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FOREWORD

The World Society for Mushroom Biology and Mushroom Products was created for bringing together scientists with an interest in mushrooms from the standpoint of any subdivision of biology and persons having economics concerns with mushrooms and mushroom products in an open exchange of information of common interest. The International Conferences on Mushroom Biology and Mushroom Products achieve this objective and are opportunities to spread information to a large public.

For the 7th International Conference on Mushroom Biology and Mushroom Products, we decided to publish articles proposed by authors of posters, in addition to the articles from oral presentations. Our objective was to provide a platform for the poster contributors from all over the world, and offering them the possibility to have their research works known by many people through a free access electronic publication.

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DNA FINGERPRINTING OF GENETIC DIVERSITY OF *AGARICUS BISPORUS*

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ABSTRACT

In this study, 305 strains of *Agaricus bisporus*, including cultivars and wild strains, were studied by using the techniques of SRAP and ISSR fingerprinting, and 32 special SRAP bands, 28 special ISSR bands were obtained. These strains were clustered using the software NTSYSpc-2.02j based on the upper 60 special bands and a tree map was generated. Clustering results showed that the overall 305 strains can be divided into two major groups at the similarity coefficient value of 0.54.One group covered all of ARP strains, while another group included two main strains clusters, Chinese wild strains and cultivation strains. ARP strains were genetically distant from cultivars and Chinese wild strains. In cultivation strains group, the 206 cultivars can be divided into 7 sub-groups at the similarity coefficient of 0.9. While according to the agronomic characters of the strains, the 7 sub-groups belong to three groups: good-quality hybridized strains, good-quality non-hybridized strains and high-production strains. In Chinese wild strains group, 41 wild strains can be divided into three sub groups, which were geographically correlated, at the similarity coefficient value of 0.93. In this study, 120 strains in overall 305 strains can be identified through 60 special bands, which indicate the genetic diversity of germplasm resources of *A. bisporus*.

Keywords: *Agaricus bisporus*, SRAP, ISSR, cluster analysis

INTRODUCTION

Agaricus bisporus (J.E.Lange) Imbach is the most widely cultivated and consumed edible fungus in the world with important economic value. The cultivation of *A. bisporus* began in France 300 years ago, and its spawn purification, preparation and strain improvement has a history spanning over 100 years.

In about 1925, *A. bisporus* was introduced into China for cultivation. In 1978, the strain improvement started in China, and the technology of compost 2-phases fermentation was introduced into China by Prof. S. T. Chang, which greatly promoted the development of Chinese

button mushroom industry. In 1989, the hybrid strain As2796, which combined the characters of both high production and good quality, was bred by Fujian Mushroom R&D Station.

Since 1999, China had become the biggest country for *A. bisporus* production. The hybrid As2796 covered the largest cultivation area in China, and became one of the commercial strains producing largest annual yield of fresh button mushroom in the world. [1, 2, 3]

In 2007, Fujian Mushroom R&D Station was reorganized as one of the institutes of Fujian Academy of Agricultural Sciences, and established a germplasm resources library of *A. bisporus*, which preserved 435 cultivation or wild strains. It has become one of the most abundant germplasm resources conservation units of *A. bisporus* in the world.

SRAP (sequence-related amplified polymorphism) technology is a new type of molecular marker developed by Li *et al.* [4]. This molecular marker amplified by the primers which designed according to the open reading frames (ORFs) and can generate polymorphic amplification products owing to different individuals, intron numbers and length between promoter and intergenic regions. It has the advantage of simple, stable, well repeatability and is a very effective tool in genetic diversity analysis, cultivar identification and phylogenetic studies [5, 6, 7, 8, 9]

ISSR(Inter-Simple Sequence Repeat) is a kind of DNA markers developed based on microsatellites sequence [10] .It semi-randomly amplifies the area between SSR(Simple Sequence Repeat) employing artificially designed simple repeat nucleotide sequence as primers , and produces rich polymorphism. It has been used in a variety of economic crops on studying comparative genomics, genetic mapping, genetic diversity analysis, germplasm identification, and genetic variation analysis or phylogenetic and so on [10, 11, 12, 13, 14, 15, 8, 9].

In this study, two DNA molecular markers, SRAP and ISSR, were used to analysis the DNA fingerprint of 305 strains including the cultivation and wild strains of *A. bisporus* collected from around the world, in order to obtain their genetic relationship, and select a number of specific bands to provide the basis of DNA level for the identification of these strains, further more, selection of hybrid parents.

MATERIALS AND METHODS

Strains. 206 cultivation strains were collected from all over the world. 41 wild strains were collected from some places of Tibet, Xinjiang, Ningxia, Sichuan, Qinghai, Gansu province and so on in which no *A. bisporus* was cultivated and a good ecological environment was maintained. These strains have been preliminarily identified as wild *A. bisporus* by isozymes electrophoresis. 58 ARP strains were collected and shared by *Agaricus Resource Program* [16]. All the strains above were provided and preserved by the Edible Fungi Institute of Fujian Academy of Agricultural Sciences, P. R. China.

Reagents. PCR kits and other reagents were purchased from Shanghai Biological Engineering Service Co., Ltd. of China

SRAP and ISSR. Four hundred SRAP primer [Invitrogen Biotech (Shanghai) Co., China] combinations were screened in three representative samples from the 305 accessions. Under the

optimized condition, 3 primer combinations, generated strong and clear amplified bands, were selected for further investigations (Table 1). The SRAP reaction mixtures (total volume 20 μL) contained 60 ng DNA, 1×PCR buffer, 0.2mM dNTPs, 2mM $MgCl_2$, 1 unit Taq DNA polymerase and 0.4 mM of both forward and reverse primers. SRAP programmes involved an initial denaturation step of 5 min at 94℃, followed by 5 cycles of 94℃/1 min, 35 ℃/1 min, 72℃/1.5 min, 35 cycles of 94℃/1 min, 50℃/1 min, 72℃/1.5 min, and a final extension at 72℃/10 min.

A total of 80 ISSR primers [Invitrogen Biotech (Shanghai) Co., China] were screened initially with three representative samples. 2 primers that produced clear and reproducible fragments were selected for further analyses (Table 2). The ISSR reaction mixture (total volume 20 μL) contained 60 ng DNA, 1×PCR buffer, 0.5 mM primers, 0.2 mM dNTPs, 2mM $MgCl_2$, and 1 unit Taq DNA polymerase. The thermal cycling profile was: 94℃/5 min, followed by 45 cycles of 94℃/45s, 55℃/40s and 72℃/70s, and a final extension step at 72℃/10 min.

Table 1: SRAP primers combination and their sequences

me1- em2	me1: 5-TGAGTCCAAACCGGATA-3, em2:5-GACTGCGTACGAATTTGC-3
me2- em4	me2:5-TGAGTCCAAACCGGAGC-3, em4:5-GACTGCGTACGAATTTGA-3
me5- em10	me5: 5-TGAGTCCAAACCGGAAG-3, em10:5-GACTGCGTACGAATTTAG-3

Table 2: ISSR primers and their sequences

808	5-AGAGAGAGAGAGAGAGC-3
809	5-AGAGAGAGAGAGAGAGG-3

Method of mycelium cultivation, genome DNA extraction and SRAP and ISSR analysis. The method of mycelium cultivation, genome DNA extraction and SRAP and ISSR analysis were the same to the references [8].

RESULTS AND DISCUSSION

DNA fingerprinting analysis of 305 strains. Primer screen of SRAP and ISSR was carried out as described above, and 32 polymorphic bands of SRAP and 28 polymorphic bands of ISSR were got. Figure 1 to 5 shows part of SRAP and ISSR amplification patterns.

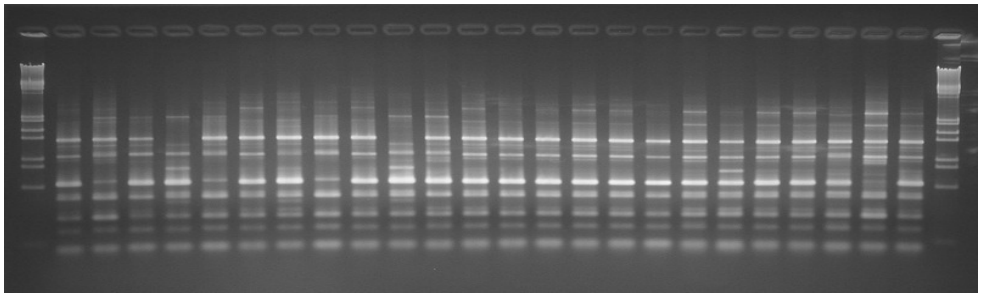

Figure 1: The SRAP patterns of 24 strains of *A. bisporus* (primers: me1-em2)
Note: Line M is Lambda DNA/EcoRI+HindIII Markers（Similar here in after）.

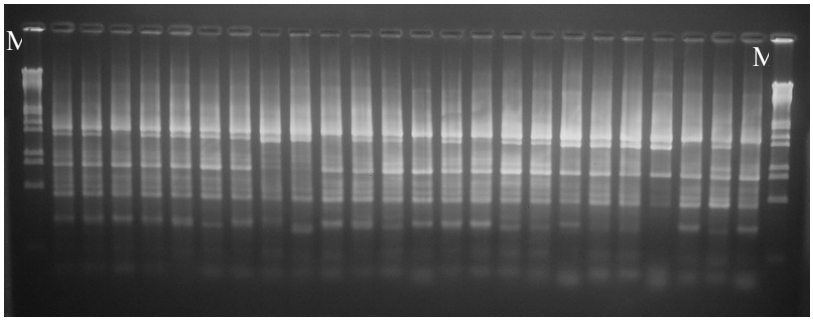

Figure 2: The SRAP patterns of 24 strains of *A. bisporus* (primers: me2-em4)

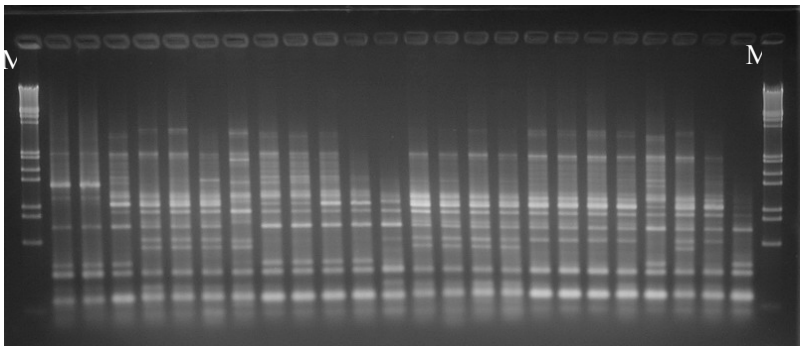

Figure 3: The SRAP patterns of 24 strains of *A. bisporus* (primers: me5-em10)

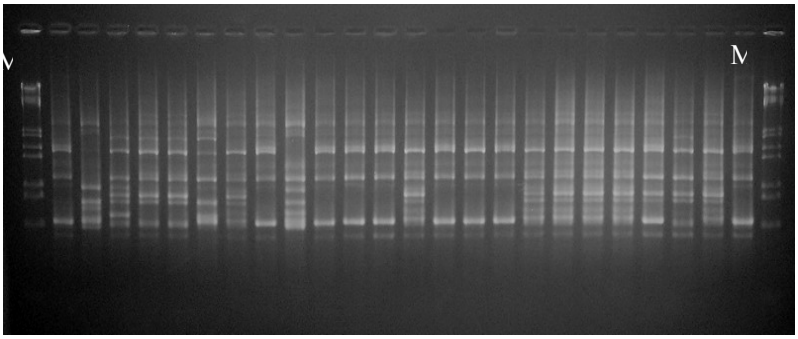

Figure 4: The ISSR patterns of 24 strains of *A. bisporus* (primer: 808)

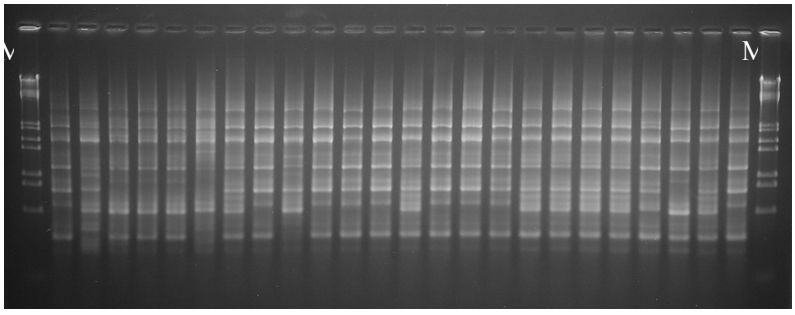

Figure 5: The ISSR patterns of 24 strains of *A. bisporus* (primer: 809)

Cluster analysis of 305 strains. Sixty specific bands obtained from SRAP and ISSR were converted into a (1, 0) data sheet (PCR positive recorded as "1", negative recorded as "0"), and clustered using NTSYSpc-2.02j software. Genetic relationship tree of overall 305 strains was

showed in Fig.6. Data showed that at the similarity coefficient of 0.54, these strains were classified into two major groups, one of which covered all of ARP strains, and another included Chinese wild strains and cultivation strains. The ARP strains completely separated from others, and were genetically distant from cultivars and Chinese wild strains. In cultivation strains group, the 206 cultivars can be divided into 7 sub-groups at the similarity coefficient of 0.9. While according to the agronomic characters of the strains, the 7 sub-groups belong to three groups: good-quality hybridized strains, good-quality non-hybridized strains and high-production strains. In Chinese wild strains group, 41 wild strains can be divided into three sub-groups, which were geographically correlated, at the similarity coefficient value of 0.93.

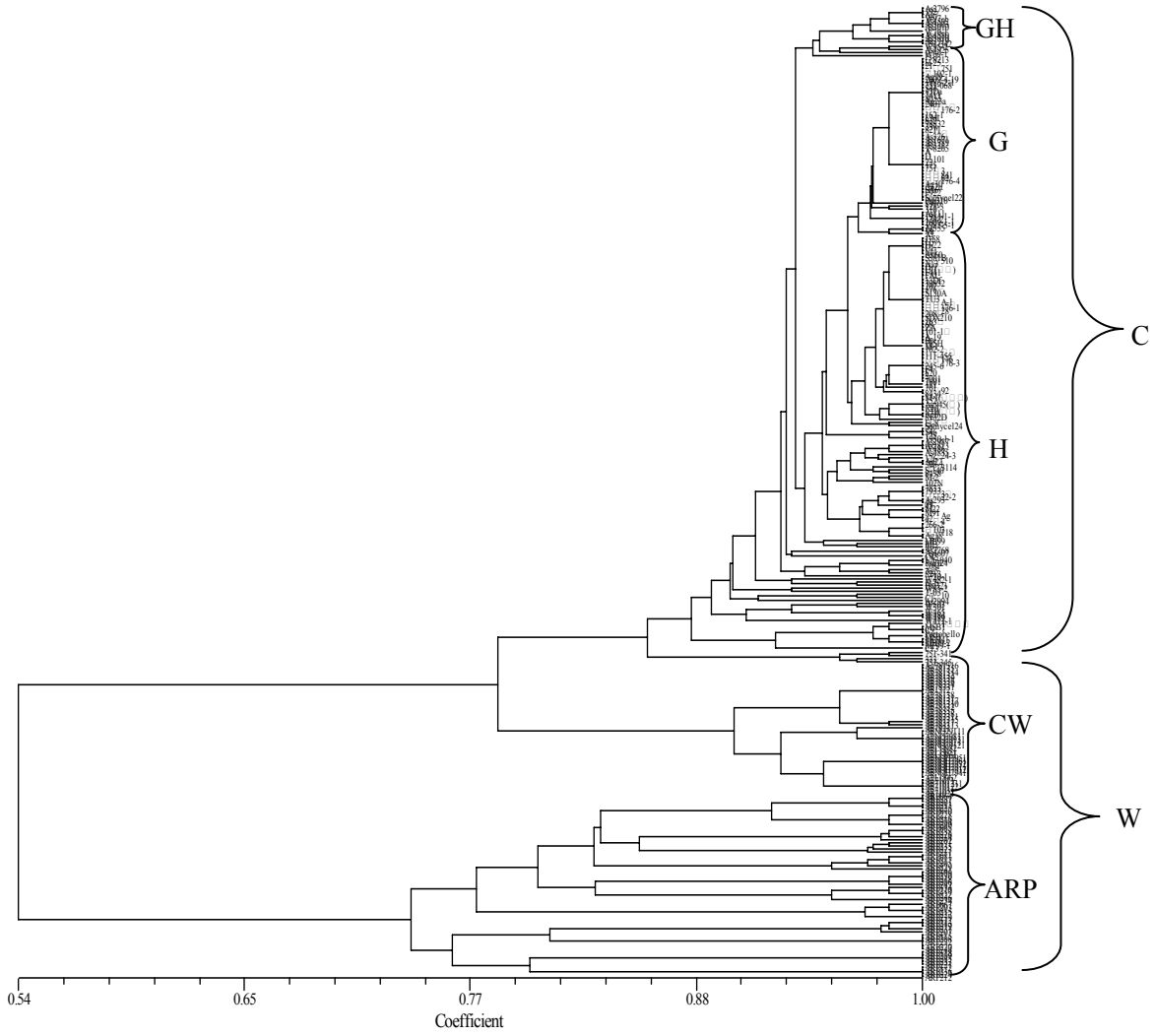

Figure 6: Clustering dendrogram of 305 strains of *A. bisporus* (W: Wild group, C: Cultivation group, H: High-production group, G: Good-quality non-hybridized group, GH: Good-quality hybridized group, CW: Chinese wild strains, ARP: ARP strains)

DISCUSSION

So far, rich germplasm resources of *A. bisporus* have been found. Since 1990s, many scientists worldwide carried out extensive research on the populations of *A. bisporus* about its distribution, behavior, community structure, gene flow dynamics and genetic variation [17, 18, 19, 20, 21, 22, 23, 24, 25]. *A. bisporus* germplasm resources were discovered one after another in Europe, America, Australia, Africa and Asia. In North America, Kerrigan had collected approximately 200 wild strains of *A. bisporus* through hosting ARP (*Agaricus Resource Program*) since 1988[16]. In Europe, a mushroom research group in France led by Callac, had collected about 250 wild strains of *A. bisporus* since 1990s [26]. In the UK, Elliott and Noble collected a lot of wild *A. bisporus* strains and also many strains of *Agaricus*. In China ,Wang *et al.* began to gather *A. bisporus* germplasm resources from all over the world since 1983[3], and had collected more than 400 strains of *A. bisporus* including 206 cultivars, 168 foreign wild strains and 61Chinese wild strains.

Genetic relationship research on *A. bisporus* has directive significance on strain identification, evaluation and breeding. Chen *et al*. carried out research on the molecular phylogenetic relationships about cultivars and wild strains respectively [8, 9]. In this article, 305 strains including cultivated and wild strains were studied together to explore their genetic relationship, and provide a scientific basis for the strain identification, evaluation and hybrid breeding parents selection. Clustering result showed that the genetic distance of wild and cultivated strains was large. Compared to cultivated strains, the PCR amplification patterns of wild strains (including Chinese wild strains and ARP strains) were more unique. In addition, 41 Chinese wild strains and 58 ARP strains clustered into individual group respectively, and separated from the cultivars. This may be caused by different source of strains. Wild strains tested are collected from China or America, while the cultivated strains were belong to European germplasm or their hybrids. The research also suggested that the populations of Chinese regional were genetically distant from European and American regional strains, which indicated that China has abundant germplasm resources of *A. bisporus* .These results were validated by isozyme electrophoresis identification (unpublished). So, isozyme electrophoresis identification is a feasible method in *A. bisporus* identification, and isozyme and DNA markers can be mutually confirmed in the identification of genetic relationships of *A. bisporus*. For the 206 cultivars, the strains with similar agronomic characters are mostly clustered together, indicating that the strains with similar agronomic properties also have relatively close genetic relationships.

CONCLUSION

Evaluation of germplasm of *A. bisporus* is very important for breeding. In this study, we used effective DNA fingerprinting tools to study the representative strains in germplasm resources library of *A. bisporus*. Results indicated that genetic distance of wild and cultivated strains was large. The genetic variation within ARP strains was in sharp contrast with the uniformity within the cultivars. This was consistent with previous studies [24, 27]. For wild strains (including Chinese wild strains and ARP strains), some of them has been confirmed that have a number of desirable traits not found in the present-day hybrids, i.e. temperature tolerance and disease resistance [28, 3]. So, full use of wild germplasm will be the key to breed new strains. For strain identification, through 5 primer pairs (combination), 60 specific markers screened in this paper,

120 species in 305 can be distinguished individually, while other strains were unable to be separated each other. This may be owing to their close genetic relationship, and more new markers or methods should be needed to identify them.

