.3), (0.62, 0.86 and 0.91), and (3.75, 3.34 and 6.42), respectively. The Ca, Na, K, P, Mg and Zn contents of these species have been studied. The important positive cations are Na, K, Ca and Mg in the body. Na plays a significant role in providing intracellular balance of blood. Black chanterelles exhibited the highest quantity of Na, 461.53 (mg/kg) wet basis (wb). White and yellow chanterelle contained 19.50 and 26.89 mg/100g. wb respectively (Table 3) [17].

Table 2: Main essential nutrients of mushrooms [17]

Essential amino acids	Leucine, lysine, isoleucine, valine, methionine, phenylalanine, threonine, tryptophan
Essential fatty acids	Polyunsaturated fatty acids, fatty acids (PUFA), linoleic acid, α-linoleic acid, omega 3 , omega 6, omega 9
Water soluble and fat soluble vitamins	Thiamine, riboflavin, pyridoxine, cyanocobalamin, pantothenic acid, niacin, folic acid, vitamin C. Fat soluble vitamins; A (retinoic acid), D (kolekalsiferol), E (tocopherol) and K (phylloquinone).
Essential minerals	Ca, P, Na, K, Cl, Mg, Mn, S, Fe, Cu, I, Zn, F, Cr, Se, Mo, Si

Derived from Reference [17]

The Eagan, Marmara and Black Sea regions of Turkey have the suitable ecological conditions. The nutritional values of edible wild mushrooms collected from the Black Sea Region of Turkey, i.e. *Cantharellus cibarıus,* (Yellow mushroom), *Lactarius piperatus* and *Boletus edulis*, were established by Çağlarırmak et al. [3]. *Boletus edulis* contains the highest protein value 7.39 %, while *L. piperatus* contains 2.67 %. The protein content of *B. edulis* is superior among the vegetables.

Table 3: Protein and vitamin contents of *Cantharellaceus* species [18]

Nutrient	*Cantharellus subalbidus* (White Chanterelle)	*Craterellus cornucopioides* (Siyah Chanterelle)	*Cantharellus cibarius* (Sarı Chanterelle)
Protein	0.24 ± 0.01	0.76 ± 0.00	0.33 ± 0.04
Vitamin C	1.64 ± 0.06	1.89 ± 0.04	1.96 ± 0.04
Folic acid	82.83 ± 1.11	17.83 ± 0.81	96.83 ± 2.47
Thiamine	0.12 ± 0.017	0.11 ± 0.04	0.12 ± 0.00
Riboflavin	0.21 ± 0.01	0.06 ± 0.01	0.11 ± 0.00
Pyridoxine	0.62 ± 0.01	0.86 ± 0.01	0.90 ± 0.01
Niacin	3.75 ± 0.13	3.34 ± 0.32	6.42 ± 0.31

Protein values expressed as % (wet weight), N x 4.38; Vitamin values expressed as mg/100g (wet weight)

Mushrooms are a well-balanced foodstuff when compared with other vegetables. Çağlarırmak [19] investigated proximate compositions, B group vitamins, minerals and volatiles of *L. edodes*, and *Pleurotus* species. In the research, the chemical composition and texture were examined according to four flush terms of *L. edodes. Pleurotus* species harvested only one flush term.

Mushroom can contribute to the human nutrition protein quality and containability of some essential amino acids. Reported mean protein values of *L. edodes*, *P. ostreatus* and *P. sajor-caju* were 2.61%, 1.76% and 0.92 % wb. Vitamin C contents of *P. ostreatu*s and *P. sajor caju* were 3.38 and 16.01 (mg/100 g wb). *Pleurotus sajor caju* can contribute to human nutrition from the point of vitamin C level. The folic acid, thiamin, riboflavin and niacin contents of these studied mushrooms found good levels for contributing to nutrition. Zn, Fe, P, Ca, Mg, K and Na quantities were determined according to flush terms. Zn, P, Mg contents of investigated exotic mushroom was high quantities [19]. In Turkey, quantity of consumption shiitake is very low since its price is very high according to approximate budget of people. Shiitake has medicinal importance [4].

Agaricus bisporus is produced in the highest quantity in Turkey. In some studies, the reported amount of total cultivated mushroom is 40,000-50,000 tonnes [20]. In rural areas, the public collects wild edible mushrooms that contribute to their economy and nutritional level.

A balanced and sufficient diet is a problem for low-income people. Wheat products are consumed in large quantities. Bread consumption is almost 450 g per day, per person. People provide 44 % of their energy and work capability and 48 % of their protein requirement. These ratios are high especially in rural areas.

The main nutritional problems are the deficiency of growing and developing because of malnutrition, night blindness, anemia, tooth problems and obesity [21]. Mushrooms contain B group vitamins and vitamin D, essential minerals and high amounts of protein. The individual mushroom quantity is between the 0.4-0.5 kg annually that is very less amount when comparing with the EU countries (2.5 kg) [20]. *Agaricus biporus* (brown) mushroom is the one of the common produced mushroom. Its detailed chemical composition and volatiles were determined in the three flush terms [5]. The mean contents of Zn, Fe, P, Mg and Na in both two harvests. (mg/kg wb) were (8.15-7.07), (7.40-7.96), (1180.93-1038.69), (88.05-76.29), (213.29-238.82), (265.0-250.89) and (534.2-554.80), respectively.

In terms of vitamin C, folic acid, thiamin, riboflavin and niacin, the mean contents (mg/kg wb) were 6.75-3.97, 0.09-0.08), 0.085-0.09, 0.27-0.29) and 3.62-2.94, respectively [4].

Agaricus biporus (white) is produced in the highest quantity among the other produced cultivated mushrooms in Turkey. The applying some of food treatments for preservation to mushroom are gained economical benefits and long shelf life. Mushroom canning is one of the common ways of preservation techniques. The nutritive values of canned mushrooms changed due to blanching and sterilization process [22]. When dehydrated mushrooms, the nutrients concentrated and preserved well. Dried mushrooms can add to other foods for enrichment [23].

Edible mushrooms also have very important health benefits. *Agaricus bisporus* (white) mushroom research has suggested that it has anticarcinogenic effects for breast and prostate cancer. White mushrooms have been found to restrain the activity of aromatase, an enzyme involved in estrogen production, and 5-alpha-reductase, an enzyme that converts testosterone to DHT. Extracts can reduce cell proliferation as well as tumor size [24, 25].

Shiitake mushroom contains lentinan, β-glucan which stimulation of the immune system and can fight the against AIDS and exhibits antitumor activity. Mushrooms have anticarcinogenic effects such as they contain β-glucans, selenium, or ether medicinal compounds. They have cardiovascular protective effects e.g. Na /K ratio high K and low Na ratio opposite of lots of plant sources. *Pleurotus* species contain bioactive compounds that can affect vein system and body [24, 25].

CONCLUSIONS

Cultivated mushroom production is an independent agriculture action since it can be carried out in closed areas for 12 months in the year. Edible wild mushrooms can contribute to the economy but collecting of these mushrooms must be done in suitable conditions without causing any hazard to nature.

In southern Turkey, Korkuteli town of Antalya is a pilot region for mushroom production. The daily mushroom production is 70 tonnes and can be enough for 60-70% of mushroom production in Turkey and 15 different cities are producing cultivated mushrooms. In that town, 40% of the population earn money from mushroom production. Mushrooms are called "white gold" in the town, and there are 1,500 mushroom producing plants. Many are small plants but some can apply modern mushroom technology processes [26].

Biochemical compositions of mushrooms may be similar to meat and contain B complex vitamins, minerals and protein, and also special volatiles [4, 5, 7]. Mushrooms are consumed in Turkey especially because of their taste (similarity to meat) and nutritional value. However, medicinal importance is not common tradition in the Anatolia. According to the Turkish Statistical Institute (TUIK), the economic contribution of cultivated mushrooms is 90-100 million Turkish Lira. The Mediterranean, Middle Anatolia, Eagan and Marmara, and Black Sea regions can produce the cultivated mushrooms, but in eastern and southeastern Turkey, production is low and less common [15].

Regular development and improvement of modern mushroom plants can help to develop the economy of countries and contribute to nutrition and health. Well-nourished individuals can become more productive and better use their mental and physical potentials for their personal development and that of their country.