ACKNOWLEDGEMENT

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AGARICUS BISPORUS CULTIVARS: HIDDEN DIVERSITY BEYOND APPARENT UNIFORMITY?

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ABSTRACT

Agaricus bisporus, commonly known as the button mushroom, is the most widely cultivated species of edible fungi. The cultivars used by growers over the world are suspected to come from the same restricted pool of strains, and the genetic base of all the present day hybrids is very narrow. The aim of this study was to assess the genetic variability among traditional and modern commercially used *A. bisporus* strains. Fourteen codominant microsatellite markers (*AbSSR*) were used to characterize 75 cultivated genotypes from European spawn makers, maintained in the Collection of *Agaricus* in Bordeaux (CGAB) since 1990. To our knowledge, it is the most extensive sample ever studied. Seven main groups were identified which corresponded to the six ancestral lineages and the hybrids belonging to either U1 or U3 sub-group of strains. Thirty-three U1-like cultivars could not be differentiated. Very few strains have a distinct and typical SSRs pattern. Based on our results, we proposed also a cultivar identification key with a limited number of markers in order to optimize forthcoming SSRs fingerprinting. For three hybrids that seemed to be genetically identical to Horst-U1 at heterokaryotic level, the analysis of each constituting nuclei has demonstrated allelic rearrangement, suggesting essentially derived varieties. The efficiency of microsatellite markers and implications of these results for germplasm management, breeding strategy and variety identification are discussed.

Keywords: genetic diversity; cultivar identification, strain protection, essentially derived varieties

INTRODUCTION

Agaricus bisporus is the most widely cultivated species of edible mushrooms. However, despite its economical importance, few efforts have been done in terms of breeding and strain improvement. As a result, all currently grown cultivars are assumed to be related to a limited number of traditional genotypes [1] and *A. bisporus* appears to be nearly a monolineage crop. Several studies using various molecular markers have demonstrated that most of the current hybrids are either identical of very similar to the first hybrids U1 and U3 released in the 80's [2, 3, 4]. Microsatellites that stand for being one of the most valuable molecular markers for genetic studies have been recently developed for *A. bisporus* [5]. We have already demonstrated that these SSR markers are useful tools for analysing intraspecific genetic variation, but the characterisation of a large cultivar set with such markers has not been done yet. The collection of Germplasm of Agaricus in Bordeaux (CGAB) encompasses at present time near 1000 strains among which we can find cultivars and mainly wild strains from various origins [6]. To improve

germplasm management, the molecular characterisation of this collection is indispensable but raises the question of duplicates. Indeed, due to the typical life cycle and the meiotic behaviour of *A. bisporus* (see the review of Sonnenberg et al. in the present issue [7]) it is impossible, at the molecular level, to distinguish vegetative copies or post-meiotic heterokaryotic mycelia.

The objective of this study was to use 14 *AbSSR* loci to evaluate the genetic diversity in 75 strains representing traditional and modern commercially cultivars. To our knowledge, this is the most extensive sample of *A. bisporus* cultivars for such analyses. Beyond the characterisation of the cultivars diversity, we have also analysed each constituting nuclei of three hybrids that seemed to be genetically identical to U1 at heterokaryotic level. By this way, we could distinguish copies from essentially derived varieties. Consequences for germplasm management, breeding strategy and strain protection will be discussed.

MATERIALS and METHODS

Fungal strains. Seventy-five fungal cultivar strains originated from various European spawn makers were analysed. Eight of them (Bs002, Bs003, Bs034, Bs061, Bs063, Bs089, U1, U3) have been already used for genetic analysis in a previous study [5] and were included in the present sampling as controls. The detailed listing of the 75 strains and their origin is available upon request.

Six homokaryons were obtained from 3 U1-like heterokaryotic strains (Bs508, Bs516, Bs681) by the protoplast method of deheterokaryotisation described in Kerrigan et al. [8].

All fungal strains are maintained in the "Collection du Germplasm des Agarics à Bordeaux" (CGAB) [6].

SSR genotyping. Total DNA was extracted from freeze dried mycelium with a classical CTAB-chloroform-isoamyl alcohol protocol. In routine use, DNA concentration was adjusted to 25ng/µl.

Microsatellite studies were based on markers previously developed in our lab [5]. The 14 SSR loci used here for genotyping 75 cultivars were chosen on the basis of their unambiguous allele scoring, their level of polymorphism revealed in the six morphotype lineages and their multiplex compatibility. Thus, 3 sets of combined SSR loci were developed according to their expected allele size range and/or fluorescent primer labels (Table 1). Repeat units, primer sequences, fluorescently dye labelling, amplification conditions, have been previously described [5, 9]. Capillary electrophoresis and fragment size determination were performed on a CEQTM 8000 Genetic Analysis System Sequencer (Beckman Coulter). The analysis of possible allelic rearrangement in the 6 homokaryotic strains was performed with 8 microsatellite markers known for being heterozygous in the U1 strain. The haplotypes of the two nuclei U1-2 (equivalent to H97) and U1-7 (equivalent to H93) were taken as reference.

Data analysis. SSR data were scored as several alleles per locus distinguished by their size. For each SSR locus, we computed with PowerMarker 3.25 [10] several genetic diversity parameters as the number of alleles per locus (*N*), the number of genotypes (*G*), the observed heterozygosity (*Ho*), the polymorphic information content (*PIC*) and the power of discrimination ($PD=1-\Sigma g_i^2$ where g_i is the frequency of the *i*th genotype). The probability PI that two distinct strains share by chance the same genetic profile was computed using Identity 4.0 (Centre of Applied Genetics, University of Agricultural Sciences, Vienna).

Genetic relationship between cultivars was assessed with the unweighted pair-group method analysis (UPGMA) using genetic distance based on the proportion of shared alleles as $DAS=1-(\Sigma S)/2u$ where *S* is the number of shared alleles and *u* the number of loci analysed.

RESULTS AND DISCUSSION

SSR informativeness. An illustration of genotyping output was shown on Figure 1. The 14 SSR markers used in the present study amplified 44 alleles, ranging in size from 107 to 332 bp. The number of alleles per locus ranged from 2 to 5 with an average of 3.14 alleles (Table 1). Allele frequencies ranged from 0.02 (*AbSSR06*-allele 196 bp) to 0.92 (*AbSSR64*-allele 107 bp) with a mean value of 0.3. The most and the least informative locus were *AbSSR36* (*PD* =0.83) and *AbSSR49* (*PD*=0.17) respectively. The average value of PD parameter over all loci reached 0.53. Several variability parameters estimated in the present study were lower than those previously reported [5]. This result was not surprising due to our sample restricted to cultivated strains. Indeed, the level of polymorphism among cultivars was described lower compared to wild strains and near 43% of *AbSSR* alleles were never found in cultivars [5].

Table 1. Locus name, multiplex and genetic parameters of the 14 SSR markers estimated in the 75 cultivar strains sample.

AbSSR locus*	Multiplex set	*N*	allele size range	*G*	*Ho*	*PIC*	*PD*	*PI*
AbSSR05	3	2	329-332	2	0.93	0.37	0.40	0.40
AbSSR06	3	3	181-202	4	0.97	0.40	0.45	0.27
AbSSR23	2	2	174-180	2	0.73	0.36	0.50	0.48
AbSSR31	2	3	160-170	3	0.00	0.20	0.27	0.55
AbSSR33	2	2	190-193	2	0.00	0.25	0.26	0.58
AbSSR36	1	5	148-161	8	0.81	0.69	0.83	0.13
AbSSR39	1	3	173-192	4	0.17	0.34	0.57	0.38
AbSSR42	1	4	160-189	6	0.66	0.50	0.79	0.19
AbSSR45	1	5	196-206	7	0.97	0.63	0.82	0.15
AbSSR49	3	2	176-184	2	0.00	0.17	0.17	0.72
AbSSR57	2	4	250-262	8	0.16	0.49	0.79	0.17
AbSSR60	3	3	206-210	4	0.83	0.57	0.34	0.29
AbSSR64	2	2	107-113	3	0.12	0.13	0.52	0.48
AbSSR65	3	4	190-215	4	0.88	0.59	0.71	0.20

* color of locus name referred to fluorescent dye (black=D2, green=D3, blue=D4)

***Agaricus bisporus* cultivars identification.** The combination of the allelic pattern obtained with the 14 SSR loci made possible to identify 13 distinct genotypes among the 75 *A. bisporus* cultivated strains analysed (Fig. 2). Thirty-three cultivars could not be differentiated and showed the same genetic profile as U1/U3 hybrid strains. Using a mitochondrial marker (data not shown), these 33 genotypes could be separated into two subgroups that correspond to either U1 or U3 as expected [11]. The small genetic difference, found at a single allele between the group of two strains (Bs005 together with Bs040) and the U1-like group, needed to be further investigated and confirmed with other markers. We could not exclude that minor differences can be due to somatic mutations that may occur in vegetative propagation system such as mycelium maintenance. Comparison of morphological and agronomical data could clarify this slight variability. Our results confirmed that most of the present day hybrids were apparently identical or closely similar to the first hybrids [3].

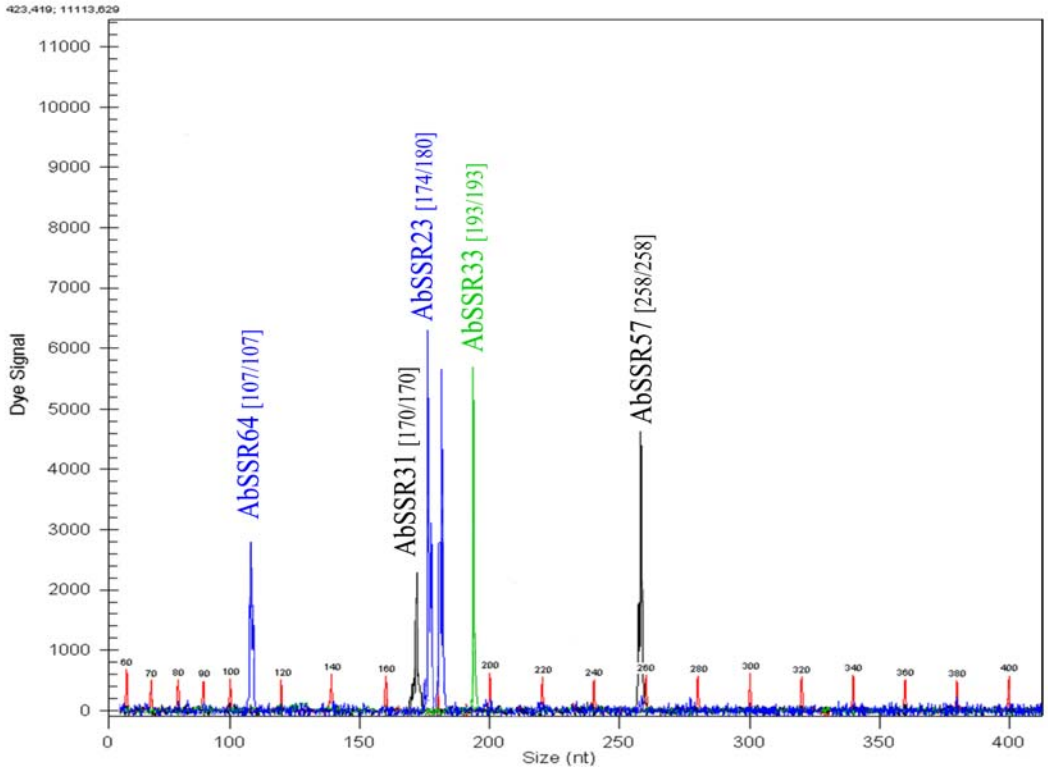

Figure 1. Amplification fragment pattern of five microsatellites (multiplex set n°2) for U1 strain (output from CEQ8000 Genetic Analysis System)

Among the 13 diplotypes, we found the six genotypes that correspond to the six morphotype lineages already described, it is to say "off white", "small white", "white", "brown", "small brown", and "golden white" [5]. However, the off white-like strains were separated into two closely related subgroups. Two strains (Bs022 and Bs668) were singled out. The present data suggested that these two strains were likely to be derived from a cross breeding scheme but it was not possible to clearly set up their pedigrees. Distance values between the 13 diplotypes ranged from 0.04 to 0.78 with an average value of 0.42. The heterozygosity levels were variable among cultivars, ranging from 7.7% (Bs006) to 57.1 % (U1-like hybrids), with an average of 42.2%.

Efficient combination of SSR markers as molecular identification key of *A. bisporus* cultivars. Considering the 14 markers, the probability that two distinct diplotypes share by chance the same genotype was estimated to be 8.3×10^{-8}. To optimize SSR fingerprinting, we would like to determine the combination(s) of *AbSSR* markers that could be sufficient to discriminate each diplotype. For some subgroups, it was possible to establish a molecular identification key by identifying specific *AbSSR* alleles. For instance, *AbSSR*36-148 bp allele was found only in small white-like strains. The signature of small-brown cultivars was the *AbSSR*39-173 pb allele (Table 2). The U1-like hybrids were characterized by specific heterozygous profiles at the two loci *AbSSR*36 (152/160) and *AbSSR*42 (179/181). The use of three selected markers (*AbSSR*36-*AbSSR*42-*AbSSR*45) appeared to be sufficient to distinguish the 13 distinct genotypes. Using this combination, we expected a probability of identity of 3.7×10^{-3} to find, by chance, two strains that showed the same profile.

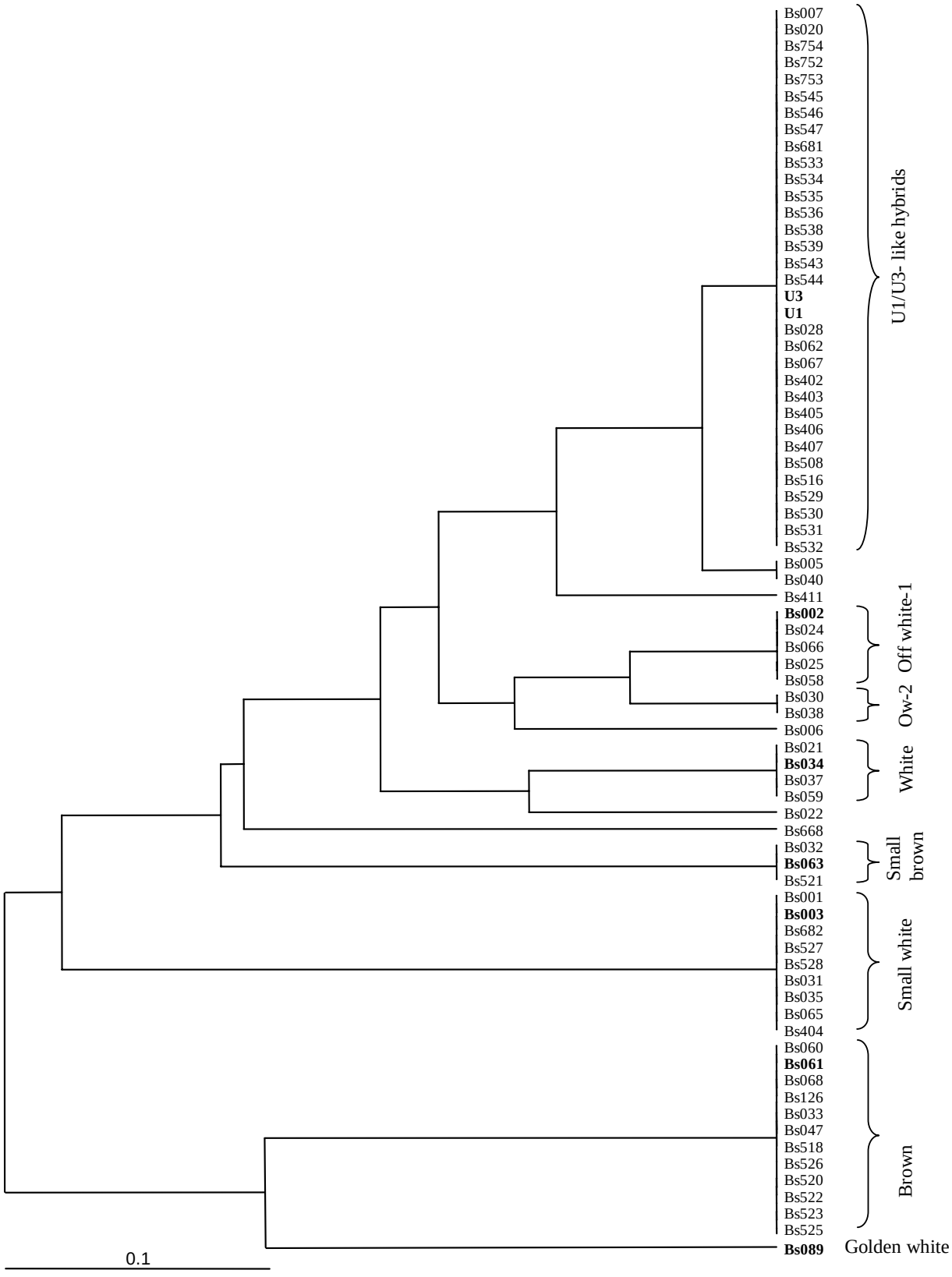

Figure 2. UPGMA dendrogram of the 75 cultivars of *Agaricus bisporus* based on distance of shared alleles estimated with 14 SSR loci. Cultivar lineage morphotypes already described in Foulongne-Oriol et al. [5] are in bold characters.

Table 2. Allelic profile at the 14 *AbSSR* loci for the traditional lineages and the U1-like hybrid.

Lineage	reference strain	AbSSR05	AbSSR06	AbSSR23	AbSSR31	AbSSR33	AbSSR36	AbSSR39	AbSSR42	AbSSR45	AbSSR49	AbSSR57	AbSSR60	AbSSR64	AbSSR65
white	Bs034	329/332	181/202	180/180	170/170	193/193	160/160	188/188	179/179	198/206	176/176	252/258	208/210	107/107	190/215
small white	Bs003	329/332	181/202	180/180	167/167	193/193	148/156	188/192	179/179	196/202	184/184	250/250	208/210	107/107	199/213
off-white-1	Bs002	329/332	181/202	174/180	170/170	193/193	152/152	188/188	181/181	196/198	176/176	258/258	210/210	107/113	190/190
off-white-2	Bs030	329/332	181/202	174/180	170/170	193/193	152/161	188/188	181/181	196/198	176/176	258/262	210/210	107/113	190/213
small brown	Bs063	329/329	196/202	180/180	170/170	193/193	156/156	173/192	179/189	196/206	176/176	256/258	210/210	107/107	190/213
brown	Bs061	329/332	181/202	174/180	170/170	190/190	156/161	192/192	160/179	196/200	176/176	252/252	206/210	107/107	190/213
golden white	Bs089	329/332	181/181	180/180	170/170	190/190	160/160	192/192	160/160	198/206	176/176	252/262	208/208	107/107	190/213
U1-like hybrid	U1/U3	329/332	181/202	174/180	170/170	193/193	152/160	188/188	179/181	196/206	176/176	258/258	208/210	107/107	190/215

Allelic rearrangement analysis. For the 3 U1-like strains, the analysis of the homocaryotic nuclei showed a re-assortment of alleles by comparison with the U1-7 and U1-2 nuclei. We observed 2, 3 and 4 allelic differences over the 8 examined loci for Bs516, Bs681 and Bs508 respectively. Furthermore, although these 3 strains are identical at heterokaryotic level, the allelic composition of their nuclei is different (Fig. 3). That indicates an independent assortment of homologous chromosomes in meiosis I and a pairing of non-sister nuclei in meiosis II. No crossing over was observed between loci shared by the same linkage group (*AbSSR*05-*AbSSR*06 on LG VI, *AbSSR*45-*AbSSR*60 on LG VII). Our results demonstrated that these 3 strains apparently genetically identical to U1 were likely to be issued from single spore isolates of U1. These results confirmed those reported by Sonnenberg et al. [7].

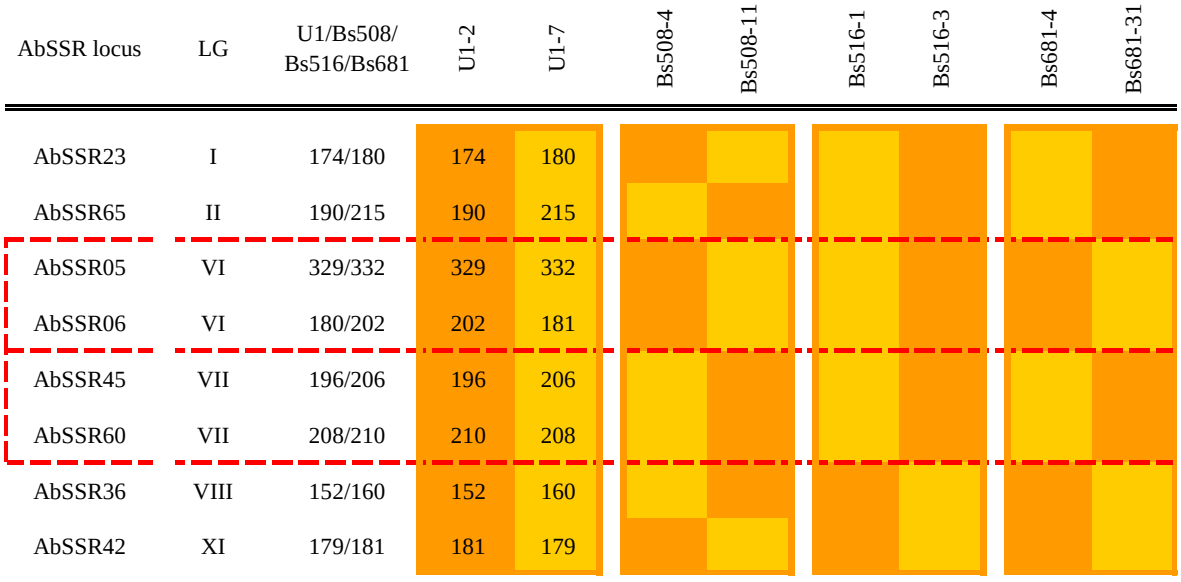

Figure 3. Genotype of homocaryotic nuclei of 3 U1-like strains at 8 *AbSSR* loci and comparison with U1-2 (dark orange) and U1-7 (light orange) nuclei taken as references. Red dotted line signifies genetically linked markers on a same chromosome.

The redistribution of chromosomes has been demonstrated to induce some phenotypic variability [12] and this could be favourable in breeding programs. Thus, the cultivars identical to U1 at the heterozygous level could present advantageous differences for phenotypic traits. Thus, phenotypic characterisation appeared to be inseparable from molecular one to optimize mushroom collection management. Consequently, in parallel to genotypic data, most of the strains in collection were also phenotyped for various agronomic traits (data not shown).

CONCLUSIONS

The SSR markers previously developed are confirmed to be powerful for genetic diversity and cultivar identification in *A. bisporus*. The molecular identification key we propose, particularly the lineage signature, may serve as an efficient tool for the *A. bisporus* community. Multiplex *AbSSR* set coupled with fluorescence-based automated detection systems make also possible the optimisation of this molecular identification key in routine use.

The narrow genetic base of the button mushroom cultivars is confirmed with the identification of six lineages that constitute the parentage of most of the available commercial strains. With this crop uniformity, the mushroom culture would be more vulnerable to pests and environmental stresses. Thus, the exploitation of wild genetic resources to broaden genetic variability is promising to develop new varieties with resistance to diseases or adaptation to climate changes [5, 6].

The homogeneity found within the actual commercial strains is in agreement with other studies but our results also demonstrate that a hidden diversity exist beyond the apparent uniformity. Several consequences should be considered. First, as it appears that the deheterokaryotisation of each strain in collection is unlikely, the molecular characterisation appeared to be needed but not sufficient to maximize the conserved diversity. It can not replace and avoid the phenotypic data, and thus, these two approaches are clearly complementary to optimize germplasm management. Secondly, it raises the question of strain protection and the definition of essentially derived varieties in this economical species, as underlined by Sonnenberg et al. [7].

Our results provide also a sound basis to manage breeding programs. The genetic data collected during this work will guide the choice of parental genotypes to cross according to their lineage belonging or their level of heterozygosity. Subsequent breeding scheme will also be facilitated through potential marker assisted selection.

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QTL FOR RESISTANCE TO *TRICHODERMA* LYTIC ENZYMES AND METABOLITES IN *AGARICUS BISPORUS.*

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ABSTRACT

Trichoderma aggressivum leads to severe crop losses in *Agaricus bisporus* cultures. The development of strain resistant to this competitor is an alternative to the use of chemicals. One of the interacting components of the putative system of resistance is the lack of susceptibility to the growth limiting compounds produced by *Trichoderma* sp. Wide variation for this trait has been previously demonstrated, with few strains able to resist to *Trichoderma* lytic enzymes and metabolites. For exploiting such a resistance in breeding programs, the knowledge of its genetic basis is a prerequisite. Therefore, QTL analysis was used to determine the number, effects and location of genomic regions associated with tolerance to *Trichoderma* lytic enzymes and metabolites in a hybrid progeny of *A. bisporus.* An *in vitro* experiment using sequential cultures on media supplemented or not with a commercial product Lysing Enzyme® was used. The mycelium growth rate in control condition, the level of tolerance and the capacity of adaptation were the traits used for QTL detection. In total for all the traits, seven QTLs were detected distributed on four genomic regions. Two clusters of QTLs related to several traits and two other trait-specific QTLs were identified. A genomic region on LGIV was detected for each trait, with the highest LOD score value and genetic effects. Our results showed that tolerance to *Trichoderma* lytic enzymes and metabolites was tightly related to mycelium growth ability. Consequences for mushroom breeding program are discussed.

Keywords: competitor, green mould disease, adaptation, fitness, breeding

INTRODUCTION

The fungal competitor *Trichoderma aggressivum* is the cause of the green mould disease in the cultivated button mushroom *Agaricus bisporus*. It develops a dense hyphal network in mushroom compost or casing materials, followed by sporulation and an almost complete lack of mushroom production. The *T. aggressivum* species compete for space and nutrients and are adapted for growth in *Agaricus* mushroom compost by resisting the inhibiting effects of bacteria in this cultivation substrate [1]. The North American *Trichoderma aggressivum* f. *aggressivum*, produces antifungal compounds in mushroom compost that inhibit mycelial growth of the commercial mushroom *A. bisporus* which results in drastic crop losses [2]. This threat affects an ever larger part of producing areas all over the world and becomes a major disorder in commercial mushroom production [3]. Prophylactic management and disinfectant treatments may limit the epidemic but the severe level of chemical residue in food product together with the increasing risk of fungicide resistance led us to consider the cultivation of resistant *A. bisporus* varieties as a promising alternative.

Little is known about the resistance to *T. aggressivum* and its mechanism in *A. bisporus*. Although brown line formation and laccases production were described when *Lentinula edodes*

or *Pleurotus* species are confronted to *T. aggressivum*, no such defense reactions of *A. bisporus* cultivars to *T. aggressivum* attack have been observed [4, 5, 6]. Wide variations within *A. bisporus* strains in response to substrate infestation by *T. aggressivum* have been demonstrated. A few strains appeared slightly susceptible but no absolute resistance was observed [7, 8].

The evaluation of green mould resistance appeared to be quite difficult and mostly influenced by the environment [1]. Many components are involved in the interaction between *A. bisporus* and *T. aggressivum*. Some of them are due to abilities of the mushroom strains to counteract growth of moulds by producing fungistatic compounds and to resist to growth limiting compounds (enzymes, volatile compounds, small non-volatile molecules) produced by *Trichoderma* sp. In a previous study, an *in vitro* test using as media supplement a commercial product (Lysing Enzyme®) containing cell wall lytic enzymes and undefined metabolites allowed to identify strains of *A. bisporus* able to resist to this growth limitation [9]. For exploiting such abilities as a component of resistance in breeding programs, the knowledge of its genetic basis is a prerequisite.

The objective of the present work was to investigate the genetic control of the resistance to *Trichoderma* lytic enzymes and metabolites in an *A. bisporus* progeny using QTL analysis. Progresses in the understanding of this particular fungal-fungal interaction and prospects for mushroom breeding are discussed.

MATERIALS and METHODS

Offspring. The population used in our study consisted of 103 second generation hybrids obtained by crossing the homokaryotic offspring (H_i) of the hybrid JB3-83 x U1-7 (H) with the homokaryon U1-2 [10, 11, 12]. The strains JB3, U1, and the first generation hybrid H were used as controls in the experiments.

Experimental design and phenotypic evaluation. The experiment was performed according to a methodology adapted from Savoie et al. [9] and described in Fig. 1.

Cristomalt (1%) and agar (1.5%) was used as the basic media. Lysing Enzyme® (LE) from *T. harzianum* (Sigma, L1412) is a lyophilized powder obtained from cultures of *T. harzianum*, containing about 80% protein, with cellulase, chitinase and protease activities. Supplementation of Cristomalt Agar with LE was made by diluting the product in water (0.75 g in 10 mL), sterilizing by filtration (0.22 µm), and adding this solution to 1 L of autoclaved media. Un-supplemented controls contained 10 mL of sterile water.

For each strain of *A. bisporus*, a mycelium agar plug of pre-culture (5 mm-∅) was placed in the centre of Petri dishes containing malt-agar with or without LE supplementation. The inoculated media were incubated at 25 °C in the dark for 14 days and at the end of this period, the diameters of mycelial colonies were recorded (two perpendicular diameters per colony). The mean of these two values stood for mycelial growth rate parameter expressed in mm. The measures obtained from this first set of supplemented (L) or unsupplemented malt agar plates were designated as Culture 1 growth rates (C1). Culture 2 mycelial growth rates (C2) were obtained as above after taking inoculation disks from the margin of 14-day old Culture 1 plates incubated under the same conditions. Four replicate Petri dishes were inoculated for each strain in both Culture 1 and Culture 2 sets. Two additional variables were calculated: C2L/C1 and C2L/C1L [9].

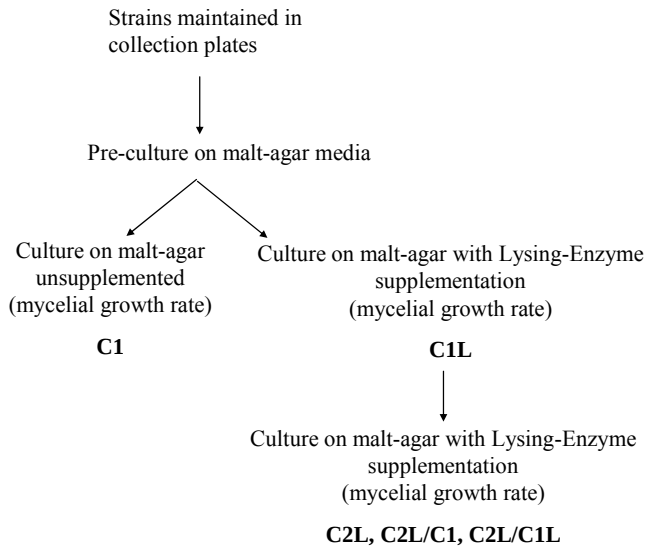

Figure 1. Methodology used to evaluate the effects of *Trichoderma* metabolites (Lysing-Enzyme) on mycelial growth rate of *Agaricus bisporus* strains, adapted from Savoie and Mata [13].

Statistical analyses and QTL detection. Means of mycelial growth were analysed by one-way ANOVAs followed by post-hoc tests for multiple comparison. Broad sense heritabilities based on genotypic mean values were estimated as $h^2 = \sigma^2_G / \sigma^2_P$ where σ^2_G represented the genetic variance, σ^2_P the phenotypic variance. Pearson correlation coefficients were calculated between each trait, based on means of each genotype. Data analyses were performed with the R-open source software.

The genetic linkage map used for QTL mapping was previously developed by Foulongne-Oriol et al. [14]. QTL detection was performed by composite interval mapping (CIM) with QTL Cartographer software [15], using B1 design. A LOD threshold of 2.5 was used to declare a QTL significant. The most likely position of each QTL was defined by the LOD score peak and a LOD-1 confidence interval. MapChart software [16] was used to produce visualisations of chromosomes carrying QTLs. The percentages of phenotypic variation explained by each QTL (R^2) and their individual additive effects (a) were given by the model. The phenotypic variation explained by all detected QTLs (R^2_T in %) was determined by multiple regression analysis, using the closest markers to the LOD score peak for each putative QTL as explanatory variables.

RESULTS and DISCUSSION

Traits evaluation. The C1 variable represented the behaviour of the mycelium in control conditions. The LE supplementation significantly affected the mycelia growth rate (Fig. 2A). Among the 103 hybrids progeny, 6% showed no significant difference between C1 and C2L, suggesting that these strains are highly tolerant to LE effects. Conversely, no lethal effect was observed. The level of tolerance of a strain could be estimated as the ratio C2L/C1 [9]. Twenty percent of the strains had similar mycelium growth rates for Culture 2 + LE and Culture 1 + LE, depicting a potential capacity of adaptation to LE effects [9]. This trait was assessed using the ratio C2L/C1L. Six percent of the strains showed higher significant mycelial growth rate on Culture 2 + LE than on Culture 1 + LE but lower than controls as illustrated in Fig. 2B.

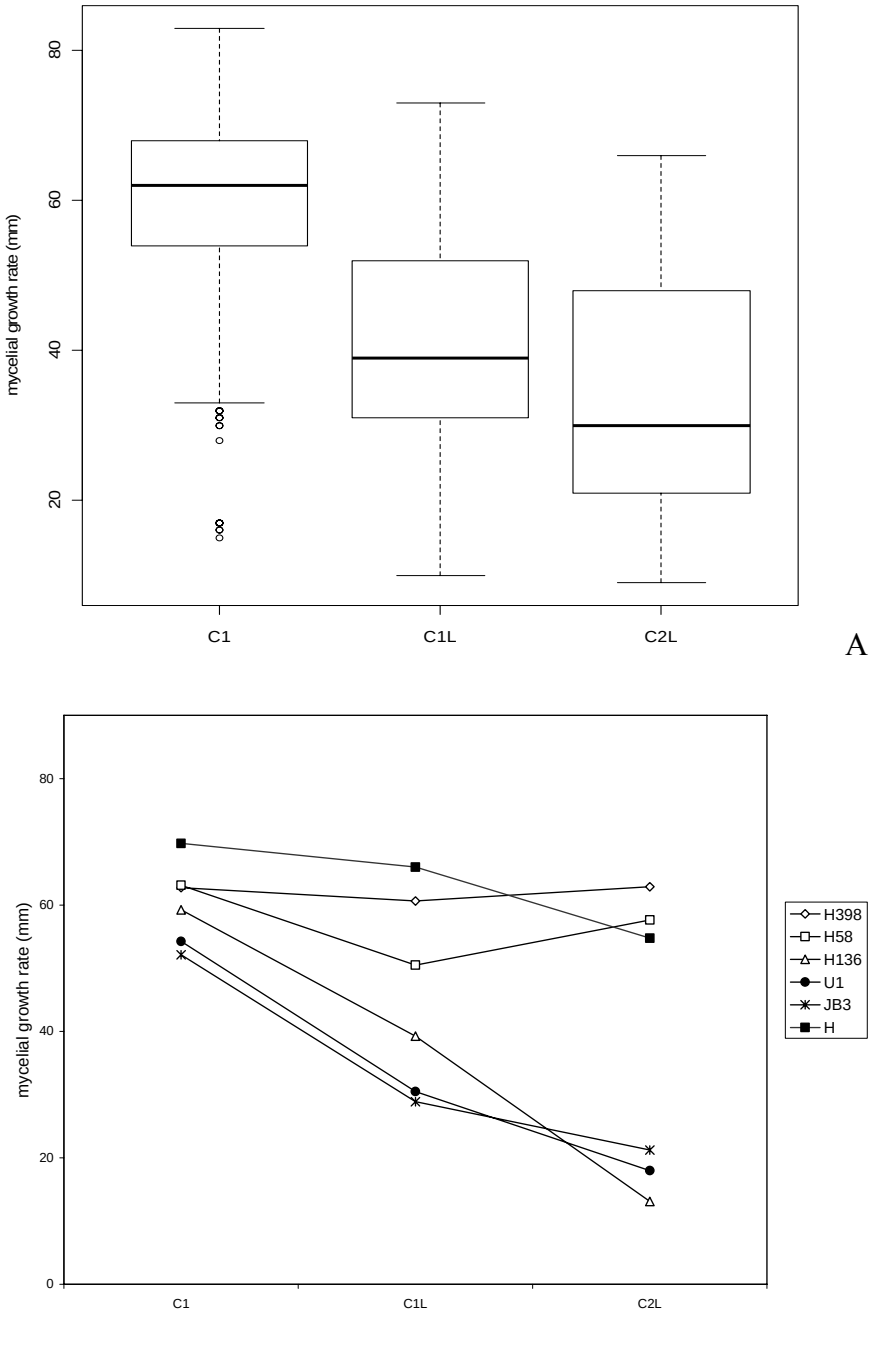

Figure 2. A- Box plot distribution of mycelial growth rate under the three tested conditions: control (C1), Culture 1 + LE (C1L), Culture 2 +LE (C2L)
B- Behaviour of some contrasted hybrids, together with the parental strains for C1, C1L and C2L

Phenotypic variation. Each trait under consideration showed a continuous distribution (Fig. 3) suggesting a quantitative inheritance. This confirmed also that the trait evaluation procedure used was reliable to assess variability in the behaviour of the hybrids progeny facing *Trichoderma* lytic enzymes and metabolites. For C1L and C2L, we observed a shift of the distribution towards the lowest values of the traits (Fig.2, Table 1), demonstrating that the LE supplementation affects mycelium growth rates. In control condition C1, 77% of the hybrids showed a mycelium growth higher than 55 mm. This percentage decreased to 38 % and 30 % for C1L and C2L respectively.

The ANOVAs revealed significant genotype effects for each trait. Post-hoc tests of ANOVAs showed that for all the traits except C2L/C1L, the two parents U1 and Jb3 were not significantly different from each other. The first generation hybrid H was found significantly better than the best parent for each trait, suggesting a strong heterosis effect. Transgressive segregants towards lowest but also in some cases highest values of trait were found among hybrids (Table 1). The broad-sense heritabilities based on genetic variance were high, ranging from 0.60 (C2L/C1) to 0.90 (C2L) indicating that the observed variation between strains is mostly under genetic control. This result was consistent with the *in vitro* experiment which allowed quite controlled environmental conditions.

The mycelial growth rates on LE supplemented media (C2L, C1L) were strongly correlated with the mycelial growth rate on control C1 (Fig. 3). The parameter of tolerance (C2L/C1) strongly correlated with the parameter of adaptation (C2L/C1L) but these two parameters were not found correlated with C1.

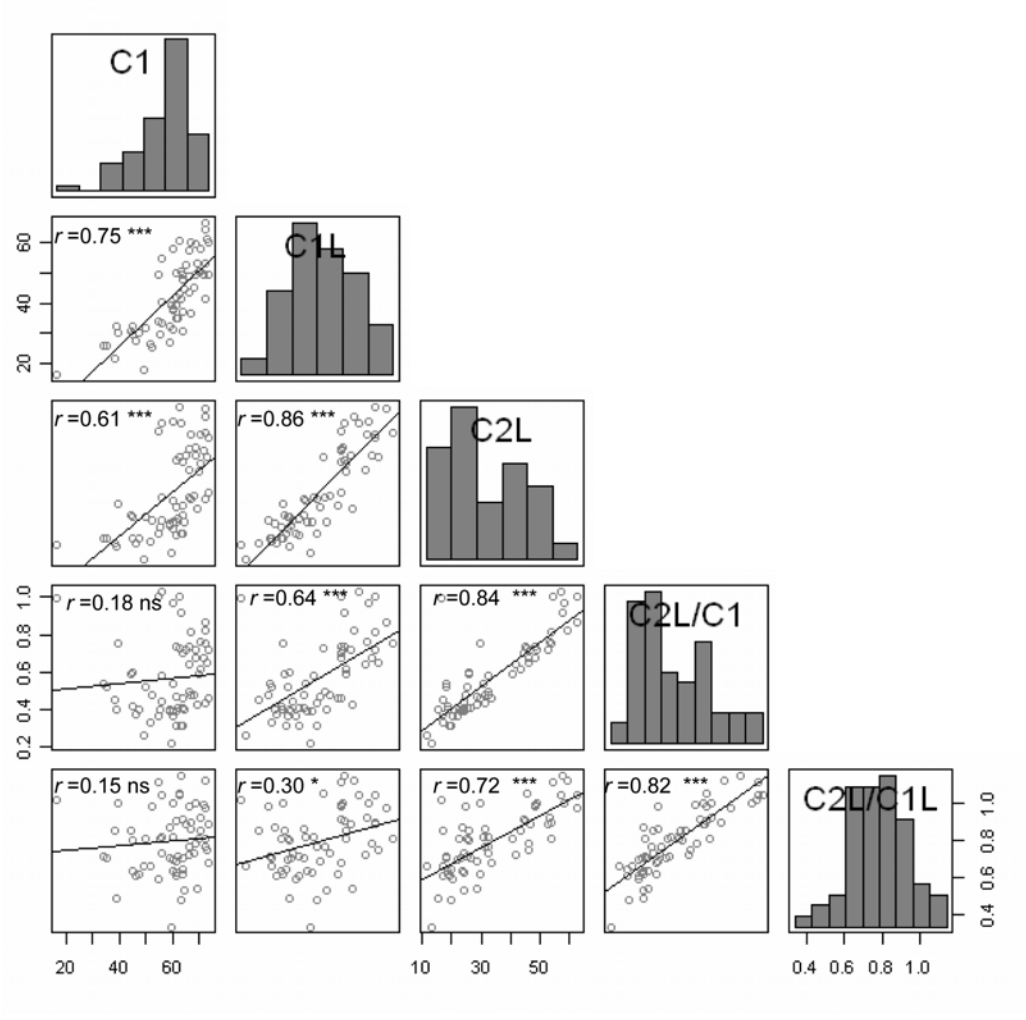

Figure 3. Frequency distribution histograms and scatter-plot matrix of *Trichoderma* metabolites resistance traits in the second generation hybrids progeny. Pearson correlation values between each pair of traits are indicated in the upper left corner of the scatter-plots (ns non significant, * significant at $p<0.05$, *** significant at $p<0.001$)

Table 1. Means, standard deviation (sd), range and heritability for *Trichoderma* metabolites resistance traits in the parental strains U1 and JB3, the H hybrid, and the second generation hybrids progeny (*n*=103).