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RESEARCH OF EDIBLE FUNGI IN SHANGHAI

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ABSTRACT

The modern edible fungi study in China was originated in Shanghai. The Edible Fungi Institute (EFI) under the Shanghai Academy of Agricultural Science has made many breakthroughs in the Chinese edible fungi study. EFI has succeeded in getting pure spawns, has diversified the cultivation varieties of edible fungi by domesticating wild mushrooms, and has developed the bag cultivation technology. The three essential scientific and technological innovations have allowed the Chinese edible fungi industry to leapfrog for three times. The IEF has been insisting on the researches on genetics and breeding of Xianggu. Up to now, the IEF has kept over 1000 wild Xianggu strains collected from domestic provinces and foreign countries, and has evaluated those strains by conducting fruiting tests and DNA Polymorphism Comparison Tests. According to the requirements of the Ministry of Agriculture of China, the IEF establishes the DUS (Distinctness, Uniformity and Stability) Testing Guidelines for new Xianggu varieties. In recent years, the IEF researchers have adopted RAPD, ISSR and AFLP to identify primary Xianggu cultivation spawns in China, and have been carrying on theoretical and technological innovation on breeding Xianggu mainly through inbreeding. Motivated by the new generation of high-throughput DNA sequencing technologies, more and more genome sequences are sequenced in fungi. The IEF has made use of the full genome sequence of Xianggu to promote the study on the genetic breeding technology. These researches include the development of molecular markers of Xianggu strains, the illustration of biological functions at the molecular level, the establishment of the linkage groups and the construction of QTL genetic map.

Keywords: The Institute of Edible Fungi (IEF); China; Xianggu

INTRODUCTION

Related research on artificial cultivation of the edible fungi in modern China can be traced back to 100 years ago. The earliest article about the cultivation techniques of the edible fungi was published in 1897 on Agricultural Study Newspaper sponsored by Shanghai Agricultural Society. From the late 1800s to 1940s, the elder generation of scholars including Zou Bingwen, Hu Changzhi, Pan Zhinong, Li Shiyi, Sun Yunwei, and Yu Xiaotie, etc. not only introduced many foreign techniques in edible fungi cultivation and disseminated scientific knowledge and cultivation techniques, but also held various edible fungi talents training courses, set up experimental bases, and conduct edible fungi cultivation experiments. Those have laid a preliminary foundation for the modernization of China's edible fungi cultivation techniques.

The modern edible fungi study in China was originated in Shanghai. After the founding of the Popular Republic of China, the Institute of Edible Fungi (IEF) under the Shanghai Academy of Agricultural Science has made many breakthroughs in the Chinese edible fungi research, mainly including the *Tremella* artificial cultivation technique, the hedgehog *Hydnum* artificial cultivation technique and the Xianggu artificial cultivation with crushed-wood material technique.

These inventions and innovations have provided solid technical support to the development of the edible fungi industry in China. The reform and opening-up policy starting in 1978 has provided a favorable environment for the development of the edible fungi industry in China. In the period of more than 30 years thereafter, the edible fungi industry in China has been developing rapidly, with the annual yield rocketing from 60,000 tons in 1978 to 20 million tons in 2009, and the proportion of the yield against total world yield growing from 5% in the past to more than 70% at present.

CONTRIBUTIONS OF EFI

In 1960, the EFI, which was founded on the former Edible Fungi Research Team of Shanghai Agriculture Experiment Station, became the first academic institute specialized in researching the science and technology of edible fungi. Thanks to great efforts of its researchers in the past decades, the EFI has made a series of remarkable achievements. Specifically speaking, the EFI has won over 50 scientific and technological awards at or above the municipal level, and what's more, it has gotten five out of eight national scientific and technological awards in the edible fungi field. The EFI has applied for 61 patents, and has received six healthy food certificates approved by the State Food and Drug Administration. 19 new varieties created by the EFI have been recognized by the Chinese government, and 39 recognized by Shanghai. More than 600 papers and over 10 monographs and popular science books have been published. The new scientific and technologic achievements derived from the EFI have generated considerable profits and ecologic benefits when they are applied at home and abroad. Obviously, the EFI has made indelible contributions to the scientific and technologic advances in Chinese edible fungi industry.

Pure spawns technology. EFI succeeds in getting pure spawns to lay a foundation for the edible fungi industry development. In 1956, the EFI researchers under the leadership of Chen Meipeng, the first chief of the EFI, adopted the tissue isolation and the spore isolation, and successfully attained pure spawns of over ten mushroom varieties, such as white mushroom, Xianggu, agaric, *Hericium erinaceus*, Lingzhi mushroom, button mushroom, and oyster-cap fungus [1-5]. Those pure spawns make the cultivation possible, laying a solid foundation for the mass cultivation of edible fungi in China.

Artificial domestication and cultivation. EFI has diversified the cultivation varieties of edible fungi by domesticating wild mushrooms. In 1959, Chen Meipeng and other EFI researchers attained the associate spawns of white fungus and *Hypoxylon* sp. On this basis, the artificial log inoculation experiment led to the white fungus fruit body [5-7]. Then, the cultivation technology of *Hericium erinaceus* was successfully developed [8].

The bag cultivation technology. EFI has developed the bag cultivation technology to promote the sustainable development of the edible fungi industry. A group of old researchers represented by He Yuansu succeeded in cultivating Xianggu with sawdust, rich bran and other necessary nutritious supplements rather than logs in the 1970s [9-10]. This technology expanded the cultivation area of the wood decay fungi from mountainous areas to different regions of China.

As three essential scientific and technological innovations, pure spawns and artificial breeding technology, artificial domestication and cultivation of edible fungi and the bag cultivation technology have allowed the Chinese edible fungi industry to leapfrog for three times.

RESEARCH OF XIANGGU

Up to now, the EFI has kept over 1000 wild Xianggu strains collected from domestic provinces, such as Hunan, Hubei, Yunnan, Gansu, Zhejiang, Jiangxi, and foreign countries, including Japan, Thailand and U.S. The majority of those strains were collected in the wild or exchanged over ten years ago. Since 2001, the EFI has evaluated those strains by conducting fruiting tests and DNA polymorphism comparison tests. In recent years, we are cooperating with relevant institutions to speed up collection, conservation and evaluation of wild Xianggu resources.

The Xianggu variety resources lay a solid foundation for the healthy development of Chinese Xianggu cultivation industry. Evaluating those resources in a correct, standard, reliable and fair manner is the basic premise to protect the intellectual property of those varieties.

DUS (Distinctness, Uniformity and Stability) Testing Guidelines. According to the requirements of the Ministry of Agriculture, the EFI establishes the DUS (Distinctness, Uniformity and Stability) Testing Guidelines for new Xianggu varieties, which are standards for the protection and recognition of new varieties [11].

Among the 239 Xianggu strains collected nationwide, 24 strains featuring clear source and traceable breeding history were selected as the standard ones in the DUS Testing. These standard strains are widely used [12], such as the Shenxiang series, No.1 Wuxiang, Cr04, 135, 9015, L26, and are provided by the EFI and other fungi institutes in Wuyi, Qingyuan of Zhejiang and Sanming of Fujian.

The Xianggu DUS Testing Guidelines, which are established on the basis of features and data in the Xianggu bag cultivation, include 36 testing specifications consisting of 34 compulsory traits (those must be tested) and 2 supplementary traits. The compulsory traits include 10 hyphae traits and 24 traits related to the bag cultivation. The hyphae traits are composed of envelope, density, growth rate, etc. The 24 cultivation traits cover those properties, such as pileus, stipe, context, scale, gill, fruit body. The supplementary traits are composed of content determination and DNA fingerprinting of special ingredients of fruit body.

DNA molecular identification of Xianggu spawns. Identification of Xianggu cultivation spawns has been the concern of EFI researchers all the time and a focus in the Xianggu genetic breeding study. Recognized as a quick, convenient, accurate and reliable method, the DNA Molecular Identification of Xianggu spawns can be divided into two kinds. One kind refers to the clustering analysis of the DNA sequence amplified by non-specific PCR primers to identify genetic polymorphism and kinship between spawns on the basis of Phylogenetic Tree, such as RAPD and AFLP markers. The other kind is to compare the DNA sequence amplified by specific PCR primers to create molecular markers which directly identify the distinctness of spawns, such as SCAR markers.

The EFI researchers have adopted RAPD, ISSR and AFLP to identify primary Xianggu cultivation spawns in China [12-14], and in addition have developed the SCAR markers for the identification of distinctness for Xianggu spawns in recent years [15]. Research results suggest that Xianggu cultivation spawn resources have small genetic differences, single parental source and narrow genetic background. All of these factors restrict the ability of Chinese Xianggu varieties to adjust to environmental changes, the potential to improve agronomic traits and the innovation in new varieties. Those result in restrictions for the Xianggu cultivation industry development from a long-term, sustainable and stable perspective. Therefore, it is necessary to make more efforts in collecting wild Xianggu resources, evaluating and utilizing the attained wild resources, and introducing more excellent strains to the cultivation resources.

SCAR stands for Sequence-characterized Amplified Regions. The SCAR Markers are suitable for the analysis of large samples because of its better specificity, stability and repeatability as well as its rapidness convenience and low cost. In recent years, a large number of SCAR markers have been developed in the molecular identification of Xianggu spawns. Some domestic

researchers have conducted development research to the SCAR markers of Xianggu in recent years. Xie Baogui Laboratory has successfully separated 14 SCAR markers from RAPD, SRAP and ISSR markers [16]. Ten SCAR markers separated by Wu Xueqian Laboratory can identify seven strains from 47 main cultivated Xianggu strains, while the other 40 strains can be divided into 11 groups [17-18]. Kwan Haishan Laboratory in the Chinese University of Hong Kong has improved the technical route of researching SCAR markers by high-throughput sequencing technologies and consequently enhanced the research efficiency [19].

Though the SCAR markers research of Xianggu has made some progress, the SCAR markers obtained through regular development technology are quite few that are worth using. In addition, most of these SCAR markers are single markers which cannot fully meet the practical needs. So separating SCAR markers through high-throughput sequencing technologies may be a novel technical route. The wide application of this route may produce plentiful SCAR markers which will pave the way for establishing a database concerning the SCAR markers research of Xianggu. As a result, the molecular identification scheme of Xianggu species can be really realized.

The breeding of Xianggu. Collecting Xianggu wild resources requires a sense of urgency. Developing the identification technology of Xianggu spawns demands a sense of crisis. The breeding of Xianggu needs the close integration of technicality, practicality and theory. Some new species have been selectively bred to meet the domestic Xianggu production in previous researches. These species are bred through technologies such as systemic breeding, symmetrical crossing and nonsymmetrical crossing [20-22]. The new development of Xianggu cultivation industry demands new varieties of Xianggu. For example, with the expanding growth area of Xianggu in recent years, it is increasingly urgent to develop ecological Xianggu species to meet local weather conditions, especially the high-yield variety adapting to the high temperature in summer.

The EFI has been carrying on theoretical and technological innovation on breeding Xianggu mainly through inbreeding in recent years. Inbreeding is the most common means to optimize and purify crossing parents. Inbreeding can improve the rate of homozygous genotype and the excellent qualities can be stable genetic as a result. In addition, the deleterious gene in parent material can be discovered. In a word, the pure lines obtained through inbreeding are of great importance to the application of hybrid vigor.

The research of inbreeding Xianggu is expected to achieve two goals: one is to get offspring strains having better characters and features which can be used as the parent for a new round of inbreeding; the other is to get relatively homozygous offspring strains having stable characters and features which can be used as parent for crossbreeding. According to the distribution of the growth rate of 931 strains' multispore self-bred progeny, the inbreeding hypha's growth rates are concentrated under 23 and 28. On the contrary, under 30, the hypha's growth rates become relatively scattered and the variation of traits of self-bred progenies was obvious. Two equal groups consisting of 20 offspring strains are established on the basis of growth rate's coefficient of variation. The growth rate refers to that of inbreeding strains in variable temperature test. The two groups are called heat resistant group (I) and the non-heat-resistant group (II). Group I displayed heat resistant characteristics in variable temperature test and can be used as the object of fruiting experiment later as well as the parent material for crossbreeding high temperature species.

The breeding of Xianggu is arduous, meticulous and long-term. It not only requires a great number of experiments and tests but also needs proper breeding theories as guidance. The EFI is determined to continue innovations concerning breeding technology and theory of Xianggu.

The prospect of Xianggu research. Motivated by the new generation of high-throughput DNA

sequencing technologies, more and more genome sequences are determined. Among these genome sequences, those belonging to basidiomycetes include *Coprinopsis cinereus*, *Laccaria bicolor*, *Schizophyllum commune*, oyster mushroom, white mushroom, *Tremella aurantialba*, button mushroom, *etc* [23-26]. Under this situation, Kwan Haishan Laboratory in the Chinese University of Hong Kong launched Full Genome Sequencing (FGS) of Xianggu and has made some progresses. As a participant in this plan, the EFI has made use of the full genome sequence of Xianggu to actively study the genetic breeding technology.

There are two markers which can be developed on the basis of the full genome sequence of Xianggu, namely, SSR markers and SNP markers. Attaining the full genome sequence of Xianggu provides a favorable premise for looking for SSR loci. By using the SSR primers (provided by Kwan Haishan Laboratory in the Chinese University of Hong Kong) designed according to the 80-pair genome sequence of Xianggu, the EFI tested the polymorphism of 36 cultivation and wild Xianggus. The research result revealed that 52-pair SSR primers demonstrated stable and obvious polymorphism. In addition, the test detected 351 polymorphic loci, that is, each pair of polymorphic primer generated 7.16 loci. Clearly, SSR primers possess high polymorphism in Xianggu, so they are relatively ideal molecular markers. A SNP, third generation molecular markers, has the advantages, such as large quantities, representativeness, genetic stability and suitability to the high-throughput test. The EFI compared the genome sequences of two protoplasted monokaryons with the coverage ratio of six times from 135 strains, and finally found that a SNP locus exist every 200bp DNA sequence on average. Such abundant SNP loci lay a foundation for the use of SNP as molecular markers.

Attaining the full genome sequence of Xianggu offers a rapid way to illustrate biological functions at the molecular level. As a result of comparing the full genome sequence and the biological functions, corresponding functional genes can be detected in the sequence. Eventually, the metabolism or response system will be constructed, such as lignocellulose degradation route, signal response process in the generative affinity match, the control procedure governing how the external factors stimulate Xianggu color change and fruit body development.

Based on the full genome sequence of Xianggu, the linkage groups analysis and the construction of QTL genetic map will dramatically differ from similar studies in conventional genetics. The Xianggu linkage group constructed on the basis of SSR or SNP markers corresponds to the full genome sequence. In the attainted linkage map, analyzing the sequence can identify the gene distribution near molecular markers, a good premise for the QTL analysis and positioning important trait genes of Xianggu. The further QTL analysis of the primary agronomic traits of Xianggu can identify major genes in the known DNA sequence linkage map, which provides the material foundation for further determination of major genes.

The new generation DNA sequencing technology will contribute to the genome information explosion of creatures. The Age of the Genome is coming, so is the age of Xianggu genome. How to make use of the full genome sequence of Xianggu to promote its genetic breeding research is a question that deserves our great attention. Scientific achievements in this area will propel the sustainable and stable development of Xianggu cultivation industry.

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RADIOACTIVE CONTAMINATION OF UKRAINIAN WILD-GROWING MUSHROOMS

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ABSTRACT

By the activity of accumulated radiocaesium mushrooms, especially mycosymbiotrophs, prevail both plants and forest litter (which during the all post-Chornobyl period is the main depot of radionuclides) by several to hundreds times. This fact allows to use species-hyperaccumulators, for instance of radiocaesium, as bioindicators for radioactive contaminated territories. They include certain representatives of *Cortinariaceae*, *Russulaceae*, *Boletaceae*, *Suillaceae*, *Hydnaceae*, *Paxillaceae*, *Tricholomataceae*, *Gomphidiaceae*. Among them, as widely spread edible species *Boletus badius* (Fr.) Kühn., and as common species *Lactarius rufus* (Scop.) Fr. and *Paxillus involutus* (Batsch) Fr., not consumed by population because of their inedibility and toxicity, had been recommended as most convenient objects for long-term radioecological monitoring of contaminated territories of Ukrainian Polissya. Accumulation of radiostrontium was in 10-10^2 times less than radiocesium. The use of mushrooms-bioindicators let give the prognosis estimation of the situation with contamination levels of soils, other wild-growing mushrooms and berries. At the same time it should be noted that it's not possible to use them for exact statistically reliable estimation of the territories contamination levels taking into account the high level of variability observed even in the samples of the same species in the same location.

Keywords: bioindication, ^{137}Cs, ^{90}Sr, the Chornobyl catastrophe.