	Parents			Offspring			
Trait	U1	JB3	H	mean	sd	range	h^2
C1	54.25 ^{B}	52.12 ^{B}	69.75 ^{A}	59.07	10.68	16.5 ^{C} - 77.16 ^{A}	0.84
C1L	30.50 ^{B}	28.87 ^{B}	66.00 ^{A}	41.27	12.43	16.12 ^{C} - 67.83 ^{A}	0.88
C2L	18.00 ^{C}	21.25 ^{C}	54.75 ^{B}	33.74	14.38	11.25 ^{D} - 64.25 ^{A}	0.90
C2L/C1	0.33 ^{C}	0.41 ^{C}	0.79 ^{B}	0.56	0.19	0.22 ^{C} - 1.03 ^{A}	0.60
C2L/C1L	0.59 ^{C}	0.74 ^{B}	0.84 ^{B}	0.79	0.17	0.33 ^{D} -1.22 ^{A}	0.63

Capital letter in exponent indicates significant means differences revealed by post-hoc ANOVA tests (p<0.05).

QTL results. A total of 3, 2 and 2 QTLs were detected using the 2.5 LOD threshold for C1, C2L/C1, C2L/C1L respectively (Table 2, Fig. 4) with individual R^2 ranging from 5.4 (QC1-I) to 15.6 % (QC1-IV). The total phenotypic variation explained by all the QTLs (R^2t) ranged from 23.4% to 26.7%. The remaining phenotypic variation which could not be explained by the detected QTL might come from other source such as QTL with too small effect to be detected in this experiment or QTL masked by differential interaction effect between segregating nuclei and the constant nucleus [17]. The number of QTL and their effects range for the mycelium growth rate in control condition (C1) were quite comparable to those found for *Pleurotus ostreatus* [17].

Table 2. Summary of QTL analyses for *Trichoderma* metabolites resistance traits

Trait	QTL name	LG	Marker[(a)]	LOD (CIM) [(b)]	additive effect	parental allele [(c)]	R^2 (%)	R^2T (%)
C1	QC1-I	I	PR007	2.9	5.33	U1-7	5.4	26.7
	QC1-IV	IV	EAAMAGt	5.3	10.3	Jb3-83	15.6	
	QC1-XIII	XIII	PR003	3.2	7.2	U1-7	8.3	
C2L/C1	QC2L/C1-IV	IV	PR041	3.7	0.2	Jb3-83	13.9	23.4
	QC2L/C1-VI	VI	ECGMCCs	2.9	0.33	Jb3-83	10	
C2L/C1L	QC2L/C1L-IV	IV	EAAMAGt	4.3	0.14	Jb3-83	15.3	23.8
	QC2L/C1L-XIII	XIII	ECGMACf	3.1	0.12	U1-7	9.4	

[(a)] nearest marker upstream to the LOD score peak
[(b)] LOD score value at the LOD score peak
[(c)] parental allele that contributes to increase the trait

Although 7 QTLs were detected for all the traits, only 4 genomic regions were highlighted (Fig. 4). Thus, the overlapping of confidence intervals revealed 2 clusters of QTLs that governed distinct traits, on LGIV and LGXIII. A genomic region of approximately 20 cM around the marker PR041 was detected for each trait (C1, C2L/C1, C2L/C1L), with the highest LOD score value. Two other genomic regions on LGI and LGVI were found each one specific to one trait, C1 and C2L/C1 respectively. These results, together with the correlation parameters suggested that either the tolerance or the adaptation were tightly linked to the growth rate ability. No significant epistatic interaction was detected.

The favourable allele that contributes to increase the trait came from either U1-7 or Jb3-83. This result was not surprising regarding the phenotypic value of each parent U1, JB3 and the H hybrid (Table 1). It suggested a complex genotypic makeup of the tolerance to *Trichoderma* lytic enzymes and metabolites. It confirmed also that potential source of resistance to green mould could be found in both wild and cultivated strains [8].

As the cap colour is a segregating trait in the studied progeny [18], our results also confirmed that the adaptation to *Trichoderma* lytic enzymes and metabolites, a trait contributing to the green mould resistance, was not related to the brown cap colour [8, 19]. Indeed, no QTL related to the traits studied here was found in the vicinity of the *PPC1* locus on LGVIII.

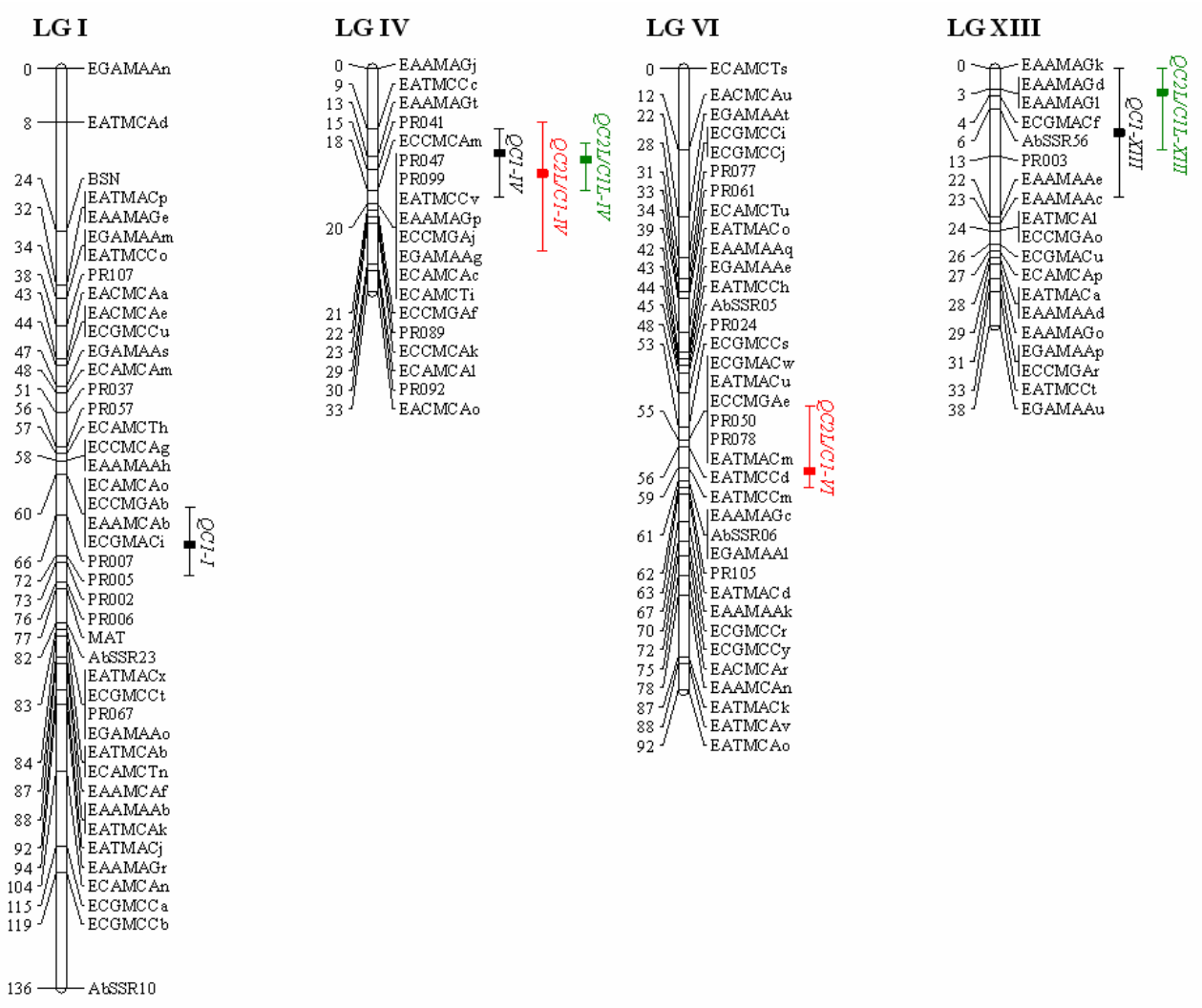

Figure 4. Map positions of the significant QTLs on the *A. bisporus* linkage map. A dash indicates LOD score peak position. The vertical bars represent the confidence intervals of the QTL (LODmax-1). QTLs for C1, C2L/C1, C2L/C1L are indicated in black, red and green respectively.

CONCLUSIONS

This was the first study dealing with QTLs related to resistance to the green mould disease in basidiomycetes. The procedure used here can be applied to other *Trichoderma*-fungus interaction to identify genomic regions involved in the resistance to this competitor causing significant damages during cultivation of many mushroom species.

We have demonstrated that the ability to resist to *T. harzianum* lytic enzymes and metabolites in *A. bisporus* was quantitatively inherited and under oligogenic control. Besides, we have identified one robust QTL on LGIV that lets presume a key role played by this particular part of the genome. The recent release of the *A. bisporus* genome sequence will help to go further in the understanding of the growth rate control.

Our results suggested that the genetic factors involved in the ability to resist or adapt to *Trichoderma* lytic enzymes and metabolites are linked to the fitness of the *A. bisporus* strains. Both traits might be involved in resistance to the green mould disease. The incidence of severity of the disease might be reduced if *A. bisporus* successfully colonizes the compost before *T. aggressivum* develops [4, 20]. During infection, at the contact point between the two fungi, the strains of *A. bisporus* having the ability to resist or to adapt to the lytic enzymes and metabolites with antibiotic effects produced by *T. aggressivum* are better armed to be less affected than others.

Further work is needed to correlate closely the present laboratory test to the resistance to *T. aggressivum* in compost based cultivation systems, but our results offer opportunities to breed and select for *A. bisporus* strains with increased potentials to outcompete the green mould.

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COMPARATIVE ANALYSIS ON THE DIVERSITY OF *AURICULARIA AURICULA-JUDAE* BY PHYSIOLOGICAL CHARACTERISTICS AND TRAP FINGERPRINTING

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ABSTRACT

Phenotypic traits (physiological characteristics) and genotypic traits (target region amplification polymorphism) were used to study the diversity of 32 main cultivars of *Auricularia auricula-judae* in China. 27 important and stable physiological indexes were evaluated; 16 pairs of TRAP primer combinations produced 535 unambiguous and reproducible DNA fragments, of these 524 (97.9%) were polymorphic. Dendrograms were constructed by Unweighted Pair-group Method with Arithmetic Averages (UPGMA) method, and the principal coordinate analysis (PCO) of the two methods exhibited similar clustered patterns, revealing that all the tested strains could be divided into three distinct groups, each of which was correlated with different geographical regions. Most strains originated from the same area were with a narrow genetic basis and could possibly be domesticated from the local wild-type strains, some strains were suspected to be synonymous. The grouping information obtained in the present work provides significant information for further genetic improvement in *A. auricula-judae*.

Keywords: *Auricularia auricula-judae*, Physiological Characteristics, TRAP, Genotypic diversity, Genetic Diversity

INTRODUCTION

Auricularia auricula-judae (Bull.) Quel. which is also known as wood ear, was subjected to Basidiomycota, Agaricomycetes, Incertae sedis, Auriculariales, Auriculariaceae, *Auricularia* [1]. *A. auricula-judae* is wildly spread in Asia, Europe and Africa, but particularly cultivated in China. Besides its nutritional attributes, *A. auricula-judae* has been used as medicine to treat angina, diarrhea, gastrointestinal and hemorrhoids upsets [2] for many centuries in China and other parts of Asia. In recent years, *A. auricula-judae* has been reported to have the functions of lowering blood cholesterol and triglycerides [3], preventing strokes and heart attacks [4], and effective in treating diabetes [5] and certain cancers [6]. It also exhibits antioxidant activities [7]. Therefore, *A. auricula-judae* enjoys popular favor in Europe, America, Japan, Korea, Malaysia, Indonesia and Thailand. At present, it is the fourth among the industrially cultivated edible fungi in the world. The officially issued statistical data reported that the estimated production of *A.*

auricula-judae in China reached about 1.905 million tons and the export reached about 7.6 million dollars in 2008.

China has abundant cultivated germplasm resources of *A. auricula-judae*. However, a major problem currently facing culativars is that identical *A. auricula-judae* cultivars are frequent introduced into different designations and randomly labeled for commercial purposes. Incorrectly designating strains may lead to negative impacts on breeding, and even cause conflicts in the protection of intellectual property rights. What is more serious is that cultivars could suffer great economic loss from the improper introduction of the strains. Therefore, it will be contributory to systematically study the main cultivar diversity of *A. auricula-judae*.

In recent years, comprehensive studies have focused on combining phenotypic traits with genotypic traits to obtain more reliable results, which have been used frequently for analyzing the diversity of cultivated germplasm sources in plants, such as in pea, wheat, common bean, almond, tomato and *Zinnia elegans* [8-12], but rarely on the cultivated germplasm of edible fungi, except *Pleurotus eryngii*, *Pleurotus ferulae* and *Agaricus subfloccosus* [13-15].

Physiological characteristics analysis is a traditional method to analyze phenotypic diversity, and has been proved to be helpful to achieve reliable results under well-controlled environmental conditions, by reliable measurement methods, and with sufficient repetition. TRAP is a fairly new marker technique which is improved on the basis of sequence-related amplified polymorphism (SRAP). It uses a fixed primer of about 18 nucleotides, which is designed from the target-expressed sequence tag (EST) or gene sequence, as well as an arbitrary primer of about the same length, with either an AT or GC-rich core to anneal with an intron or exon, respectively [16]. As a simple yet powerful technique for estimating genetic diversity, TRAP has been successfully used for sugarcane, sunflower, *Porphyra*, *Pelargonium*, *lettuce*, and *Spinacia oleracea* germplasm diversity [17-22]. Although TRAP has been introduced into fungi and proved to be reliable for analyzing genetic diversity of *Lentinula edodes* in China [23], it was for the first time adopted in the present study for analyzing the diversity of *A. auricula-judae*.

The present study aimed to survey the phenotypic and genotypic diversity of 32 main cultivars of *A. auricula-judae* in China. The study was performed in phenotypic traits (physiological characteristics) and genotypic traits (TRAP molecular marker), the usefulness and reliability of the two methods were also compared.

MATERIALS AND METHODS

Mushroom strains. Thirty-two main cultivars were collected from local professional research institutes in different geographical regions of China; and maintained at the Institute for Edible Fungi, Shanghai Academy of Agricultural Sciences. All strains were divided into five populations according to different floristic regions (Table 1).

Physiological characteristics. All strains were incubated on CYM (complete yeast media) for 7 d at 25 ^{o}C and then cultivated by wood-log method in Suixian, Hubei Province to generate fruiting bodies. Table 2 shows the detailed evaluation list of 27 stable and important physiological characteristics indexes, each index was repeated 10 times.

Table 1: Designation, source, and floristic regions of *A. auricula* tested strains

No.	Cultivar	Source[1]	Floristic Fegion	No.	Cultivar	Source[1]	Floristic Fegion
1	Hei-29	MIHL	NE China	17	DZ-1	BIH	Central China
2	8808	MIHL	NE China	18	XE-987	GEMC	North China
3	CBS-7	JAU	NE China	19	XE-887	GEMC	North China
4	YM-1	JAU	NE China	20	HE-9	EMIS	North China
5	HEI-916	JAU	NE China	21	JY-1	MIHB	North China
6	9809	DCH	NE China	22	ZJ-310	EMIC	North China
7	DA-1	DCH	NE China	23	ME-6	EMIC	North China
8	DA -2	DCH	NE China	24	ZHI-5	HIB	North China
9	DA –3	DCH	NE China	25	97-1	HIB	North China
10	139	HAU	Central China	26	C21	MIS	North China
11	YE-K3	HAU	Central China	27	173	XFH	North China
12	SN-A8	HAU	Central China	28	186	XFH	North China
13	XP-10	HAU	Central China	29	HE-3	SAAS	SE China
14	8129	HAU	Central China	30	DP-5	HAU	SE China
15	SHAN-1	HAU	Central China	31	XK-1	HSCS	SE China
16	Au110	BIH	Central China	32	HME-1	EMIK	South China

[1]MIHL, Heilongjiang Microbiological Institute; JAU, Jilin Agricultural University; DCH, Dongning County, Heilongjiang Province; HAU, Huazhong Agricultural University; BIH, Biological Institute of Henan Scientific Academy; GEMC, Guangda Edible Mushroom Center, Jining; EMIS, Edible Mushroom Institute of Shouguang; MIHB, Microbiological Institute of Heibei Province; EMIC, Edible Mushroom Institute of the Chinese Agricultural University; HIB, Hanzhong Institute of Botany, Shanxi Province; MIS, Microbiological Institute of Shanxi Province; XFH, Xixiang Edible Fungi Institute, Shanxi Province; SAAS, Shanghai Academy of Agricultural Sciences; HSCS, Haibing Spawn Center of Suizhou; EMIK, Edible Mushroom Institute of Kunming

TRAP analysis. DNA extraction was conducted by following the method of Tang et al. [24].Sixteen fixed primers were derived from nucleotide sequences of the *Auricularia* genus in Genbank and designed by the web-derived software "Primer 3" (http://frodo.wi.mit.edu/primer3/) (Table 3). The main design parameters were as follows: primer optimum size, maximum size, and minimum size were all set to 18 nucleotides; primer optimum Tm, maximum Tm, and minimum Tm were set to 53 ^{o}C, 55 ^{o}C, and 50 ^{o}C respectively. Sequences of the arbitrary primers with an AT or GC-core sequence were selected from SRAP primers of Tang et al. [24].

PCR amplification of each pair primer was repeated 3 times and then analyzed in 6% denaturing polyacrylamide gel as described by Xiao et al [23]. The gels were directly analyzed to well record the genotypes of the tested strains instead of being analyzed on the photographs. To ensure the reproducibility and reliability, the bands with low intensity were excluded, and the scored polymorphic bands were rescored manually for several times. DNA bands were scored with "1" for presence, and "0" for absence in each genotype.