INTRODUCTION

The Chornobyl catastrophe is unprecedented both by the territory of radioactive contamination and by intensity of doses absorbed by biota objects. Even 25 years after the accident radioactive contamination of mushrooms is quite significant, in some cases reaching very high levels, and creates human health problems resulting from their nutritional and medicinal use. The capacity of mushrooms to be concentrators of heavy metals and radionuclides of natural and technogenous origin is well documented in special literature [1-8]. A strong argument in favor of use of macromycetes as bioindicators is a clear prevailance of radiocaesium contamination for the whole post-Chornobyl period in some macromycetes species relative to forest litter (by several to hundreds times), which, in turn is the main

radionuclides depot [9-11]. It must be noted that the radioactively polluted areas of Ukraine are characterized by the mosaic pattern of contamination that complicates the estimation and forecast of radionuclides uptake by biota objects. ^{137}Cs (during the first post-catastrophe period in combination with ^{134}Cs) was confirmed to be the main doze-forming radionuclide in the contaminated area, and that conditioned a priority given to the uptake of this element by mushrooms. However, fruiting bodies having a high activity are only an insignificant part of the total mycelial biomass which is situated in the organic soil layer. An essential contribution of the macro-and micromycetes mycelia biomass and its ability to uptake and retain radionuclides play a very important role in its migration and distribution processes.

MATERIALS AND METHODS

^{137}Cs activity in fruiting bodies of wild growing mushrooms (207 species) and substrates from their habitats, collected in 1990-2010 at 159 locations of Kyiv (including the Chornobyl zone), Chernihiv, Zhytomyr, Cherkassy, Volyn', Rivne, Ivano-Frankivs'k, Poltava, L'viv, and Zakarpattia regions of Ukraine have been studied using gamma-spectrometry. Some samples were analyzed on ^{90}Sr using radiochemical methods on the basis of the accumulation of ^{90}Y. Samples weighed from 1 to 200 g and had 3 to 30 fruiting bodies. Soil samples were taken at the depth of 0-5 cm. Mushroom samples cleaned from soil and plant particles as well as samples of soil (substrate) were dried at 40-50^{o}C and ground to fine-dispersed condition. Then they were dried at 80^{o}C for 24 hours and placed in plastic Petri dishes, or plastic bags. The counting time was 6-36 hours. Counting errors for the measurements of ^{137}Cs were usually lower than 20%. Average levels of soil surface contamination with ^{137}Cs were defined during field measurements, according to the maps presented in the National report [12], Atlas of radioactive contamination of Ukraine and data in General dosimeter certification.

RESULTS AND DISCUSSIONS

Researches of radionuclide accumulation in mushrooms of Ukrainian Polissya which is the most polluted region as a result of failure on Chornobyl Nuclear Power Plant (ChNPP) in 1986, have shown that during all period after catastrophe mushrooms constantly demonstrated high levels of radiocesium contamination**.** It is clear that mushrooms contamination levels is mainly correlated with soil contamination levels. The maximum radiocaesium levels (up to millions Bq per kg of dry weight) in species taken from the Chornobyl exclusion zone were observed in mycosymbiotrophic species - *P.involutus* – 31 MBq/kg d.w.(Shepelychi forestry, 1996), *Boletus subtomentosus* (L.) Quél.– 20 (Shepelychi forestry, 2004), *Gomphidius glutinosus* (Schaeff.)Fr. – 17 ("Red"forest, 1993), *Lactarius turpis* (Weinm.) Fr. – 19 and *Suillus luteus* (L.) Roussel – 15 (Kopachi, 2004), *B. badius* – 12 ("Red" forest, 1996), *B. edulis* Bull. - 11 MBq/ kg d.w. (Janiv, 1998). In general, ^{90}Sr uptake in wild-growing mushrooms is not as intensive as that of radiocesium. Samples from that zone were shown have a ratio of ^{137}Cs/^{90}Sr within the range 10-10^{3} (mean - 10^{2}).

However, it is not the reason to ignore ^{90}Sr, that element presenting a great danger for the human organism. In cultivation condition it was shown increasing of radiostrontium accumulation in edible and medicinal species such as *Lentinula edodes* (Berk.) Pegler and *Pleurotus ostreatus* (Jacq.) Kumm. [13]. This phenomenon can be connected with higher biological availability of ^{90}Sr from mixed and watered substrates which are used in mushroom industrial culture. It can be assumed that an increase of ^{90}Sr activity in wood (observed last years at the contaminated territories) will inevitable cause increase of its content especially in lignotroph species. Therefore, the selective control of ^{90}Sr accumulation in lignotrophs and especially cultivated ones even at the areas with low surface soil contamination is strongly recommended.

The most numerous group of mushroom specimens was taken from region with the surface contamination of 37 – 185 kBq/m^2 (i.e.zone of an intensified radiation control). Presently over 1.5 million people of Ukraine live at such contamination level [12]. Here, despite the considerable variability of the obtained data, that is typical for radiobiological research, quite high levels of radiocesium accumulation were observed. Coefficients of accumulation (which are equal to the ratio between activity of radiocesium in mushrooms and its activity in the substrate/soil in the location) reach tens, hundreds, and during the first post-accident years even thousands. For instance, during the period from 2000 to 2005 the maximum levels of radiocesium in the studied locations with this average levels of surface contamination of soils with ^{137}Cs have been observed in *L. rufus* – 375294 (vil.of Kolentzi), *P. involutus* – 282764 (vil.of Fenevychi), *Cortinarius sp.* – 269692 (vil. of Lutizh), *Pluteus cervinus* (Schaeff.) P.Kumm.– 161643, *B. badius* - 120711, *S. luteus* – 117771 (near city of Ivankiv), *Hebeloma crustuliniforme* (Bull.) Quél.– 100519 Bq/kg dry weight (settl.of Klavdievo-Tarasove). High levels of radiocaesium uptake constantly were shown by *B. badius* from different locations (Fig. 1). A considerable decrease in levels of radiocaesium was observed only in drough-affected years. During all period of investigations accumulation of radiocesium in wild growing mushrooms represents a damped wave process with rises in wet years and lowest values in dry years.

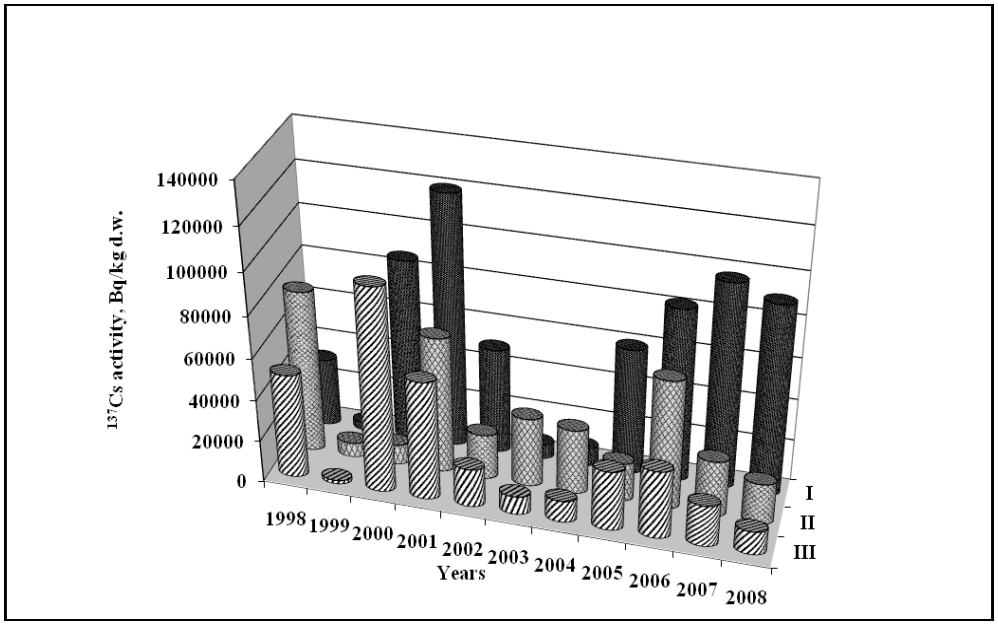

Figure 1. Activity of ^{137}Cs in fruiting bodies of *B.badius* from locations with average soil surface contamination 37 – 185 kBq/m^2 (I – city of Ivankiv, II – vil. of Shpyli, vil.of Lutizh, Kyiv region).

The ratio ^{137}Cs/^{90}Sr was quite high (Fig.2).

On the whole, during the last years the levels of ^{137}Cs contamination of up to 80% of edible species samples (*Boletus* spp., *Suillus* spp., *Leccinum spp., Cantharellus cibarius*, *Lactarius deliciosus*, *Tricholoma* spp. etc.) traditionally included into a diet of Slavic people exceeded the maximum permissible norms accepted in Ukraine (2,5 kBq/kg d.w.).

At levels of ^{137}Cs contamination $\leq$ 1 Ci/km^2 in macromycetes also quite high level of radiocaesium were observed.