Table 2: 27 evaluation index of physiological characters

No	Physiological Characteristics	Evaluation Method
1	hyphal density degree under 25 °C	sparse=1/ medium=2/ dense=3
2	mycelium growth rate under 25 °C	≤0.3 slow=1 / 0.3-0.5 medium =2/≥0.5 fast =3
3	mycelium endurance to 40 °C	absent=0/present =1
4	optimum temperature for mycelium growth	24 °C =1/26 °C =2/ 28 °C =3
5	optimum pH for mycelium growth	pH6=1/pH7=2
6	optimum moisture for mycelium growth	55%=1/ 60 %=2/ 65%=3/ 70%=4
7	mycelium assimilation of nutriment	≤ 22 slow=1/22-24 medium=2/≥ 24 fast=3
8	time from inoculation to harvest in log cultivation	≤ 75d short=1/75-90d medium =2/ ≥ 90d long =3
9	occurrence status of fruiting body	≥ 70% tufted =1/ tufted or solitary =2/ ≥ 70% solitary =3
10	shape model of fruiting body	cupped =1/ flaky=2/earlobe=3/ chrysanthemum=4
11	edge model of fruiting body	flat=1/curved=2/nicks=3
12	ventral color of fresh fruiting body	cinnamon=1/brown=2/ brownish black =3
13	ventral color of dried fruiting body	brown=1/ brownish black =2
14	reverse color of fresh fruiting body	cinnamon=1/dust color=2/brown=3
15	reverse color of dried fruiting body	gray=1/ dust color=2/ brown=3
16	wrinkle number of fresh fruiting body	0 absent=1/1-3 light=2/ ≥ 4 heavy =3
17	wrinkle depth of fresh fruiting body	≤ 100 shallow=1/100-300 medium =2/≥ 300 deep =3
18	length of fresh fruiting body	≤ 45.0 short=1/45.0-50.0 medium=2/≥ 50.0 long=3
19	width of fresh fruiting body	≤ 65.0 narrow=1/65.0-80.0 medium=2/≥ 80.0 wide=3
20	longitude to width ratio of fresh fruiting body	≤ 0.6 small=1/0.6-0.8 medium=2/≥ 0.8 large=3
21	root size of fresh fruiting body	≤ 3.0 small=1/3.0-5.0 medium=2/≥ 5.0 large=3
22	back-scrolling degree	absent=1/medium =2/obvious=3
23	thickness of fresh fruiting body	≤ 1.0 thin=1/1.0-2.0 medium=2/≥ 2.0 thick=3
24	The quality of fresh fruiting body	soft=1/medium=2/hard=3
25	single weight of fresh fruiting body	≤ 5.0 low=1/5.0-8.0 medium=2/≥ 8.0 high=3
26	ratio of dry weight to fresh weight	≥ 1/10 low=1; 1/10-1/15 medium=2; ≤ 1/15 high=3
27	dried weight of harvested fruiting body per 100kg logs	≤ 1.5 low=1/1.5-2.5 medium=2/≥ 2.5 high =3

Data analyses. For phenotypic traits data, the original matrices based on physiological characteristic indices were standardized by the STAND option, and then constructed into the Euclidean distance matrices by using the SIMINT option [25], while for genotypic traits data, the SIMQUAL option was applied to calculate the simple matching (SM) coefficient and the pairwise genetic similarity (GS) matrix [26]. The construction of Unweighted Pair-group Method with Arithmetic Averaging (UPGMA) dendrograms and Principal Coordinate Analyses (PCO) were carried out by following Tang et al. [24].

Data analyses were performed using Numerical Taxonomy Multivariate Analysis System (NTSYS-pc), version 2.10 (Exeter Software, Setauket, New York) software package [26].

Table 3: 16 pairs primers combinations used in TRAP

Primer name	GeneBank accession no.	Primer sequence		Number of loci	Percentage of polymorphism
		Fix-primer	Arbitrary[a]		
Ras1-L	GQ244321	TCGGAAATACCAGAGGCAGC	EM8	33	100%
Ras2-L	GQ244319	ATCCTGACATTGCAGCCACA	EM8	24	100%
Ras3-L	GQ244318	GGTGGTAAAGTGGAAGCCC	EM5	16	100%
Ste1-L	FJ756942	GGCATGAGAGCACCCACATA	EM5	41	100%
Ste2-L	FJ756941	CCTGGAACTCGGGTAAGTGG	EM5	37	100%
Ste3-L	DQ303127	TCTGAGCCTCGTCTGTCTCA	EM5	38	100%
Fks-L	AY254574	TACGCCCGGAATCAAGAGTG	EM5	25	100%
Lac1-L	AY450405	TCCGAAGTGGATCCAGACCT	EM1	28	100%
Lac2-L	AY450405	CACTTGAGGGTCACGCAAAC	EM8	70	94.59%
Mul-L	AY485828	GAGCAACTTGTCCAGCCAAC	EM8	29	100%
Lac-L	AY616035	GGAACGTACTGGGTCCACTC	EM1	41	95.34%
Cla-L	AY225999	GAGCACAATCAGCTCGAGGA	EM8	27	100%
Ste1-R	FJ756942	GCAGAGGACACAGAGGATGG	ME2	25	92.59%
Ste3-R	DQ303127	AAGGATGGATGAGCTCGCAG	ME2	37	94.87%
Fks-R	AY254574	ATGTTCGCCCATACCAGACC	ME2	26	100%
Lac1-R	AY450405	AGTACGGTCGTCTCGAGGAT	ME3	28	100%

[a] detail information cited from the paper of Tang et al. (2010)

RESULTS AND DISCUSSION

Diversity analysis based on physiological characteristics. The physiological characteristics were found correlated with geographical region where the strains are originated from. The strains from the same regions always had more similar physiological traits, while more divergence was found between those from different regions. The mycelium morphology of majority strains (such as DA-2) from the Northeast grew densely and had the fastest nutriment assimilation; the fruiting body was thick, solitary, cupped or earlobe-shaped in brownish black or brown, with a flat edge and obviously wrinkled with a hard and crispy texture (Fig.1 a-c). In contrast, the mycelium morphology of majority strains (such as DP-5) cultivated in the Southeast region grew sparsely and had the slowest mycelium nutriment assimilation; the fruiting body was thin, solitary or tufted, earlobe-shaped in cinnamon, curved or nicked on its edge, and slightly wrinkled with a soft and smooth texture (Fig.1 d-f). In addition, the mycelium morphology of majority strains (such as XE-887) cultivated in the North region grew densely and had the middle mycelium nutriment assimilation; the fruiting body was moderately thick, solitary or tufted, earlobe-shaped or flaky in brown, curved on its edge, and moderately wrinkled with a moderate hard and smooth texture (Fig.1 g-i).

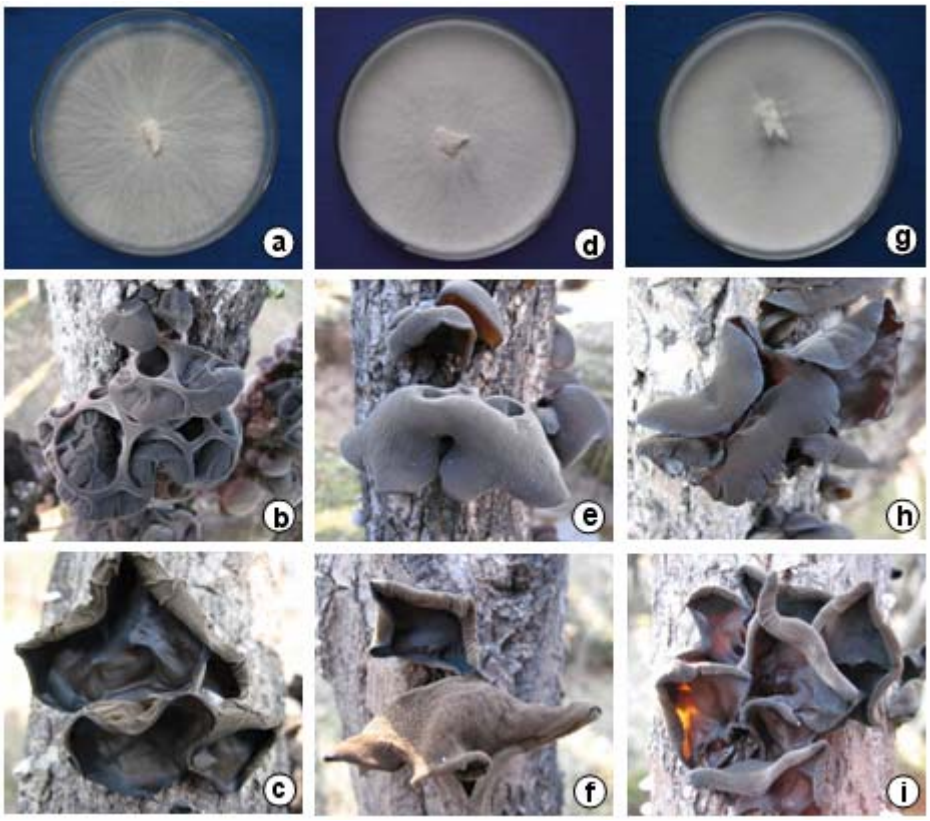

Figure 1: The mycelium and morphology characters of the DA-2 (a-c), DP-5 (d-f) and XE-887 (g-i) strains. Strains DA-2, DP-5 and XE-887 originated from Northeast, Southeast and North region respectively.

Euclidean distance similarity coefficients among the 32 tested strains was ranged from 4.27 (XK-1 and DP-5) up to 11.33 (C21 and DP-5); the co-phenetic correlation between clustering and the data matrix was estimated at 0.77, corresponding to a good fit. UPGMA dendrogram grouped all strains into six main clusters at a Euclidean distance index value of 6.78 (Fig. 2). Cluster II, III and IV comprised of 7, 12 and 10 strains respectively, while Cluster I, V and VI respectively contained a single strain. Partial strains demonstrated higher similarity coefficients and clustered into sub-clusters, such as DA-1 and DA-3, 97-1 and SHAN-1, 9809 and 139, DP-5 and XK-1, XE-987 and XE-887, XP-10 and YE-K3, and ME-6 and ZHI-5.

PCO was performed to more directly visualize the association among accessions (Fig.3). It showed that the three most principal coordinates explained 45.18% of the total variation. 32 tested strains were divided into 3 groups while Group I contained 7 strains, Group II contained 14 strains and the rest 11 strains were in Group III. The results of PCO analysis closely corresponded to those obtained through UPGMA cluster.

Because of the relatively simple structure of the *A. auricula-judae* fruiting body, the use of morphology characteristics to analyze the germplasm diversity was naturally limited. In our research, 27 relatively stable and highly heritable physiological characteristics were chosen for the first time to explore the phenotypic diversity of *A. auricula-judae* (Table 2), and to minimize subjective error and ensure the accuracy of analyses. The physiological characteristics comparison proved that the strains in the same group had more similar physiological traits than in different clusters, and this result could be the useful reference for breeding.

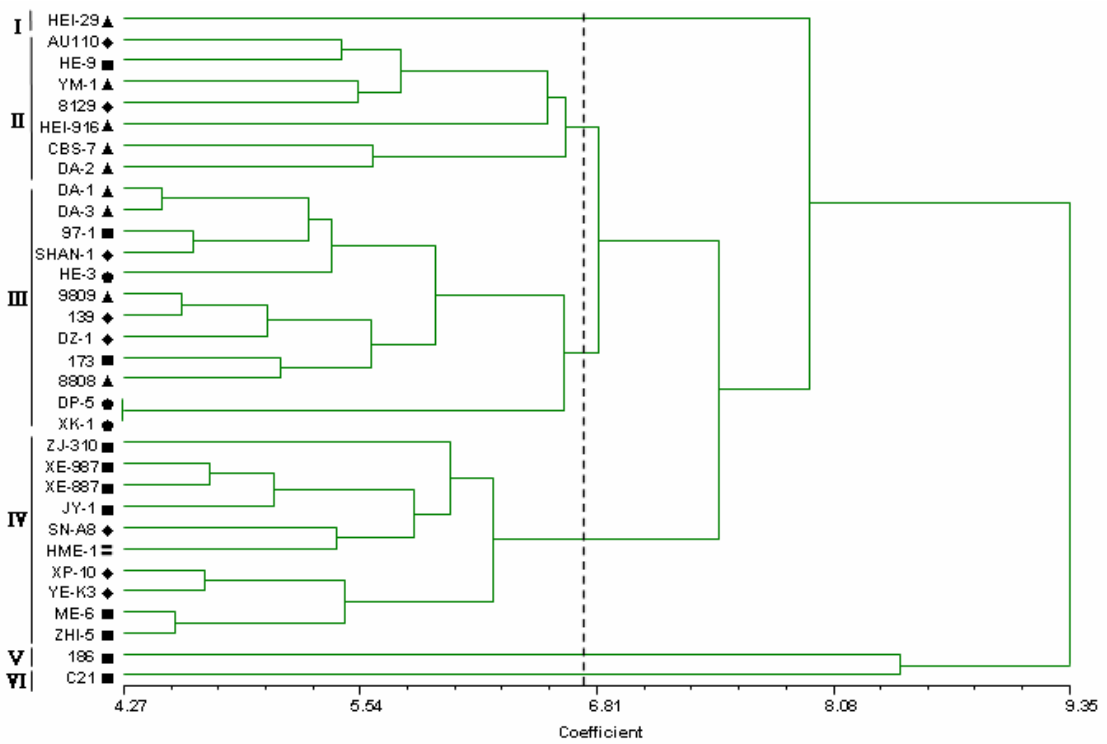

Figure 2: Dendrogram based on the physiological characteristics cluster analysis of *A.auricula-judae*. ▲ represented the Northeast region,• indicated the Southeast region, ◆ stand for the Central region,■ represented the North region and = indicated the South region.

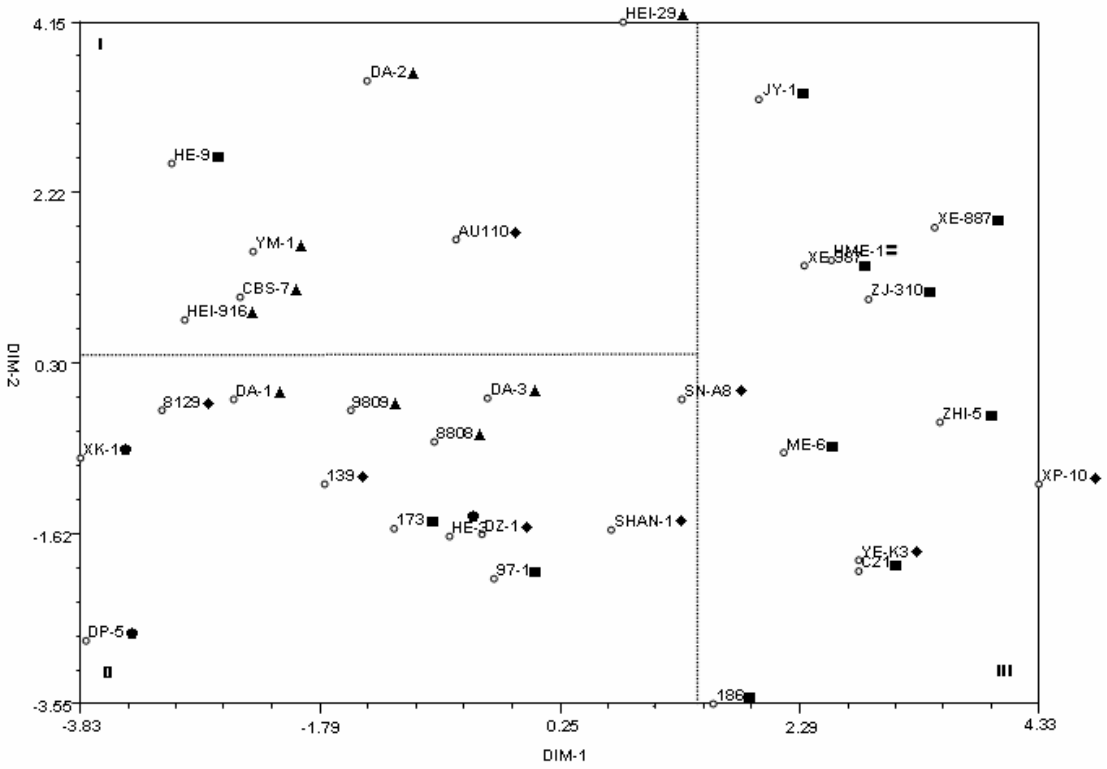

Figure 3: Relationships visualized through the PCO of physiological characteristics data. ▲ represented the Northeast region,• indicated the Southeast region, ◆ stand for the Central region,■ represented the North region and = indicated the South region.

Diversity analysis based on TRAP data. A total of 535 unambiguous and reproducible DNA fragments, of which 524 (97.9%) were polymorphic, were scored from the 32 genotypes, using the 16 TRAP primer pairs listed in Table 3. Most amplified fragments were ranged in different sizes from 100 to 1000 bp, and the number of fragments detected by each primer combination were ranged from 16 to 70, with an average of 34 (Table 3).

Genetic similarities by SM coefficients among the 32 genotypes varied from 0.567 (8808 and SHAN-1) to 0.922 (139 and 8129). The co-phenetic correlation between the clustering and the data matrices was estimated at 0.92, corresponding to a very good fit. UPGMA dendrogram grouped the 32 genotypes into four main clusters at a similarity index value of 0.67 (Fig. 4). Cluster II consisted of 14 genotypes and was further divided into 2 sub-clusters at a similarity index value of 0.70; Cluster I and III each comprised of 7 and 10 genotypes, while Cluster IV composed of a single strain SHAN-1. Several strains demonstrated higher similarity coefficients and were further delineated into sub-clusters, such as AU110 and ME-6, 139, 8129 and 9809, DP-5 and XK-1, and C21 and XE-987.

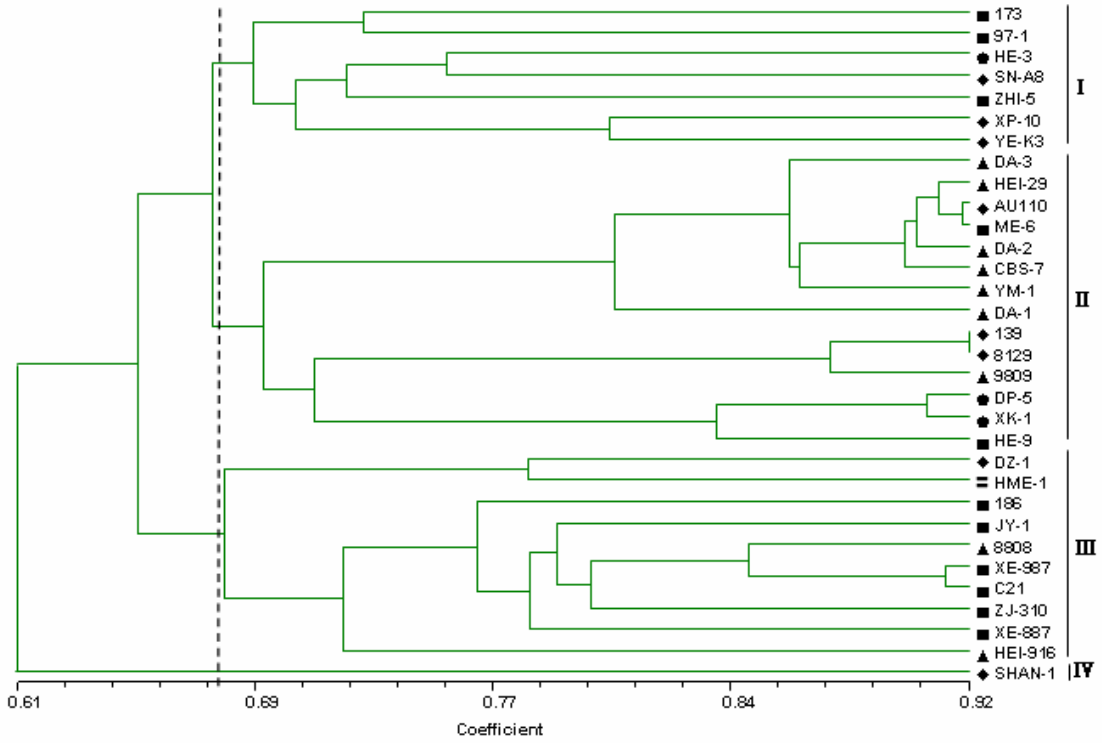

Figure 4: Dendrogram based on TRAP fingerprinting cluster analysis of *A. auricula-judae*. ▲ represented the Northeast region,• indicated the Southeast region, ◆ stand for the Central region,■ represented the North region and = indicated the South region.

Groupings identified by UPGMA analysis were confirmed by PCO data in Figure 5. The three most principal coordinates accounted for 35.40 % of the total variation. Similar to cluster analysis, the PCO result showed that the 32 strains were distinctly divided into 3 groups. Group I contained 8 strains, Group II contained 14 strains and the remaining 10 strains were in Group III.