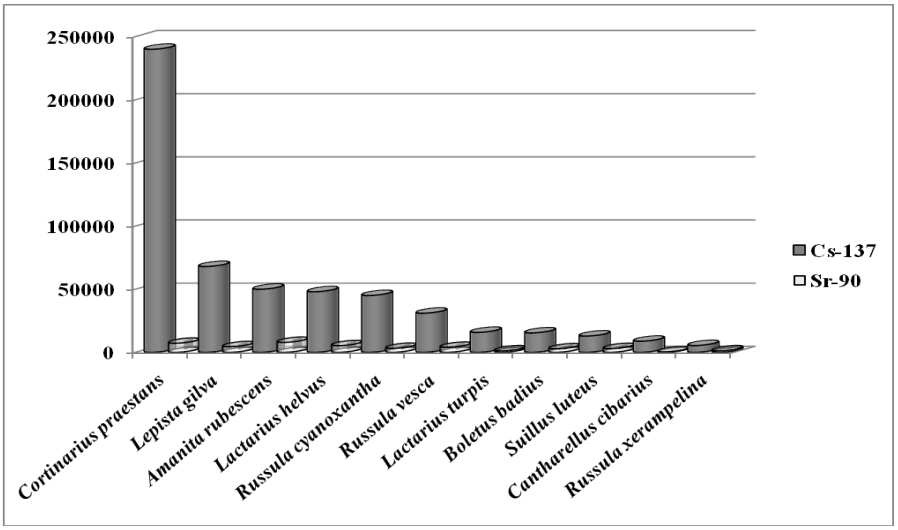

Figure 2. ^{137}Cs and ^{90}Sr activities in mushrooms in 2010 (vil.of Karpylivka, Chernihiv region).

Analysis of literature and our results of 1990-2010 monitoring allows concluding that level of radionuclide accumulation in mushroom depends both on specific radioecological situation in the area of mushroom sampling (quantitative and qualitative composition, forms of radionuclides present in soil, moisture, pH, type of soil, climatic factors, landscape peculiarities etc) and on mushroom species specificity, belonging to ecological trophic groups, depth of mycelia location in soil. It must be noted that the tendency of increase of accumulation ability in the sequence from lignotrophs →humus saprotrophs →litter saprotrophs →mycosymbiotrophs, described earlier [9, 11] apparently concerns only the accumulation of radiocesium. It is clear that in case of ^{90}Sr this sequence will be of other kind.

In spite of positive correlation is marked between the level of contamination of soils, humidity and level of contamination of mushrooms on the whole, mosaic pattern of contamination of territory of Ukraine, complex of the controlled and non-controlled factors, and especially extraordinarily high variability of mushrooms contamination levels (Table 1) complicate the prognosis estimation of the dose loadings on the human as a result of consumption of wild growing mushroom species.

Table 1. The estimation of variability of mushrooms contamination levels in 1990-2010

The average level of soil surface contamination with ^{137}Cs, kBq/m^2	The range of mushrooms contamination with ^{137}Cs, kBq/kg d.w.
3,7-18,5	BDL – 150
18,5-37	BDL – 300
37-185	BDL – 350
185-370	100 – 350
370-555	600 – 1 300
555 – 1480	4 000 – 17 000
>1480	Up to 32 000

BDL = below detection limit

The conducted investigations on wild growing mushrooms of Ukrainian Polissya made it possible to single out species with hyper-accumulative ability. Certain mycosymbiotrophic representatives of families *Cortinariacea*e [*Cortinarius* spp., *Rosites caperata* (Pers.) P.Karst., *Hebeloma crustuliniforme* (Bull.) Quél.], *Russulaceae* (especially *Lactarius* spp.), *Boletaceae* [*Boletus* spp., *Leccinum scabrum* (Bull.) Gray, *Tylopilus felleus* (Bull.) P.Karst.], Suillaceae (*Suillus* spp.), *Paxillaceae* [*Paxillus involutus* (Batsch) Fr.], *Hydnaceae* [*Sarcodon imbricatus* (L.) P.Karst., *Hydnum repandum* L.], *Tricholomataceae* [*Tricholoma flavovirens* (Pers.)S.Lundell], *Gomphidiaceae* [*Gomphidius glutinosus* (Schaeff.) Fr. and *G.rutilus* (Schaeff.) S.Lundell] have shown steadily high levels of contamination that makes it possible to use them for bioindication purposes. Among them widely spread and common species in Ukraine *L.rufus* and *P.involutus* may be regarded as the most convenient bioindicators since inedibility of the first and toxicity of the second allow to reduce the influence of the antropogenic factor in the estimation environmental radiocesium contamination. Use of *B. badius* as the bioindicator is of some interest in terms of comparison with the data obtained for other countries**.** The coefficients of accumulation of these three species reach tens and even hundreds.

Mapping that involved bioindicative mushroom species (Figure 3) shows an evident gradient of radioactive-induced contamination effects on mushroom fruiting bodies from northern and northwest part to southern and central part of Kyiv region of Ukraine. Figures show a coincidence, in general, of contamination intensity with the soil contamination levels presented on maps published in the National report [12].

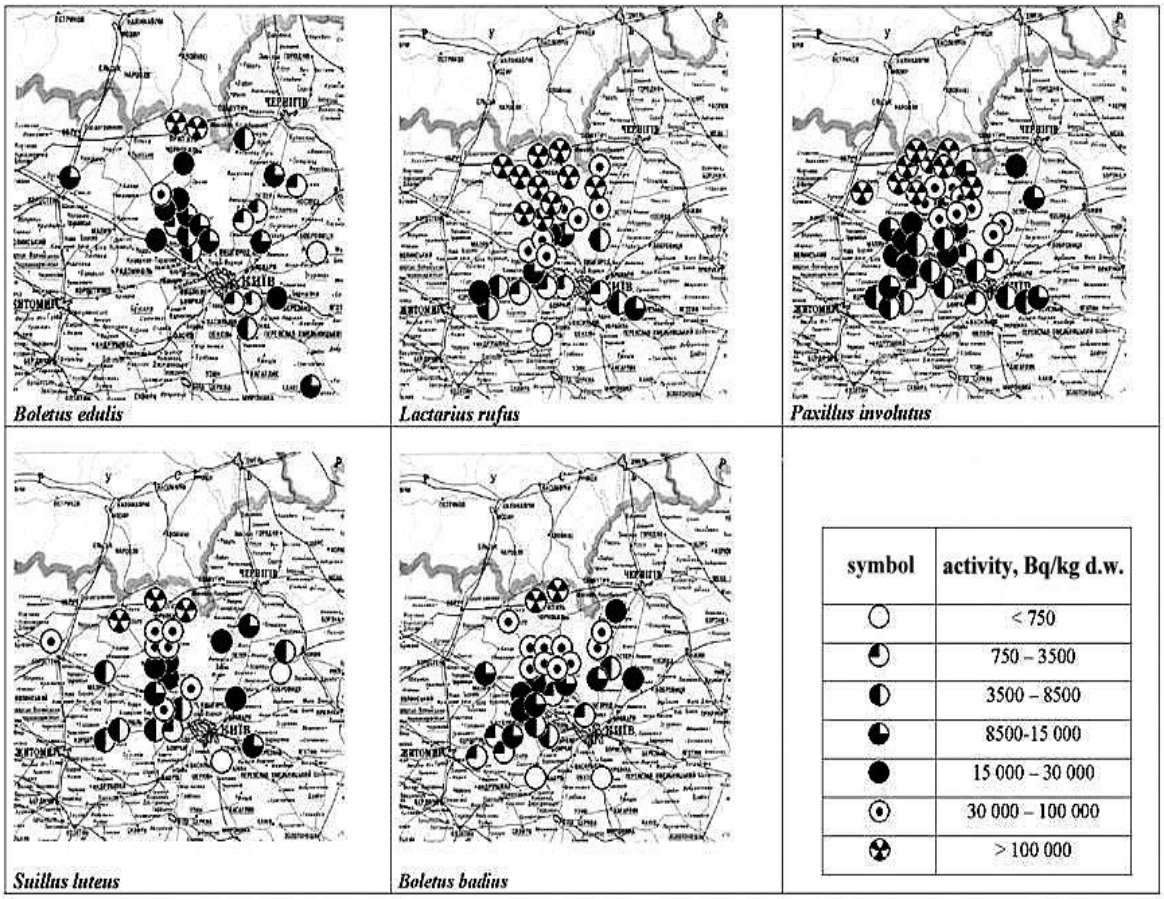

Figure 3. Mapping of Kyiv and part of Zhytomyr region with species-bioindicators.

The use of selected mycoindicators is an express-technique that enable to assess and extent of risk of mushroom consumption in that area, to forecast the development of situation regarding contamination levels of soils, other wild-growing mushrooms and berries. At the same time it should be noted that it is not possible to use them for exact statistically reliable estimation of the territories contamination levels due to the high level of variability observed even in the samples of the same species in the same location.

Literature data regarding a radioactive danger of mushrooms are numerous and as a result it is quite natural to ask a question about the reasons of a mass character of wild-growing mushrooms consumption by the population of Ukraine. In our opinion the reasons are several. They lie in the traditional perception of «the gifts of forest», in lack or complete absence of information concerning mushroom radioactive contamination in each area, in the syndrome of «tiredness» of negative information published in mass media and in sense of being doomed that in combination with a finance modest means of the majority of people living in the contaminated areas form that vicious circle that is hard to get out from. It should be mentioned that for some country people wild growing mushroom collection for sale forms a significant part of their family's budget. Thus, a specific scientific problem has deep social grounds.

CONCLUSIONS

The analysis of radiocesium accumulation dynamics in wild growing mushrooms gives evidence of a long-term radiological risk resulting from their consumption as food and medicine on the territory with ^{137}Cs contamination more than 37 kBq/m^2. It can be said that this situation will remain dangerous for many years. In these conditions the only reasonable alternative is to increase the industrial mushroom cultivation involving new perspectives with species having high nutritional and medicinal properties on tested free from ^{137}Cs and ^{90}Sr substrates.

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DIVERSITY OF WILD MUSHROOMS FROM JAMMU AND KASHMIR (INDIA)

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ABSTRACT

Jammu and Kashmir state is stretched between 32°17'-37°03' N latitude and 72°03'-80°20' E longitude, and covers a total area of 222,235 km², with an average annual rainfall between 60-80 cm. It is bordered to the north and east by the main Himalayan ranges and Punjab plains to the south. The state exhibits varied climatic and topographic conditions and provide pleasant environment for the lavish growth of diverse group of plants. However, information on the species of wild mushrooms from this state is limited. In this backdrop, a systematic study of wild mushroom diversity from various locations of Jammu and Kashmir was undertaken. During the course of intensive field research over the last four-five years in the forests of some regions of the state, the authors collected a number of wild mushrooms belonging to Ascomycetes, Basidiomycetes and Gasteromycetes. During survey, it has also been noticed that the state has the largest concentration of forest dwellers, comprising of about one-fourth of the population of the state. Several tribes and villagers subsist largely on non-traditional and wild food sources especially wild edible mushrooms. Keeping this in view, the ethnomycological information related to these fungi was gathered from several tribal men and women, village heads, and other local informants as well as ayurvedic hakims in order to gain better understanding of the relationship between the fungi, the local people, and the economy. Collection was mainly concentrated in the dense coniferous and mixed forest of *Cedrus deodara* (Roxb.) G. Don, *Pinus wallichiana* A.B.Jackson, *Picea smithiana* (Wallich.)Boiss. *Abies pindrow* Royle, *Quercus* sp. L., *Juglans regia* L., *Alnus nepalensis* D.Don, *Ulmus wallichiana* Planch. etc. Standard methods of collection, preservation and identification have been followed.

Key words: Wild, Diversity, Mushrooms, Edible, Ethnomycology

INTRODUCTION

Mushrooms are cosmopolitan heterotrophic organisms that are quite specific in their nutritional and ecological requirements. As such, they have been generally divided into humicolous, lignicolous, coprophilous, fungicolous, parasitic or saprophytic or may show some mycorrhizal associations with both broad-leaved forest trees and gymnospermous taxa. They constitute the most relished food commodities amongst the number of non-conventional foodstuffs primarily because of their unique flavor and texture. The Indian state of Jammu and Kashmir, which lies in the north-west Himalaya, is a rich repository of the unexplored macrofungal wealth due to its varied climatic and topographic conditions, thus providing congenial environment for the lavish growth of this heterogenous group of fungi.

Wild edible mushrooms have been collected and consumed by people since thousands of years. Archaeological evidences reveal edible species associated with people living 13000 years ago in Chile [1] but it is in China where the eating of wild fungi was first reliably noted several hundred years before birth the of Christ [2]. Many cultures, especially in the Orient, identified that certain mushrooms could have profound health-promoting benefits [3]. Mushrooms have been exploited commercially world over and may be cultivated or gathered from the wild. The size of the gathered wild edible fungus market globally has been estimated as several million tones with a value of at least US$2 billion in 2004 [4].

Of the 14,000 mushroom species, nearly 7000 species are well studied to possess varying degree of edibility, and more than 3000 species spread over in 31 genera are regarded as prime edible. Thus far, only 200 of them are experimentally cultured, 100 economically cultivated, approximately 60 commercially grown and about 10 have reached an industrial scale [5]. The rate of consumption of fleshy fungi in many countries has increased in recent years and hence it becomes imperative to explore the treasure of wild mushrooms.

Several mycologists have reported ethnomycological usage of this natural resource wealth from some regions of India [6-12]. However, indigenous knowledge about edible and medicinal mushrooms has not been given significant attention in Jammu and Kashmir State and presently no literature on this vital aspect exists in this State.

MATERIALS AND METHODS

Wild edible mushrooms were collected from various locations in North West Himalaya of Jammu and Kashmir state. Standard methods of collection, preservation, and identification were followed. Ethnomycological information was recorded from reliable sources such as hakims, tribals, and local inhabitants who were considered to have good knowledge of the wild resources of the region. They were taken to the forests as guide cum informants. In order to gain better understanding of the relationship between the fungi, the local people, and the economy, field investigations and interviews were conducted in different local languages *viz*., Bhadarwahi, Kishtwari, Gadaishi and Kashmiri. The interviews were semi-structured having a set of questions which were put forth to the selected locals to ascertain their views on historical background, traditional usage, edibility status, folk taxonomy, methods of drying and preservation, commercial importance etc. of fleshy fungi. An effort was made to reach the key informants, people who were known to collect and sell this natural resource wealth. Repeated interviews were conducted to substantiate and authenticate the information. At times, additional information regarding fleshy fungi was gathered by showing the specimens itself. The photography was accomplished using digital camera (Sony DSC-P92). Each specimen was collected and labeled, indicating number, date of collection, locality and uses. All collections have been deposited in the herbarium of Botany Department, University of Jammu, Jammu with accession numbers.

RESULTS AND DISCUSSION

As many as 150 collections of wild mushrooms were made and worked out for their macro- and micro-morphological and ethnomycological features. A total of 66 taxa of wild mushrooms belonging to 33 genera spread over 22 families, 10 orders and 3 classes were identified. The identified species and varieties spread over in following genera viz., *Agaricus* (1), *Astraeus* (1), *Amanita* (1), *Auricularia* (1), *Boletus* (4), *Bovista* (2), *Cantharellus* (2), *Chalciporus* (1),

Clavaria (1) *Clavulina* (2), *Coprinus* (1), *Flammulina* (1), *Geopora* (2), *Gyromitra* (2), *Helvella* (3), *Lactarius* (1), *Lentinus* (1), *Leucopaxillus* (1), *Lycoperdon* (4), *Macrolepiota* (1), *Morchella* (5), *Otidea* (1), *Peziza* (2), *Pleurotus* (2), *Ramaria* (6), *Rhizopogon* (4), *Russula* (3), *Schizophyllum* (1), *Scleroderma* (1), *Sepultaria* (1), *Sparassis* (2), *Termitomyces* (4) and *Verpa* (1).

Indigenous knowledge of edible mushrooms and their utilization by local population is an important component of ethnomycology. Data were gathered during the ethnomycological survey related to collection of wild mushrooms. It was found that the collection of wild mushrooms was undertaken early in the morning, as there was intense competition for mushroom gathering, especially for the morels because of their high commercial value. Women and children from 'Gaddi and Shippi' tribes were frequently involved in these activities then men. Children frequently accompany the women, as they were good at locating mushrooms because of their sharp eyes and proximity to the ground and crevices where the occurrence of the mushroom is highest. A special basket called 'Tokri', 'Keed' or 'Chounllee' or a 'Cotton cloth' (Duppatta) was used for collecting mushrooms (Fig. 1 a-e).