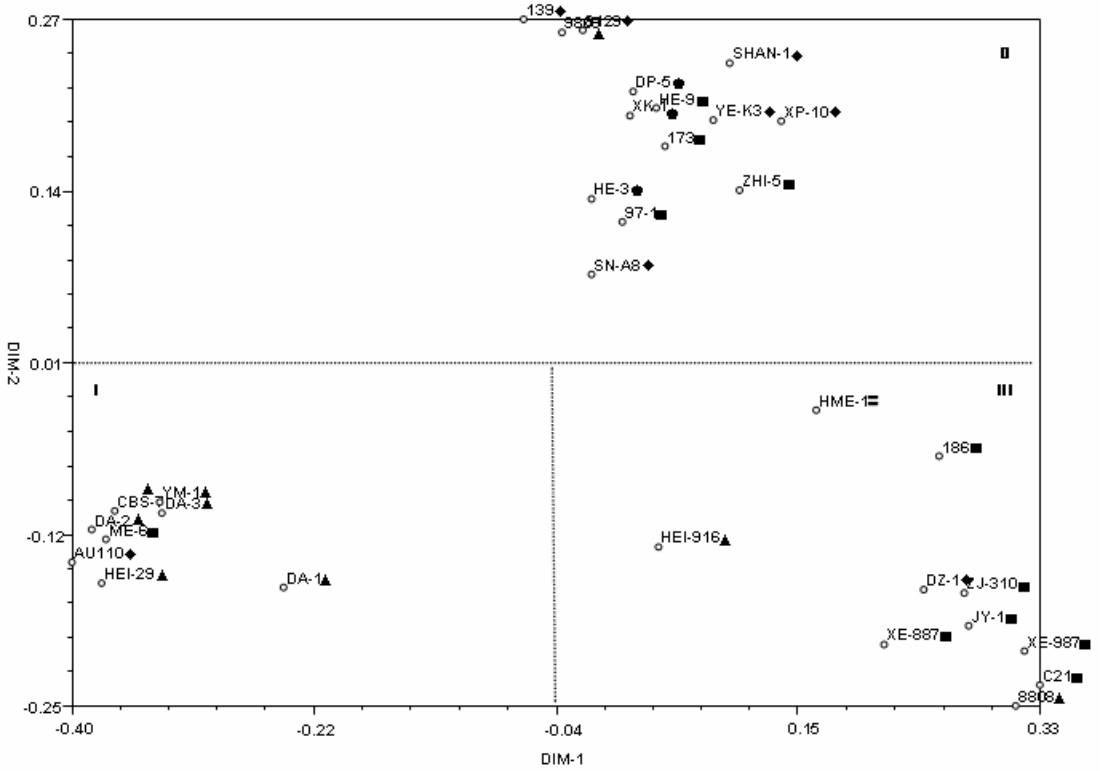

Figure 5: Relationships visualized through the PCO of TRAP date. ▲ represented the Northeast region,• indicated the Southeast region, ◆ stand for the Central region,■ represented the North region and = indicated the South region.

Inter-simple sequence repeats (ISSR) and SRAP had been used to analyze the similar tested strains in our previous study [24], in which ISSR and SRAP generated 129 and 154 polymorphic bands of 34 strains by all 13 ISSR primers and 11 SRAP primer pairs. The percentage of polymorphism of ISSR and SRAP were 96.9% and 96.1%, and the related co-phenetic correlation was 0.85 and 0.90. Compared with ISSR and SRAP, TRAP had a much higher value for evaluating genetic diversity of *A. auricula-judae*. Its advantages presented not only in the total amplified DNA fragments (535 bands), but also in the percentage of polymorphic fragments (97.9%). Although the strains of the Central region have been divided into 2 groups in the clustering, PCO analysis revealed that all the tested strains could be distinctly divided into three groups. The value for estimating genetic diversity was represented by TRAP (0.92) > SRAP (0.90) > ISSR (0.85) in *A. auricula-judae*, which is similar with vegetative biotype buffalograss [27] and *Lentinula edodes* [23].

The comparison and combined diversity analysis based on two methods. The co-phenetic correlation was represented by TRAP (0.92) > physiological characteristics (0.77). The combined PCO analysis of the two methods exhibited similar clustered patterns and revealed that all the tested strains could be divided into three groups: Group I, Group II and Group III, which contained the majority strains originated from the Northeast, the Southeast and the Central, and the North and South regions, respectively.

High correlations between physiological characteristics and molecular marker have been proven in common bean, and Tunisian winter barley [10, 28]. The present study compared the

usefulness and reliability of the two methods, and suggests that to accurately estimate the diversity solely, TRAP demonstrated the higher value in *A. auricula-judae*. Genetic traits analysis could provide more diverse information and help to obtain more reliable results than phenotypic traits. Therefore, in order to achieve a more reliable evaluation and robust characterization of the species diversity, this study suggested that phenotypic traits and genotypic traits should be analyzed and complemented with each other, which is meaningful for the further fungal diversity analyses.

On the other hand, PCO analysis largely corresponded to those obtained through cluster analysis, but could provide more effective and visible information than the UPGMA clustered dendrogram, which could be widely applied in analyzing the fungal diversity in the future.

CONCLUSIONS

In the present study, all the two analytical methods provided important information on phenotypic and genetic diversity of *A. auricula-judae* germplasm. By various methods, all the tested strains could be divided into three groups corresponding to the Northeast, the Southeast and the Central, as well as the North and South regions, respectively, which also proved that the result was reliable.

Most strains originated from the same area clustered together at a high similarity level, for example from Northeast China and North China, which may indicate that these strains were with a narrow genetic basis and could possibly be domesticated from the local wild-type strains. In contrast, the diversity in the Central regions of China was relatively greater due to the frequent introduction from broad scales. The correlations between the diversity and geographical data could further demonstrate the tendency, which was in accordance with our previous results based on ISSR and SRAP [24].

However, partial strains demonstrated higher similarity coefficients in all analysis methods and were suspected to be synonymous, strain doublets DP-5 and XK-1, XE-887 and XE-987 originated from the same region, probably were domesticated from the same fruiting body, but were labeled by different names; strain doublets such as 139 and 9809 originated from different regions could be probably caused by sharing strains in human communication. Herein, the phenotypic and genetic diversity of main cultivars in China were comparatively low, which indicates that the domestication of wild-type strains should attract more attention.

As is well known that, for the improvement of strains, selecting parents in hybridization is very important. Hybridization program involving phenotypic and genetic diverse belonging to different distant clusters will facilitate breeding program. Fortunately, the grouping information obtained in the present work provides significant information for further genetic improvement in *A. auricula-judae*, and is expected to be the reference for the similar studies in the other countries.

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PHENOTYPIC VARIABILITY IN CULTIVARS AND WILD STRAINS OF *AGARICUS BRASILIENSIS* AND *AGARICUS SUBRUFESCENS*

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ABSTRACT

In recent years, there is continued commercial interest in the cultivation of mushrooms with medical and pharmacological value. In Brazil, research and development of cultivation techniques, selection of strains to increase mushroom yield and production of bioactive molecules is needed as the industry is relatively young (1990's) and is focused on the mushroom formerly known as *Agaricus blazei* or *A. brasiliensis*. Recent studies have clarified the taxonomic status of these fungi and they are considered to be synonyms of *A. subrufescens* however the name *A. brasiliensis* is used in many publications on the Brazilian medicinal mushroom. In this paper we evaluate medicinal strains presently cultivated in Brazil, strain ATCC 76739 and wild strains of *A. subrufescens* from various countries for i) mycelial growth at different temperatures, ii) mycelium efficiency to colonize the substrate, and iii) mushroom yield under various cultivation conditions (spawn rate, light, cold shock). Most of the medicinal cultivars showed higher mycelial growth rates than the wild *A. subrufescens*. A temperature of 35°C was not lethal for the cultivars, but seemed so for two *A. subrufescens* strains. Cultivation experiments were performed using commercial compost used for *A. bisporus*. The efficiency of compost colonization was estimated by measuring H_2O_2 after 20 days of incubation. Cultivars produced variable concentrations of H_2O_2 (39-217 nmoles/g of compost) whilst low concentrations were found in the group of wild *A. subrufescens* (47-91 nmoles/g). There was no relationship between compost colonization and others parameters studied (yield, time to fruiting and sporophore mean weight). When taken as a whole, the group of cultivars differed from the group of wild strains for the time taken to first fruiting, yield and sporophore mean weight. Cap colour also separated the cultivars from the wild strains. ATCC 76739 grouped with the cultivars for the various traits analysed. Light and cold shock had no significant effect on the time to first fruiting and yield. Valuable wild material useful for productivity and breeding was identified.

Keywords: Mycelial growth; Compost colonization; Yield; Morphology.

INTRODUCTION

Edible mushrooms are appreciated for their gastronomic, nutritional and medicinal values. The mushroom cultivated in Brazil, formerly known as *Agaricus blazei* Murrill, is widely used and

studied for its medicinal and/or therapeutic properties [1]. Several works have been published to clarify its taxonomic status [2-6], and two new species names were proposed, *Agaricus brasiliensis* and *Agaricus subrufescens*. Kerrigan [2] considers *A. brasiliensis* Wasser et al. and *A. blazei* Murrill to be synonomous with *A. subrufescens* as they interbreed and produce fertile offspring. Wasser [3, 4] claimed that they are different species and proposed *A. brasiliensis* for the medicinal mushroom. Currently many publications refer to the Brazilian cultivar as *A. brasiliensis* and it is believed to originate from Brazil [7]. Wild mushrooms referred to as *A. subrufescens* have been found in the wild in California, Israel, Taiwan and Hawaii [2, 6], Mexico and European countries. These mushrooms are not currently cultivated at commercial scale, but recent work performed by Moukha *et al.* (this issue) proved that wild strains of *A. subrufescens* can show medicinal properties.

Several studies describe specific techniques and parameters for cultivating the Brazilian commercial strains [8-14] but substrates, casing materials and procedures are adapted for tropical countries. Brazilian strains have been reported to have a high genetic similarity [15-18] and a typical morphology [3, 19, 20].

Our target was to assess phenotypic variability among currently cultivated Brazilian strains and wild strains of *A. subrufescens* from various origins. We also wanted to identify individuals giving good yields when cultivated using substrates and procedures similar to those used for *Agaricus bisporus* production in France. With this aim, we evaluated both groups of strains for: i) mycelium growth rate at different temperatures, ii) mycelium efficiency to colonize the substrate, and iii) morphology, time to fruiting and yield under various cultivation conditions.

MATERIALS AND METHODS

Fungal material. Twenty five strains from the "Collection du Germoplasm des Agarics à Bordeaux" (CGAB), including 14 cultivars, the Brazilian strain ATCC 76739, 9 wild *A. subrufescens* and a hybrid between ATCC 76739 and a French *A. subrufescens* were evaluated in this study (Table 1). The strains were preserved in tubes on compost extract medium submerged with mineral oil. Before being used for the experiments, the strains were sub-cultured on malt agar (MEA) medium (pH 5.75) for 20 days at 25°C.

Table 1: Commercial and wild strains with reference to origin and code in collection.

Cultivars		Wild strains	
Origin	Code	Origin	Code
Brazil	CA 455	Brazil	ATCC 76739
Brazil	CA 560 = ABL-99/28	Mexique	CA 603
Brazil	CA 561 = ABL-99/30	Taiwan	CA 276
Brazil	CA 562 = ABL-03/44	USA	CA 462
Brazil	CA 563 = ABL-04/49	Belgium	CA567
Brazil	CA 564 = ABL-05/51	France	CA 487
Brazil	CA 565 = ABL-03/48	France	CA 516
Brazil	CA 566 = ABL-06/53	France	CA 643
Brazil	CA 570 = ABL-01/29	Spain	CA 438-A
Brazil	CA 571 = ABL-98/11	Italy	CA 536
Brazil	CA 572 = ABL-07/58	Hybrid	ATCC 76739-3 x CA487-100
Brazil	CA 574 = ABL-07/59		
Mycelia Co	CA 646 = 7700		
Mycelia Co	CA 647 = 7703		

Radial mycelial growth. Inoculum plugs (7 mm diameter) were removed from edge of 20-day-old cultures and placed at the centre of Petri dishes filled with MEA medium. The strains were grown in the dark for 14 days at 25°C, 28°C, 30°C, 32°C and 35°C. Three replications per strain were made for each treatment. Radial mycelial growth was estimated by two perpendicular measurements of the colony diameter. The linear growth period common to all strains (d5 to d10) was identified from the kinetics of radial growth and used to calculate the mycelial growth rate (mm day^{-1}). At the end of the experiment (d14), the strains of the 35°C treatment were changed to 25°C and measurements were performed as previously described.

Compost. The substrate used to assess mycelial colonization and mushroom yield was commercial compost prepared for *A. bisporus* cultivation, and provided by Renault SA, Pons, France.

Ability of mycelium to colonize commercial compost. Small crates were filled with 150 g of compost and the whole surface of the substrate was covered with mycelium of the studied strains on agar medium (content of three Petri dishes per crate). After 21 days of incubation at 25°C and 85% humidity, the substrate was freeze-dried and colonization by the mycelium was estimated by measuring H_2O_2 as described by Savoie et al. [21]. Compost samples from 12 cultivars, ATCC 76739 and four wild strains of *A. subrufescens* were analysed, with 2 replicates per strain.

Small scale cultivation. Crates filled with 500 g of compost were inoculated as described for compost colonization. After incubation for 20 days at 25°C and 85% humidity, a casing layer (1/3 limestone, 1/3 peat, 1/3 thin sand) was added, and the crates were left under the same environmental condition for a 7-day post-incubation period. To initiate fruiting, the room temperature was maintained at 22-25°C with 95-97% humidity and low CO_2 concentration. Time to fruiting was calculated as the time period between casing and the first pick of mushrooms. The number and fresh weight of the fruiting bodies were recorded for up to 65 days after casing. Nineteen strains (12 cultivars, ATCC 76739, 5 wild strains of *A. subrufescens* and the hybrid) were cultivated in duplicate in a completely randomised design experiment. Yield data are mean values of the total weight of biomass produced per kilogram of substrate.

Medium scale cultivation. The substrate (8 kg) was inoculated with 2% spawn and incubated at 25 °C, 85 % humidity, for 15 days. Standard conditions for casing, post-incubation, fruiting conditions, and collection of data were as described above. Fourteen strains (8 cultivars, ATCC 76739 and 5 wild strains of *A. subrufescens*) randomly chosen among those screened in the small scale experiment were cultivated in a completely randomised design experiment with four replicates per strain.

Effect of climatic and biological factors. Strains were cultivated on 8 kg substrate according to standard conditions, except where otherwise stated. The experiments were performed according to a completely randomised design with four replicates per strain.
Light and cold shock: At the end of post-incubation, three strains (ATCC 76739, CA 487 and the hybrid) were submitted to four different treatments, namely A: 12 h light / 24h, cold shock (4 h at 18 °C twice a week); B: 12 h light / 24h, no cold shock; C: no light, cold shock (4 h at 18 °C twice a week); D: no light, no cold shock.
Spawn rate: Spawning at 1% and 2% were compared for their effect on the time to fruiting and biomass production for three Brazilian cultivars (CA561, CA565 and CA 570), ATCC 76739 and *A. subrufescens* CA 487.

Data representation and statistical analyses. The box-plot representation [22] was used to show data distribution for radial mycelial growth. The Cramer–Von Mises's and Kolmogorov-Smirnov's non parametric tests were performed to compare data distributions.

Data recorded for time to fruiting, biomass production and mean weight were analysed using ANOVA followed by Duncan's test to identify statistical differences. The Pearson coefficient was calculated to find correlations between treatments.

RESULTS AND DISCUSSION

Mycelial growth rate. When all Brazilian cultivars were considered as a whole, no significant differences were observed between the distributions of radial growth rates at 25, 28 and 30 °C. A significant move toward slower growth rates was observed at 32°C, and to a greater extent, at 35 °C. Similar results were obtained with the group of wild strains. Although distributions at 28 and 30 °C did not differ significantly, growth rate data for the wild isolates tended to be highest at 30 °C (Fig. 1). At each temperature, except for 30°C and 35°C the distributions for the group of cultivars and the group of wild strains differed significantly. The fastest growth rates were observed among the cultivars and the slowest among the wild strains. The mycelial growth rates of the Brazilian strain ATCC 76739 were in the range of those observed for the cultivars, whatever the incubation temperature, whilst those of the hybrid differed from the cultivar distributions.

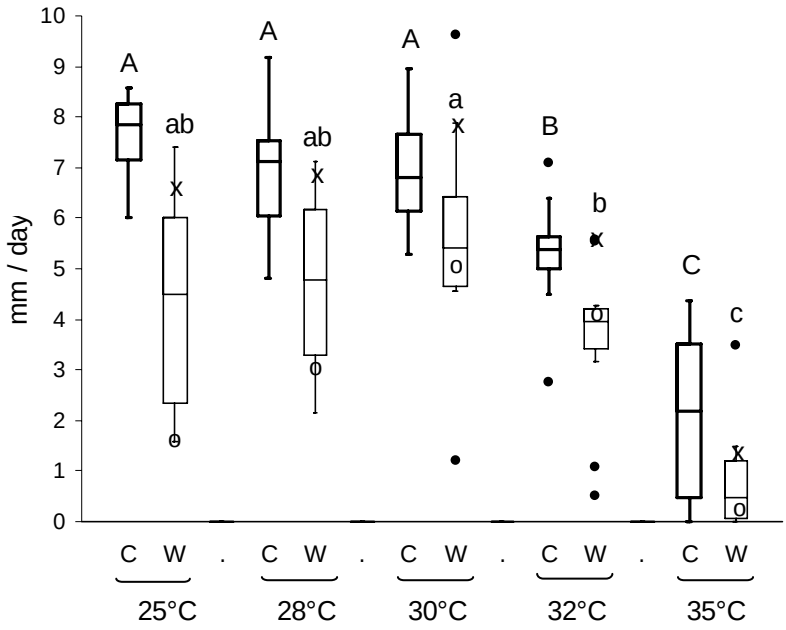


Figure 1: Distribution of the mycelium growth rate at five incubation temperatures.
C: Brazilian cultivars (C), W: wild strains. ●: data outside the distribution, x : ATCC 76739, o : hybrid.
Within a same group of strains, growth rate distributions with a same letter did not differ significantly at $p = 0.05$.

In the treatment at 35°C, the cultivar CA 574 failed to grow, 4 cultivars had non-significant growth ranging from 0.23 to 0.90 mm d^{-1}, and the others showed poor to medium growth rates ranging from 1.47 to 4.37 mm d^{-1}. The wild strains were more severely affected by incubation at 35 °C. Three strains failed to grow, four strains had non-significant growth ranging from 0.10 to 0.53 mm d^{-1}, and three others showed poor growth rates of 1.17 - 1.50 mm d^{-1}. The only one with a medium growth of 3.47 mm d^{-1} was outside the distribution (Table 2 and Fig. 1).

Neves et al. [16] reported that all but one of six strains provided by spawn makers, or isolated from fruiting bodies collected in Brazilian mushroom farms, showed optimal growth temperatures of 28 or 30 °C. More recently, Colauto et al. [8] observed that five strains can develop mycelium at temperatures between 22 and 34 °C. The temperatures tested in our experiments did not enable us to identify optimal growth temperatures for the cultivars but we found that 25–30 °C was suitable for their mycelial development; this is in accordance with the observations described above. The growth rate of the Brazilian ATCC 76739 did not differ from the cultivars whilst the wild *A. subrufescens* strains tended to grow better at 30 °C.

Table 2: Mycelial growth rate of the commercial and wild strains at 35°C

Radial growth rate (mm day^{-1}) *					
Cultivars			Wild strains		
CA570	4.37	A	CA516	3.47	A
CA563	3.90	AB	CA438	1.50	B
CA647	3.63	ABC	ATCC 76739	1.23	BC
CA562	3.57	ABC	Hybride	1.17	BC
CA646	3.37	ABC	CA487	0.53	BCD
CA565	3.00	BC	CA462	0.47	BCD
CA572	2.70	CD	CA643	0.33	CD
CA560	1.67	DE	CA276	0.10	CD
CA561	1.47	EF	CA567	0.00	D
CA566	0.90	EFG	CA536	0.00	D
CA571	0.33	FG	CA603	0.00	D
CA564	0.30	G			
CA455	0.23	G			
CA574	0.00	G			

* Within a column, data followed by a same letter did not differ at $p = 0.05$.

When placed at 25°C after 14d incubation at 35°C, all the cultivars began or continued to grow. With the exception of CA 563, growth rates at 35 °C and growth rates after change to 25°C were significantly correlated (r = 0.770, $p = 0.002$) (Fig. 2A). However, the growth rates of cultivars at 25°C after a first incubation period at 35 °C did not correlate with the growth rates at 25°C with no pre-incubation (r = -0.491, $p = 0.075$; line not shown on graph) and they were always lower than those observed for direct incubation at 25°C (Fig. 2B). Similarly, ATCC 76739 developed a slower growth rate when changed from 35°C to 25 °C compared to direct incubation at 25 °C.

When placed at 25 °C after incubation at 35 °C, two of the three *A. subrufescens* wild strains that failed to grow at 35 °C developed no mycelium; the other strains began or continued to grow, but growth rates were not correlated to those observed at 35 °C (r = 0.170, p = 0.663; line not shown on graph) (Fig. 2A). However, there was a significant correlation (r = 0.860, p = 0.001) between growth rates of *A. subrufescens* wild strains at 25 °C, and growth rate at 25 °C after a first incubation at 35 °C, although mycelial development was slower in the latter treatment (Fig. 2B).

Incubation at 35°C proved that this temperature was not lethal for the cultivars, but was lethal for two wild strains. Similarly, this temperature was lethal for *in vitro* mycelial development of several strains of *A. bitorquis* [23] but only rendered inactive four strains of this species [24]. Experiments with more strains and temperature (35 °C and above) are necessary before conclusions on the lethal temperature for both Brazilian cultivars and wild *A. subrufescens* can be drawn. However, both groups of strains were less susceptible to high temperature than *A.*

bisporus. Indeed, all the six *A. bisporus* strains tested by Lemke [24] failed to develop mycelium at this temperature.