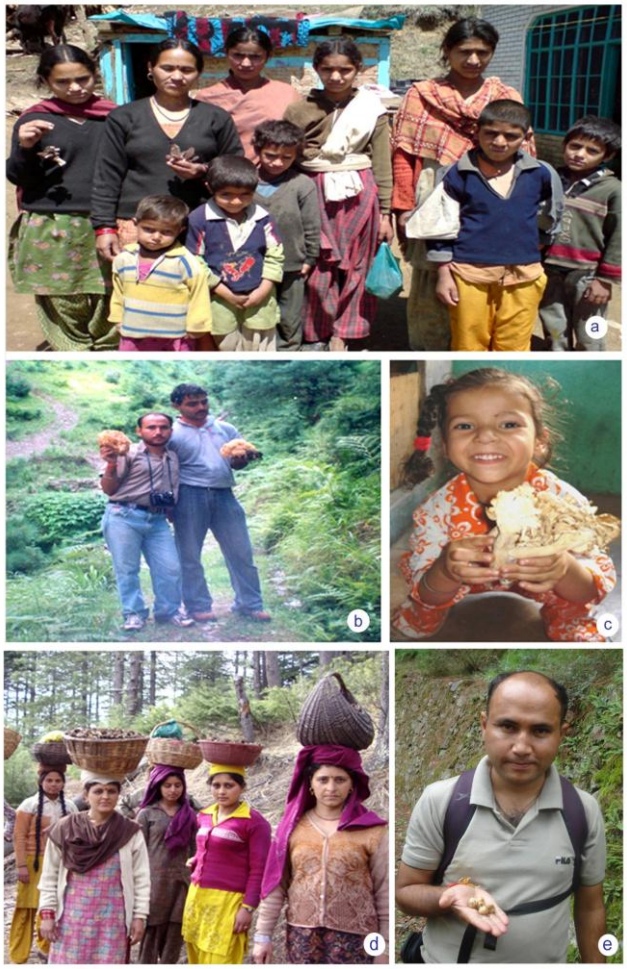

Figure 1 : Wild Edible Mushrooms Harvest : Collectors Delight
a- A Bhadarwahi family of Dugga area displaying morels
b- *Sparassis* spp. in the hands of author and his friend during a collection foray
c- A little girl holds *Pleurotus* sp. in her hands with a pleasant smile

d- Womenfolk on their way back to homes after rich mushroom gathering from the woods.
e- *Rhizopogon* sp. in the hands of author

Collection of wild edible mushrooms. Collection forays were more frequent in March and April and July and August months. However, the best period for wild mushroom collection in the study area starts with the onset of rains, the period when the conditions are conducive for the mushroom growth and they are available in plenty. This activity also coincides with the gathering of fallen pine needles used in roof topping of mud houses and firewood to be stored for winter months as the weather conditions during this period are harsh due to snow and fuel shortage.

The study also indicated that several reasons probably enabled rural folks to participate successfully in the harvest of wild edible mushrooms. These were:

1) Open and unrestricted access to the forests and grasslands.
2) No expenditure was involved for mushroom gathering.
3) Economic benefits in which income generated from the sale of collected mushroom resources goes directly to these inhabitants. As a result, majority of the rural people (e.g. Gaddis, Shippis etc.) besides practicing traditional subsistence herding and agriculture also participate in wild mushroom collection.
4) As a nutritional supplement, mushrooms could help diversify an otherwise monotonous diet during the rainy season when there was a paucity of other food resources.
5) There could be several other social and cultural benefits including the healthy bonding between the families and the market place.

Edibility status. The ethnomycological survey related to edibility status of mushrooms was also undertaken and the results revealed that sixty two potential wild edible fleshy fungi from different locations of study area were recorded. Out of these, as many as 41 mushrooms were preferentially consumed by the native populations of the area while remaining 22 species, although having reported edibility elsewhere, did not find place in the list of edible mushrooms.

While confirming the edibility status of these mushrooms, the consumer's preference (range of palatability) was ominous**.** Species such as *Agaricus arvensis, Boletus luridus, Geopora arenicola*, *Gyromitra* spp., *Morchella* spp., *Pleurotus* spp., *Rhizopogon* spp., *Sepultaria sumneriana, Sparassis* spp. and *Termitomyces* spp. are considered highly delicious, while *Boletus edulis, B. granulatus, Helvella* spp., *Ramaria* spp. have good acceptability for consumption. Remaining species namely, *Clavaria vermicularis, Clavulina* spp., *Coprinus comatus, Macrolepiota procera, Ramaria* spp. are not much sought after mushroom species in the region. Edibility of some of these most popular and widely consumed wild macromycetes has been reported throughout the northern hemisphere, South Africa and New Zealand [13, 14]. The usage of these fruiting bodies both commercially and domestically may be in part a result of their appealing taste, their frequent occurrence and the fact that they are easily identifiable by the locals as safe for consumption.

Several mycologists in India have also reported the edibility of these species from various states [15- 21].

Drying and preservation. Fresh wild edible mushrooms have a short period during which they can be eaten or consumed. Owing to their perishable nature, they quickly deteriorate, rot, or shrivel up. On questioning local people about this aspect, it was realized that they consumed

large number of the mushrooms in fresh form and only a few are preserved after sun-drying, smoke drying or salting. Mushroom species such as *Geopora arenicola, Sepultaria sumneriana*, *Morchella* spp., *Pleurotus* spp., *Russula* sp. and *Sparassis* spp. are sun-dried in open and then stored in gunny bags, polythene bags or jars. In addition, a unique method for the preservation of *Geopora arenicola* and *Sepultaria sumneriana* is followed. These hypogeous mushrooms are thoroughly washed in water to remove soil debris adhering to the apothecia, sun-dried, salted and then mixed with turmeric powder for enhancing shelf-life in storage and off-season consumption (Fig. 2 a-h and Fig. 3 a-e).

Figure 2: Wild Edible Mushrooms : Traditional Drying and Storage methods.
a- Drying of morels in open
b- Fruiting bodies of *Geopora* spp. & *Sepultaria sumneriana* collected in baskets (Tokries)
c&d- Fruiting bodies of *Rhizopogon* spp. gathered from the forests
e- *Geopora arenicola* & *Sepultaria sumneriana* in 'Chajjh' for removing soil
f- Dried form of *Sparassis* spp. and morels
g- *Geopora arenicola* & *Sepultaria sumneriana* ready for cooking after proper washing

h- Morels packed in jars and polythene bags in a local market

Figure 3: Wild Edible Mushrooms: Drying and Sale
a- Sun drying of morels on a cow dung coated floor
b- Elderly women putting morels in a string for drying. Also seen are the fruiting bodies of *Geopora* spp. & *Sepultaria sumneriana* in 'Chajjh' for cleaning
c-e- *Pleurotus* spp. for sale at local shops in the study area

Culinary potential. Outcome of the survey on the culinary potential of the edible mushrooms revealed that mushrooms in the study area represented a longed for culinary innovation and find a remarkable utility in the culinary traditions of the area. Majority of the people questioned showed deep affection for the taste and preparation of wild mushrooms while only a few respondents showed aversion towards them. It was observed that the larger quantities of mushrooms are being consumed in the area and are regarded by many as wholesome food and in certain cases, an equivalent of meat. The inventory of consumption pattern of wild edible mushrooms extracted from the inhabitants of Bhadarwah, Kishtwar, Poonch, Jammu, Rajouri etc. The mushrooms that are consumed as fresh vegetables include *Agaricus arvensis, Boletus* spp., *Coprinus comatus, Peziza badia, Clavaria vermicularis, Clavulina* spp. *Geopora arenicola, Gyromitra* spp., *Helvella* spp., *Macrolepiota procera, Morchella* spp., *Otidea leporina, Pleurotus* spp., *Ramaria* spp., *Sparassis* spp., *Sepultaria sumneriana, Russula* sp. and *Termitomyces* spp. These are usually cooked with tomatoes and onions while others are dried (*Morchella* spp., *Geopora arenicola*, *Sepultaria sumneriana*, *Sparassis* spp. *Pleurotus* spp., *Verpa conica* etc.) and consumed in off-season particularly during winter months during which

the availability of vegetables is scarce in the hilly inhabitations and movement of the local people is restricted due to snowfall and harsh weather.

Several mushrooms (*Rhizopogon* spp.) are eaten uncooked after cleaning and washing or after brief roasting on fire (*Boletus luridus*). Likewise, the morels are used in making several traditional recipes prominent amongst which are 'Chaschni' (a local dessert), 'Thunthoo Pullow' (rice + morels), 'Thunthoo Kheer' (milk +morels), and 'Thunthoo Yakhni' (curd + morels). Therefore, the study indicates that wild mushrooms play an important dietary role as they are considered as a substitute of 'meat', a key ingredient in vegetables, flavouring agent or even as a condiment. Like spices and sauces, they could transform a routine monotonous diet into a feast.

Descriptive vocabulary and folk taxonomy of wild edible mushrooms. Folk biological systems have been in practice throughout the world and they play an important role in local taxonomy. Research was carried out in various locations using four local dialects (Bhadarwahi, Kishtwari, Gaddaishi and Kashmiri). Young informants, usually under the age of 25 years were able to recognize on an average 33% of the mushroom species while middle aged people (usually below 50 years and above 25 years) were familiar with nearly 50-60% of mushrooms, while the people in older age categories could distinguish maximum of collected wild mushrooms locally. Overall, women recognized more species as compared to men. This slightly greater recognition of mushrooms may be attributed to their greater involvement in collecting, cleaning and cooking of these mushrooms.

In English vernacular ethnomycology, mushrooms have been grouped as agarics, morels, puffballs, earthstars, truffles, coral fungi and many more based on their general morphology. Consequently, an extensive descriptive vocabulary relating to mushroom morphology, growth and habit was recovered during the interviews. It was inferred that the descriptive vocabulary used in the area was found to be remarkably comparable to the macro morphological features used in scientific groupings of mushroom.

As many as 37 vernaculars indigenously used for 71 wild mushrooms are recorded and transcribed. Some of the species are monotypic having only single names while others are polytypic i.e. having more than one vernacular. These vernacular names could be categorized based on gross morphology and life forms into main seven types or folk genera. Most of these categories, inadvertently used though, referred to clearly defined biological groups such as 'Chaltee' or 'Sirer' for agarics; 'Bhutol', 'Bhutoo' or 'Dailoo' for boletes; 'Shairee', 'Gaub' or 'Gabur' for coral fungi. Similarly, puffballs are locally referred to as 'Dudh Katt'; earthstars as 'Sapp Nasvar'; morels as 'Thunthoo' and cup fungi are locally recognised by the folk genus 'Kundii'**.**

Several other miscellaneous vernaculars (folk taxa) based on varied attributes were also recorded during the survey. For instance, *Peziza vesiculosa* is recognized as 'Kann Kutch' which means ear like fungus in Kishtwari and Kashmiri. Similarly, *P. badia* is recognized as 'Chaber Kann' (meaning ear like fungus growing in marshy area) in Bhadarwahi. *Morchella conica* and *M. elata* is referred to as 'Paien Loj' meaning straight stick like morel in Kishtwari and Kashmiri whereas, *Morchella esculenta* and *M. rotunda* is fondly called as 'Batt Kutch' (mushroom consumed with rice) in Kishtwari and Kashmiri. *Geopora arenicola* and *Sepultaria sumneriana* is identified as 'Kundii' meaning mortar (made of hard stone) shaped in Bhadarwahi and Gaddaishi. *Boletus* spp. are called 'Dailoo' (the fungus that breaks easily into pieces) in Bhadarwahi or 'Bhutol' and 'Bhutoo' (edible after roasting on fire) in Bhadarwahi and Gaddaishi dialects respectively. *Pleurotus* spp. are named as 'Saroori' (meaning growing on

different host plants) in Kishtwari; 'Chur Sirer' (i.e growing on *Juglans regia*) in Kishtwari and Kashmiri languages.

However, a minor variation in the use of vernaculars in four different dialects (Bhadarwahi, Gaddaishi, Kishtwari and Kashmiri) was also observed. The vernacular 'Shairee' is commonly used for many species of *Clavaria*, *Clavulina,* and *Ramaria* in Bhadarwahi and Gaddaishi languages. Similarly, *Agaricus arvensis* is referred to as 'Chaitar' in Kishtwari and *Agaricus arvensis* and *Macrolepiota procera* together as 'Chaltee' in Bhadarwahi and Gaddaishi; *Boletus edulis* and *B. granulatus* as 'Bhutoo' in Bhadarwahi and Gaiddaishi; 'Dailoo' in Bhadarwahi; *Boletus luridus* as 'Bhutol' and 'Dailoo' in Bhadarwahi and 'Bhutoo' in Bhadarwahi and Gaddaishi dialects. Like wise, *Geopora arenicola* and *Sepultaria* sp. has various names in different dialects such as 'Kundii' in Bhadarwahi and Gaddaishi; 'Kutch' in Kishtwari; 'Gav Padur' or 'Khuduz' in Kashmiri and 'Kann Kutch' in Kishtwari and Kashmiri parlance. Species of *Helvella* are referred to as 'Thunthoo' in Bhadarwahi and Gaddaishi while *Rhizopogon* spp. are identified as 'Dudh Katt' and 'Moraii Dudh Katt' in Bhadarwahi and Gaddaishi and 'Matij' in Kishtwari and Kashmiri. Two species of *Sparassis viz*., *S. crispa* and *S. radicata* were referred to as 'Bedth Shairee' in Bhadarwahi and 'Rao Gaub' and 'Rao Gabur' in Kashmiri and Kishtwari jargon.

It is apparent from these results that extensive descriptive vocabulary and folk nomenclature for wild edible mushrooms is in practice in the area. Though a few researchers from other states of India have conducted similar kinds of studies yet the intensity of vernaculars usage was quite rare. Sagar *et al*. [22] made ethnobotanical survey in tribal district of Kinnaur of Himachal Pradesh and found the recognition of some epigeous gilled agarics on the basis of colour only. In addition, a few agarics like *Russula brevipes* were identified locally as 'Kaithno' or 'Kaithmuh'. Kamat [11] while working on ethnomycology of Goa recorded that local wild edible mushroom were commonly called as 'Olmi'; bolete species which sprout with forest showers as 'Bhuifod' (earth boil) or 'Fuge' (baloon mushroom) and termitophillic species were known as 'Roen Olmi' (termite hill mushroom). Many such significant contributions in the ethnomycological classification of the macrofungi world over have been reported recently [23-26]. Therefore, the study indicates the cultural importance and long traditional use of wild mushrooms in the studied area.

Market sale of edible mushrooms. Traditionally, fleshy fungi were being collected for home consumption and any commercialization was strictly at the local market except for *Morchella* spp. During survey, it was observed that wild edible fleshy fungi are usually available in the village shops or town markets for sale in monsoon season. Most of the edible species are sold in fresh form while others such as *Morchella* spp. and *Pleurotus* spp. *a*re put up for sale in both fresh and dried forms (Fig. 2 a-h & Fig. 3 a-e). Several economically downtrodden natives also vend these mushrooms to well-to-do families in exchange for goods such as used woollen clothes, rice, flour etc.

These species are marketed at different rates. *Agaricus arvensis, Clavaria vermicularis, Clavulina* spp. *Otidea leporina, Macrolepiota procera, Rhizopogon* spp., are sold @ rupees 20-30 per kg while *Coprinus comatus*, *Ramaria* spp., are available at rupees 30-40 per kg. Correspondingly, *Geopora arenicola*, *Pleurotus* spp., *Sepultaria sumneriana, Sparassis* spp. and *Termitomyces* spp. were sold at marginally higher price of rupees 40-50 per kilogram.

Many of these observations follow the earlier studies of Atri and Kaur [27] who observed that in Punjab and the adjoining border areas of Himachal Pradesh, the local people collected the

mushrooms in bulk and further sold these through their sale counters @ Rs. 60-80/kg. Harsh *et. al.* [8] observed *Termitomyces heimii* alone was sold by the tribals of Madhya Pradesh in 15 local markets during the season fetching a total price of Rs. 25,000 (approx.). Sharma *et al.* [28] made a socioeconomic study in the Amarkantek Plateau of Madhya Pradesh and found that the sale of edible fungi contributes about 2% to the annual income of tribal family signifying the role of macrofungi in alleviating poverty and serving as a vital ingredient of the nutrition supplements.

CONCLUSION

In conclusion, the present investigation stresses upon a great need for thorough, careful and comprehensive macrofungal forays for further collection of these important group of organisms existing in various locations of the state. The study also recommends regular surveys over an extended period in order to assess the patterns of abundance of mushrooms in different seasons. From such information, harvesting strategies and management plans can be formulated and implemented to ensure the lasting presence of these socially and economically important species.

In view of the increasing commercialization of the wild edible mushrooms, more studies on the ethnomycology of mushrooms in the State are called for. Further, it will be worthwhile to gather different views of the local populace about the value of mushrooms, which would pave a way for the introduction of some known wild edible mushrooms in the diet of rural population. In addition, introduction of simple and appropriate low cost technology for utilization and production of some of these socio-economically important indigenous species can be undertaken in the area. This becomes even more relevant when Food and Agricultural Organization has recommended the use of edible mushrooms as food supplement for protein deficient populations of developing and underdeveloped countries.

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