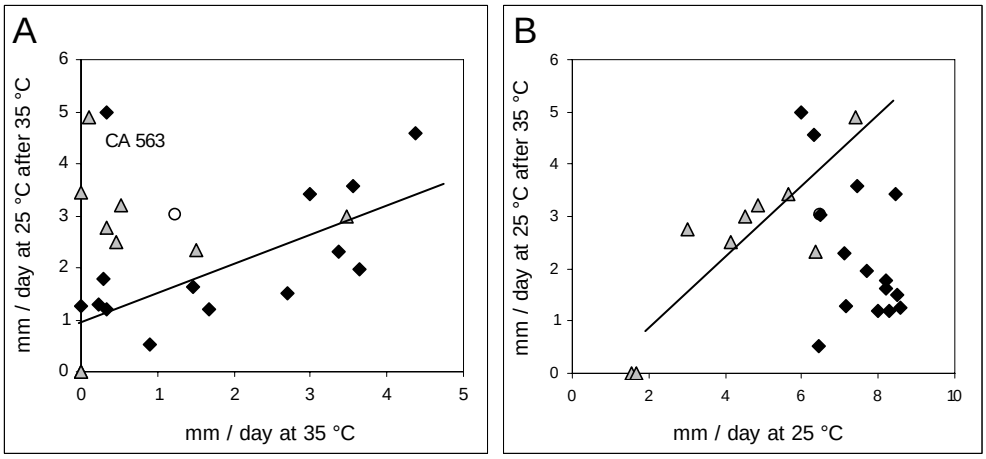


Figure 2: Radial growth rate observed at 25°C after previous 14-day incubation at 35°C compared to (A) radial growth at 35 °C and (B) radial growth at 25°C with no pre-incubation.
♦ Cultivars, ○ ATCC 76739, △ *A. subrufescens*

Compost colonization. Five cultivars showed high ability to colonize and transform the substrate with H_2O_2 concentrations ranging from 217.5 to 560 nmol g^{-1}, whilst low concentrations (39.5 - 78 nmol g^{-1}) were measured in substrates colonized by the other cultivars. A similar range of low H_2O_2 concentrations was obtained with the *A. subrufescens* strains (47.5 – 91.5 nmol g^{-1}) and the hybrid (74 nmol g^{-1}). ATCC 76739 produced a medium concentration (129 nmol g^{-1}). The H_2O_2 levels observed are in the same range to those measured for another Agaricus species, *A. bisporus*, producing 300 - 600 nmol g^{-1} substrate 15 days after spawning [21]. In contrast to *A. bisporus* [21], neither the group of cultivars nor the group of wild strains showed a correlation between the H_2O_2 concentration and the mushroom yield (Fig. 3), time to fruiting or sporophore mean weight (not shown).

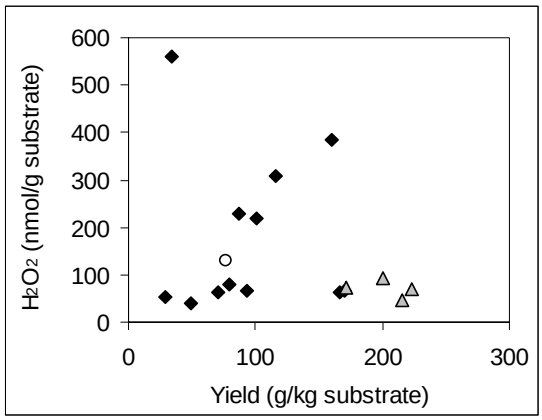


Figure 3: Concentrations in hydrogen peroxide and biomass produced by the Brazilian cultivars and the wild strains.
♦ Cultivars, ○ ATCC 76739, △ *A. subrufescens*

Mushroom yield. The cultivars and the wild strains showed a wide range of mushroom yield in commercial compost commonly used to grow *A. bisporus*. In the small scale experiment, the cultivars yielded 29 - 171 g kg^{-1} substrate showing a great variation in adaptation to commercial

compost produced for *A. bisporus* cultivation (Fig. 4A). The Brazilian ATCC 76739 was comparable to cultivars showing average biomass production. *Agaricus subrufescens* from Spain and France showed little differences in mushroom yield and were highly productive (197.5 – 215.7 g kg^{-1} substrate). The yield of the hybrid was between those observed for its two parents (Fig. 4A).

The fourteen strains screened in medium scale experiments under the standard conditions confirmed the high variability in biomass production found in the small scale experiment. Mushroom yields at both experiment scales were significantly correlated, either for the cultivars (r=0.796, $p = 0.018$) or the wild strains (r = 0.870, $p = 0.024$) (Fig. 4B).

Four medium scale experiments confirmed that the hybrid yield (116.7 ± 32.4 g kg^{-1} substrate) fell between the yields of its two parents, ATCC 76739 (41.9 ± 12.2 g kg^{-1}) and CA 487 (207.9 ± 47.9 g kg^{-1}).

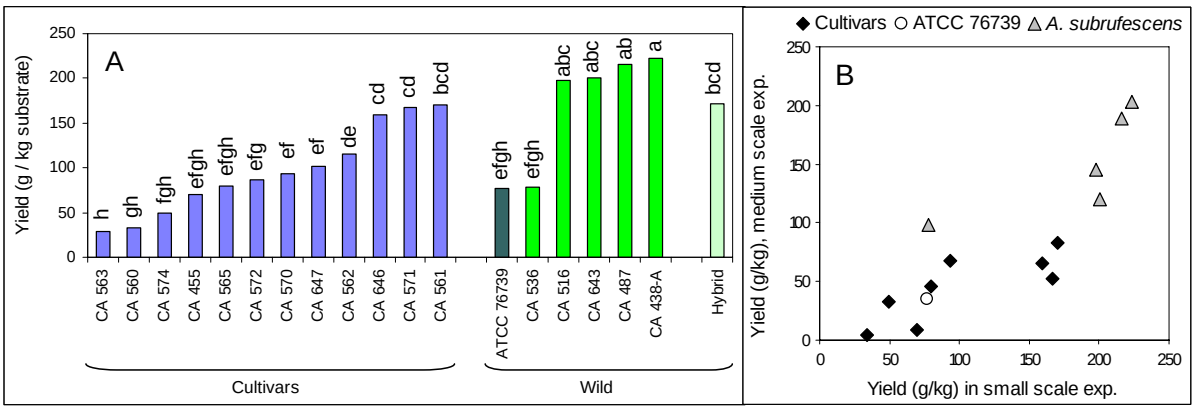


Figure 4: Mushroom yield after a 65-day fruiting period.
A: small scale experiment; B: comparison of the small and medium scale experiment.
Data for medium scale are means of 2 to 4 experiments of 4 replicates

Compared to other cultivated *Agaricus*, the productivity of the Brazilian cultivars reported in the literature is low and depends to a high degree on the strain and the cultivation conditions. Eira [1] reported yield from 3 to 25 kg of fresh mushroom /100 kg of substrate based on local material. More recently, the average production in Brazil was estimated at 8 - 16% after 120 days of cultivation [25]. In this study, three cultivars showed productions between 6.5 and 8.2% after the 65-day fruiting period and would therefore be considered valuable material for cultivation using commercial compost produced for *A. bisporus* cultivation. The French and Spanish *A. subrufescens* strains and the hybrid adapted well to this substrate.

Time to fruiting. In the small scale experiment, the time to first fruiting varied from 26 to 40 days after casing for the group of cultivars. ATCC 76739 began to fruit on day 34, and consequently did not differ from the group of cultivars for this trait. Four of the wild strains were early fruiting (19 - 25 d) (Fig. 5A). The medium scale experiment confirmed these observations (Fig. 5B). The literature indicates that cultivar first flush occurs approximately 15-20 days after casing. Under our cultivation conditions, cultivars began to fruit later. However, time to primordial onset is dependent on the casing materials [26]. We used a single casing mixture, derived from that prepared for *A. bisporus* cultivation. The casing composition seemed suitable for *A. subrufescens* cultivation, but the time to first fruiting of cultivars might be improved by the use of different casing mixtures.

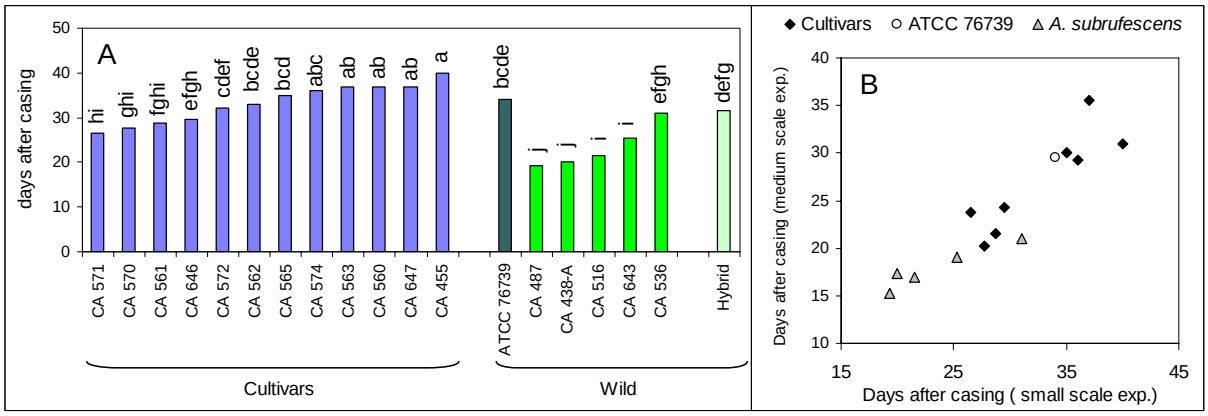
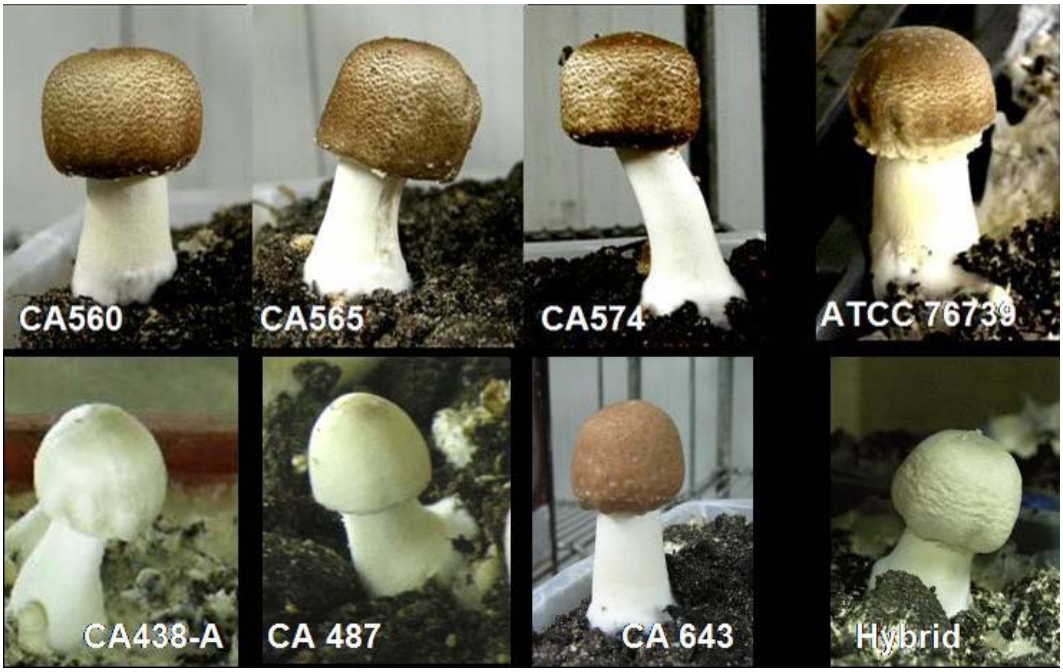


Figure 5: Time to fruiting (in days after casing) of the cultivars and the wild strains: (A) small scale experiments; (B) comparison of small scale and medium scale experiments.

Sporophore morphology. Under the standard cultivation conditions described above, Brazilian cultivars showed a cylindric, brownish-gold cap, as described in the literature for the cultivated strains [3, 20]. The Brazilian wild strain ATCC 76739 showed the cultivar morphology, whilst the wild *A. subrufescens* strains exhibited different morphology. The French strain CA487 and the Spanish strain CA438-A exhibited a cream cap whilst the French strain CA643 showed a brown cap, but without the gold appearance characteristic of the Brazilian cultivars (Fig. 7). All strains bore a white stipe, and an elastic flocculent veil (Fig. 8).



Fgure 7: Examples of aspects of young sporophores of cultivars (CA 560, CA565, CA574), ATCC 76739, wild *A. subrufescens* (CA 438-A, CA 487, CA 643), and the hybrid ATCC 76739-3 x CA 487-100)

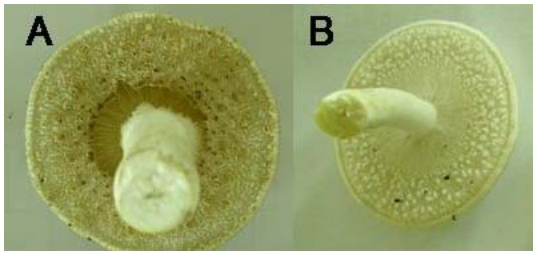


Figure 8: Veil of cultivar (A) and wild strain (B).

Sporophore mean weight was used as a rough estimation of sporophore size. In the small scale experiment, the cultivars showed a wide range of sporophore mean weight (14.0 – 36.6 g) whilst the wild strains produced small sporophores (11.0 – 19.1 g) (Fig. 6A). This observation was confirmed with the medium scale experiment. When taken as a whole, the group of cultivars produced larger sporophores compared to the group of wild strains. The Brazilian ATCC 76739 did not differ from the cultivars (Fig. 6B). The morphological traits of mushrooms grown on the same substrate clearly separated the Brazilian cultivars and the ATCC 76739 from the wild *A. subrufescens*.

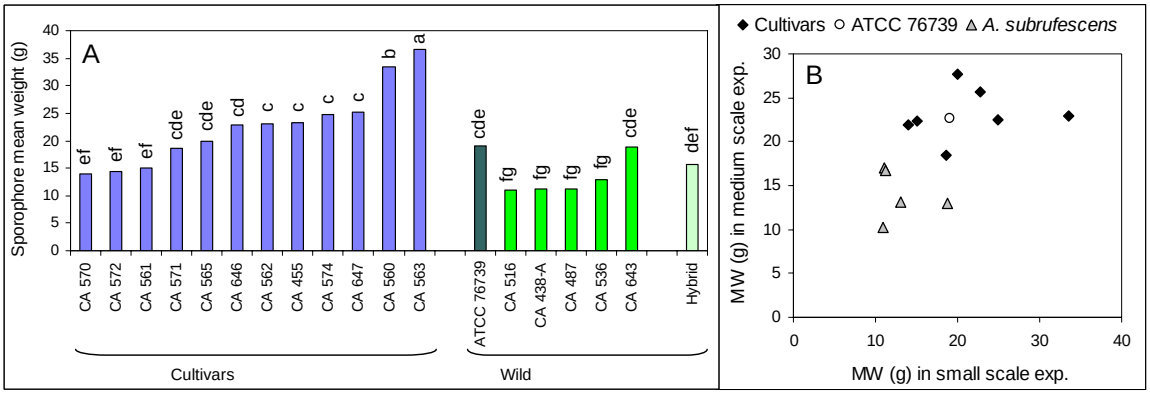


Figure 6: Mean weight of sporophores: (A) produced in small scale experiment and (B).comparison of small scale and medium scale experiments

Effect of light and cold shock. Neither the light period nor the cold shock had a significant effect ($p = 0.05$) on the biomass production and time to fruiting of the three strains tested (ATCC 76739, CA 487 and the hybrid). As previously observed, ATCC 76739 was later fruiting and produced less biomass but larger sporophores compared to CA 487. The time to fruiting, yield and sporophore size of the hybrid were between those of its two parents.
The literature reports the production of Brazilian cultivars in the dark [13], with light/dark photoperiod [27], under day/night periods in glasshouse [9] and outdoor [10], but no study focused on the effect of light on the mushroom yield. From our observations, it is clear that the Brazilian ATCC 76739, the *A. subrufescens* CA 487 and the hybrid developed perfectly in the dark. The three strains studied by Zied et al (This issue) on 3 different composts with variations in temperature during the cropping period showed the same levels of yields and time to fruiting.

Effect of spawn quantity. The time to first fruiting was significantly affected by the reduction of spawn from 2% to 1%, but the effect was highly dependent on the strain. On substrate spawned

at 1%, average time between casing and first fruiting increased significantly by 5 and 8 days for CA 561 and CA 570, respectively. No significant difference was observed for the other strains (Tables 3 - 4). Despite the effect on the time to first fruiting, no significant variation in biomass production was detected in relation to spawn rate (Table 3).

Table 3: Effect of spawn rate on fruiting earliness and mushroom yield.

Source	DDL	Fruiting earliness			Yield		
		Mean squares	F	Pr > F	Mean squares	F	Pr > F
Strain	4	264.013	61.237	< 0,0001	4501115.055	32.793	< 0,0001
Spawn rate	1	53.635	12.440	0.002	425483.469	3.100	0.092
Replicate	3	0.522	0.121	0.947	192902.776	1.405	0.268
Strain*spawn rate	4	19.864	4.607	0.007	93777.654	0.683	0.611

Table 4: Comparison of the time to fruiting at the two spawning rates.

Strains		Days after casing [1]	
		1% spawn	2% spawn
Cultivars	CA561	28 A	20 B
	CA 565	26.2 A	26.7 A
	CA 570	24.2 A	18.5 B
Wild	ATCC 76739	29.5 A	29.7 A
A. subrufescens	CA 487	14.2 A	13.7 A

[1] Data were means of four replicates.

Spawning at 1-2% is commonly used for commercial production of Brazilian cultivars [10, 13, 28, 29]. Spawning at 2% rate increased the yield of strain 7700 (Mycelia Co) by 26% on average, compared to yields obtained with 1% spawn, in a cultivation substrate composed of wheat straw and chicken manure [30]. Such yield improvement were not observed with the three cultivars and the two wild strains cultivated on French commercial compost based on horse manure reported here.

CONCLUSION

We evaluated 14 Brazilian cultivars, ATCC 76739 – the presumed source material of many strains cultivated in Brazil, and 9 wild strains of *A. subrufescens* from different geographic origins for phenotypic variability. All the studied traits (mycelium growth rate, compost colonization, time to first fruiting, yield, sporophore macro-morphology) clearly separated the cultivars and the wild *A. subrufescens*, whilst ATCC 76739 did not differ from the cultivars.

Significant phenotypic diversity was found among each group, cultivars and wild *A. subrufescens*. Several strains appear to have valuable characteristics for cultivation on commercial compost. Future work with selected strains will focus on investigating the following biological and climatic conditions: spawning at 1 % for economic consideration as this rate did not reduce yield, no light to reduce energy cost and no cold shock to limit manipulations. Different casing mixtures may improve yield of the selected strains. This species is of interest for cultivation during the warmest months in Europe to reduce energy costs,

The medicinal properties of *A. subrufescens* strains is also an important consideration. Detection of biomolecules in *A. subrufescens* (Moukha et al., this issue) and the results presented here suggest that interesting strains among wild *A. subrufescens* may be identified that have both medicinal properties and high yield on commercial compost for *A. bisporus.*

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TAXONOMICAL SIGNIFICANCE OF MICROSTRUCTURES IN PURE CULTURES OF MACROMYCETES

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ABSTRACT

For the correct identification of the taxonomic position of cultures, a complex of morphological criteria are proposed by authors: presence and morphology of teleomorth stage; colour, morphology, and growth rate of mycelial colony; type of anamorph; presence, dislocation, and morphology of clamp connections; special hyphal structures and other characteristics. Using scanning electron microscopy, new data were obtained on microstructures of vegetative mycelium in more than 100 species.

Keywords: scanning electron microscopy, clamp connections, conidial sporulations

INTRODUCTION

Mushroom cultures are widely applied in biotechnology (production of fruit bodies, cultural mycelium, pharmaceutical substances, enzymes, etc.) and in various aspects of fundamental mycological studies [1, 2, 3, 4, 5, 6a]. Like in most other fungi, the vegetative mycelium of mushroom cultures is a complex of branched hyphae, which differ only within narrow limits of width, length, number of nuclei, thickness of cell walls, and the character of branching. On the basis of statistical evaluation, some authors [7] conclude that the vegetative mycelium is similar in different groups of fungi, but its characteristics cannot be used as reliable taxonomic features. However, continuous accumulation of information on an increasing number of fungal species provides new material for study and allows the comparison of morphological characters and for the estimation of their potential use for taxonomic purposes and purity control in biotechnological processes [1, 8, 9, 6b].

Culture collections today store important components of medicinal mushrooms for carrying out fundamental studies and biotechnological application of medicinal as well as culinary mushrooms. A long-term study was carried out using strains from the Culture Collection of Mushrooms (IBK) of the M.G.Kholodny Institute of Botany, National Academy of Sciences of the Ukraine (Kiev), and the Culture Collection of Higher Basidiomycetes of the International Center for Cryptogamic Plants and Fungi, Institute of Evolution, University of Haifa (HAI), Israel. [10, 11].

Good representation of various species in these culture collections include the following genera: *Agaricus*, *Pleurotus*, *Lentinus*, *Coriolus*, *Coprinus*, *Morchella*, *Ganoderma*, *Lycoperdon*, *Piptoporus*, *Oudemansiella*, *Flammulina*, *Hericium,* etc. [10].

MATERIALS AND METHODS

Vegetative mycelial microstructures were studied using a scanning electron microscope (SEM). Mushroom cultures were grown on wort agar or malt-extract agar in Petri dishes. On inoculation of a Petri dish, five-to-seven sterilized square 4×4 mm cover glasses were aseptically placed 1–6

cm away from the inoculum. Petri dishes were incubated at 26□C. When the cultural mycelium grew over the cover glass surfaces, the cover glasses were removed from the surface of the agar media and transferred to microscope slides. The microscope slides were then placed into a sealed glass vessel to fix the mycelium with osmium tetroxide vapour (1% solution) for 96 h. On fixation, the slides were transferred to an empty Petri dish to dry for 72 h. After drying, samples were covered with gold in a vacuum spray gun JII–4X with rotation. The specimens were examined using a Scanning Electron Microscope JEOL JSM-6060 LA (Jeol, Japan) and studied at a magnification from × 100 to × 18.000 [8].

RESULTS AND DISCUSSION

Vegetative mycelium of mushroom species investigated in pure culture consisted of thin-walled, septated, and branched hyphae. The diameter of generative hyphae varied between 1.5 to 7.5 μm. In *Agrocybe aegerita* (V. Brig.) Singer, *Auricularia auricula-judae* (Bull.) Quél, *A. polytricha* (Mont.) Sacc., *Coriolus zonatus* (Nees) Quél., etc., cultures were thin (≤ 1μm wide), with no branching hyphae. In the younger part of *Grifola frondosa* (Dicks.) Gray mycelial colonies that branched, thin (≤ 1μm wide) hyphae (dichohyphidia) were formed. In the older parts of a mycelial colony, thin (≤ 1μm wide) non-branched hyphae and generative hyphae 3-7 μm wide were observed. Also, non-branched, aseptate, or secondary septa sceletal and sceletoid hyphae without clamp connections and with thick-walled cells occurred.

In mushroom cultures, a great diversity in hyphal morphology was described and some of the forms observed may have taxonomic importance (Fig. 1, 2). A few suggestions for classification of hyphae on the basis of their physiological role, type of branching, cell wall thickness, presence of aggregates on the surface or inside the cells, etc., were made. On the mycelia, different types of bristles, spines, swellings, bulbs, hyphal tangles, monilial hyphae, and gloeocystidia formed, some of which may be useful for the morphological characterization of cultures permitting the identification of fungal species. Stalpers [9] presented a description of 26 types of hyphal modifications, though many of them, in our opinion, are hardly distinguishable.

Figure 1: *Oudemansiella brunneomarginata* Lj. N. Vassiljeva. Teleomorph on agar media (MEA)

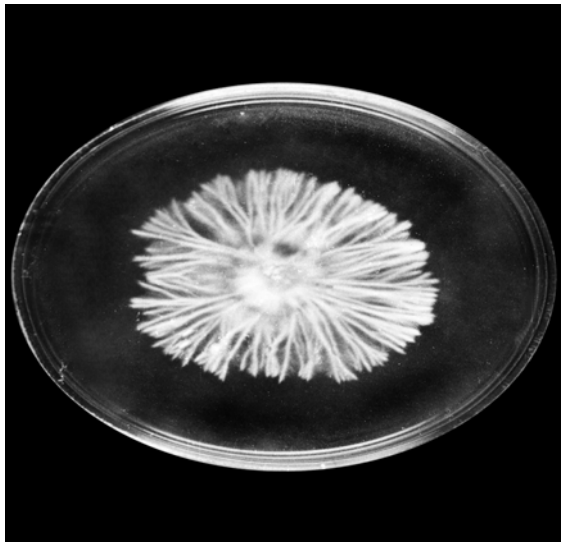

Figure 2: *Lacrymaria velutina* (Pers.) Konrad & Maubl. Mycelial colony on agar media (MEA)

The presence of dolipore septa was an important criterion for the identification of cultures belonging to the higher Basidiomycetes. Dolipore septa were present between the cells. For the dikaryotic mycelia, the occurrence of clamp connections was typical. Clamp connections were

absent on primary monokaryotic mycelia that started from single spores or may have disappeared under favorable conditions of cultivation in liquid media [1].

Anastomoses formed between hyphae in all investigated species and strains. In some cases, numerous anastomoses were expected. In old parts of mycelial colonies, anastomoses between hyphae and clamps were typical. In our opinion, anastomoses are of no taxonomical significance.

Hyphal ornamentation from the genus *Lyophyllum,* observed under SEM, may serve as taxonomic characters. Warty ornamentations were detected in *Oudemansiella brunneomarginata* Lj. N. Vassiljeva and *O. mucida* (Schrad.) Höhn. on hyphae forming loops. Very typical lacunose structured hyphae were described in some species of *Morchella* (Ascomycetes) *M. angusticeps* Peck, *M. conica* Pers., *M. crassipes* (Vent.) Pers., (*M. esculenta* (L.) Pers, *M. semilibera* DC.

In cultures of *Coprinus cinereus* (Schaeff.) Gray, *Crinipellis shevczenkoi* Buchalo, *Agaricus gennadii* (Chat. et Boud.) P.D. Orton, *Leucocoprinus bresadolianus*, etc. sclerotia of various shapes, sizes, and structural forms were present.

Strand-like mycelial cords were found in cultures of some species of *Agaricus*, *Macrolepiota*, *Omphalotus olearius*, and some Gasteromycetes species (*Phallus impudicus* L., *Tulostoma brumale* Bertero).

So called hyphae coils were detected in the *Oudemansiella brunneomarginata*, *O. mucida,* and *Tricholoma mongolicum* S. Imai mycelial cultures. The occurrence of coils in the mycelium is a new characteristic that has not yet been described in the literature. It is possible that similar structures will be found in other groups of fungi.

The presence of crystals on hyphae of mushroom cultures was reported in the literature [1, 8, 11, 12, 9]. Calcium oxalate crystals were formed on hyphae under cultivation in different nutritional media (agar and liquid media, grain, compost, etc.) and represented a relatively stable characteristic of the cultures. Oxalic acid represents one of the main metabolites of the Krebs cycle in living organisms [12]. Crystal formation was observed in all species of *Agaricus* investigated. Two types of cristals on a hyphae of *Agaricus brasiliensis* Wasser, M. Didukh, Amazonas & Stamets crystals were obsirved (Fig. 3 A,B). The density of crystals on the surface of hyphae may vary. Different stages of crystal formation could also be observed. As a rule, crystals cover the hyphae and were rarely found separated from the cells.

The morphology of the crystals was very different. We observed cubic, hexahedral, pyramidal, bipyramidal, prismatic, rod-shaped, and acicular crystals.

Polygonal crystals and crystals of other shapes were observed in *Armillariella mellea* (Vahl) P. Karst., *Hericium erinaceus* (Bull.) Pers., *Hypsizygus marmoreus* (Peck) H.E. Bigelow, *Kuehneromyces mutabilis* (Schaeff.) Singer & A.H. Sm., *Lentinus edodes*, *Omphalotus olearius*, *Pholiota adiposa* (Batsch) P. Kumm. etc.

On *Agaricus subfloccosus*, *Coprinus comatus* (O.F. Müll.) Gray and *Montagnea arenaria* (DC.) Zeller hyphae, thin filamentous hair-like crystals were observed.

In *Lentinus edodes*, crystals formed on the hyphae under cultivation on different nutritional media (agar and liquid media) and presented a relatively stable characteristic of cultures. The morphology of the crystals varied, and could be rhomboid and amorphous. Variously shaped crystals (needle-like, rod-shaped, cubic-like, etc.) formed on hyphae in different, mainly aged, parts of *Omphalotus olearius* colonies.

Clamp connections are characteristic features of dikaryotic mycelia of many Basidiomycetes. The presence and dislocation of clamp connections on hyphae are essential taxonomic characteristics for some species. In addition to the presence of clamps in identifying Basidiomycetes cultures, form, size, and frequency of occurrence were also considered. Clamps can be divided into large or small, long or short, gentle or abrupt, and curved or medallion-types based on the ratio of clamp size to hyphal diameter, the angle of a clamp and hyphae, and the

presence or absence of a slit between a clamp and a septum [9]. Some species have clamps of an original form, namely, *Auricularia auricula-judae*, *Lyophyllum decastes* (Fr.) Singer, *Lyophyllum ulmarium* (Bull.) Kühner (Fig. 4), *Oudemansiella mucida*, *Panus tigrinus* (Bull.) Singer, *Piptoporus betulinus* (Bull.: Fr.) P. Karst. etc., and were characterized with clamp connections of various forms and sizes. In *L. decastes* and *Piptoporus betulinus*, besides single clamps, whorls of clamps, coupled clamps, and sprouted clamps were observed. Clamps on *P. betulinus* hyphae were rather variable in shape and size. Sprouted, coupled, and single clamps were also found on the mycelium of *Pleurotus ostreatus* (Jacq.) P. Kumm. In *Panus tigrinus*, the clamps were mostly single or coupled, and only seldom sprouted clamps were present; some clamps were asymmetrical.

In *Coprinus comatus*, a representative of Agaricales, clamps were mostly single, of medallion type and seldom without a slit. Their form was rather stable and uniform. In *Marasmius oreades* (Bolton) Fr., belonging to the same order, mostly single clamps of a relatively stable form were observed. The medallion type clamps occurred rather frequently.

Single clamps (except in pairs and whorls) were characteristic for the mycelium of *Cyathus olla* (Batsch) Pers. and *C. striatus* (Huds.) Willd. Some clamps forming anastomozes with adjacent hyphae occurred.

It is widely accepted, however, that clamps are not common in all species of Agaricales. They are constant in cultures of *Pleurotus*, *Coprinus*, *Oudemansiella*, *Panus*, *Lentinus*, and *Pholiota*. Clamp connections were observed in *Agaricus campestris* L., *A. subperonatus* (J.E. Lange) Singer, *A. arvensis* Schaeff., *A. bernardii* Quél., *A. comtulus* Berk. et Broome. The majority of authors noted that clamp connections occurred very rarely in vegetative mycelium of *Agaricus*. Clamps were found in *Agaricus brasiliensis* Wasser et al.,

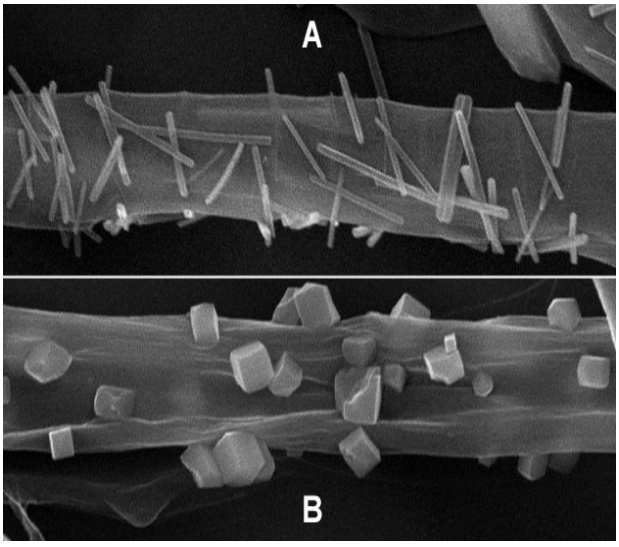

Figure 3: *Agaricus brasiliensis* Wasser, M. Didukh, Amazonas & Stamets. SEM (× 4800)

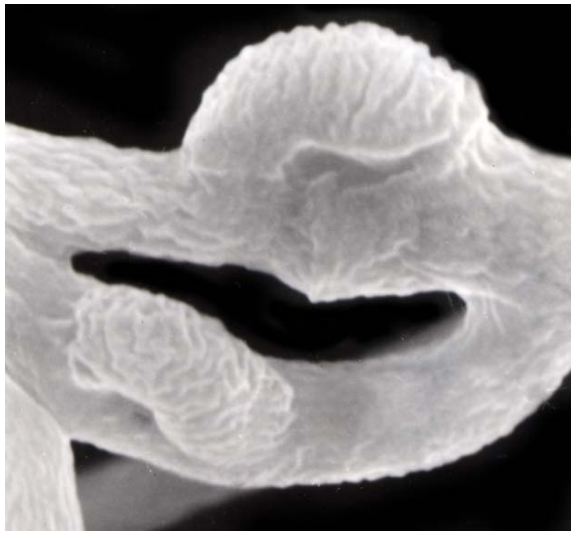

Figure 4: *Lyophyllum ulmarium* (Bull.) Kühn. Clamp connections. SEM (× 10000)

A. campestris L., *A. gennadii*, *A. nevoi* Wasser etc. Clamps seldom occurred, they were mostly of a classical shape, often without a slit. However, in many species, even the SEM is unable to detect any specific morphological features of a clamp.

Species of macromycetes form different structures of asexual reproduction (anamorphs). Anamorphs, mainly conidial sporulation, may serve as taxonomic criteria for species or

sometimes at higher taxonomic levels. Even though the first study on asexual reproduction of mushrooms was provided by Brefeld [1], more detailed studies only began relatively recently. For most macromycetes species, anamorphs have not been revealed yet. Taking into account various taxons, the most attention was given to Aphyllophorales, whereas Agaricales, Boletales, Gasteromycetes and Pezizales were not studied in detail. In general, chlamydospores and arthrospores were the most common asexual reproduction structures of Basidiomycetes [13, 14, 15, 9, 16].

Anamorphs in cultures of macromycetes have been studied mostly using light microscopy. We presented a more detailed description of anamorphic structures using scanning electron microscopy [1, 17, 18, 19, 10, 8, 20, 21].

Arthroconidia have been found in the mycelial cultures of *Oudmenasiella brunneoincarnata*. Conidial sporulations (arthroconidial structures) were also found for *Agaricus arvensis*, *A. cupreobrunneus* **(**Jul. Schäff. & Steer) Pilát, *A. fisuratus* (F.H. Möller) F.H. Möller, *A. maskae* Pilát, *A. silvaticus*, *Hypsizygus marmoreus*, *Lepista nuda*, *Lyophyllum ulmarium* (Bull.) Kühner, and others (Fig. 5, 6, 7). Arthroconidia were formed by the increase in age of the protoplast.

Two coremia-forming species (*Pleurotus abalonus* Y.H. Han, K.M. Chen & S. Cheng and *P. cystidiosus* O.K. Mill.) were studied using the SEM. In our ultrastructural study, we found no difference between the imperfect states of *P. abalones* and *P. cystidiosus*, which were identical to *Anthromycopsis broussonetiae*. Coremia formation on colony surfaces began as a little tangle of sterile hyphae which turned into a clavarioid form. In the following stages, the growing coremia differentiated into the head and the stipe, and on the head chains of alantoid conidia 12-20×4-7 μm formed. No conclusive difference in the process of coremia formation; their size and shape as well as the shape and size of conidia between the two studied organisms were determined [21].

The blastic type of the anamorohic stage was more common in Aphyllophorales than Agaricales. Blastoconidia in *Fistulina hepatica* Schaeff.: Fr. were formed on conidiophores either individually or in a chain (Fig. 8); chlamydospores were intercalary, lemon-shaped. *Pholiota adiposa* (Batsch) P. Kumm. anamorphs were similar to the conidia described from *Ph. aurivella* Singer and *Ph. nameko* (T. Ito) S. Ito et S. Imai cultures [15, 16]. *Ph. adiposa* conidia developed on short branches arising from hyphae. Our observation of conidia formation in *P. adiposa* confirms the opinion that they are arthroconidia.

In *Coprinus spp* the branched conidiophores bearing conidial structures were described (Fig. 9). We studied the fine structure of those "conidia" and showed that at the tips of conidiophore branches, no real conidial cells were formed. The tips of conidial branches ended with a tuft of fine, radially outgrowing hairs that gave at low magnification the impression of round verrucose conidia. However, the real nature of those structures was revealed at higher magnification. The possible role of the structures has yet to be elucidated.

Single globose conidia, which are termed by some authors as blastoconidia, pseudoconidia, or excretory conidia [22] on simple conidiophores, resembling a sterigmata of the basidium, were laterally formed on hyphae in cultures of *Pleurotus* spp. and *Schizophyllum commune* Fr.: Fr. They were globose, 3-5 μm in diameter.

Terminal and intercalary chlamydospores in dicaryotic *Hericium erinaceus* cultures, and dichohyphidia and intercalary chlamydospores in the dicaryotic vegetative stage of *Grifola frondosa* were of taxonomic significance [18, 8].

Conidial sporulations of the oidium type (budding cells) were characteristic of some species belonging to Morchellaceae (Ascomycota) (Fig. 10).

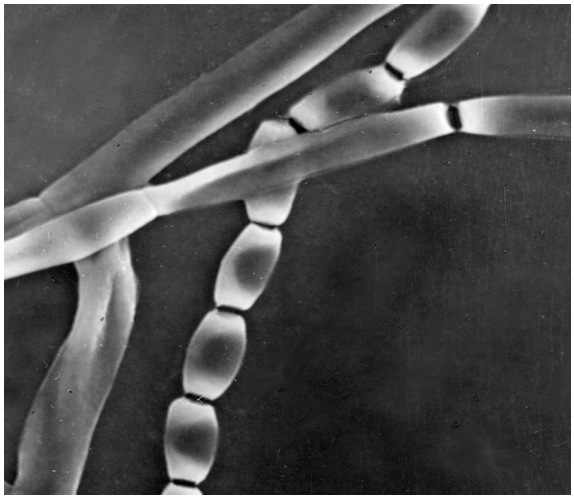

Figure 5: *Lepista nuda* (Bull.) Cooke. Conidial sporulation. SEM (× 1600)

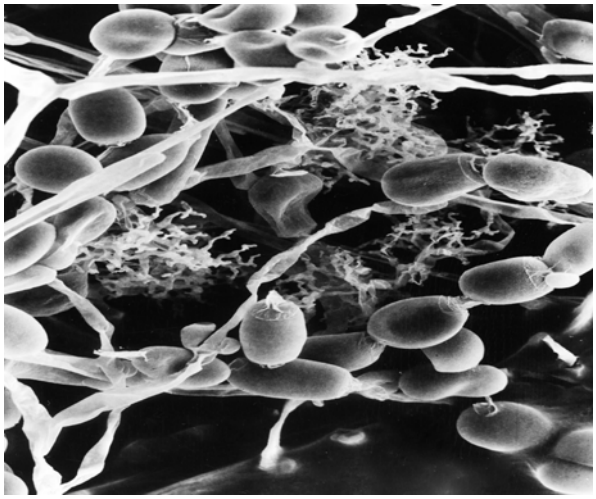

Figure 6: *Laetiporus sulphureus* (Bull.) Murr. Conidial sporulation. SEM (× 1800)

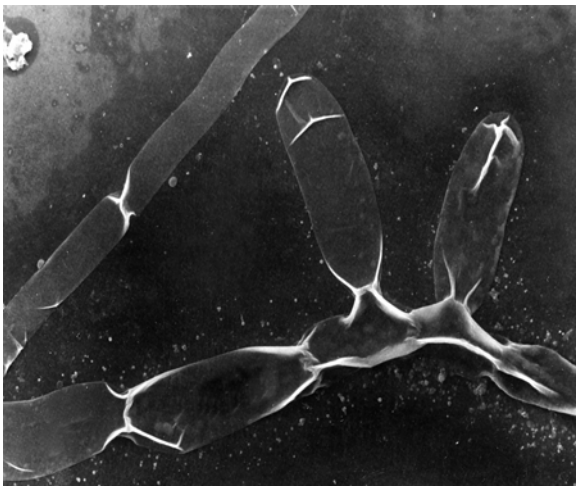

Figure 7: *Morchella esculenta* (L.) Pers. Conidial sporulation. SEM (× 940).

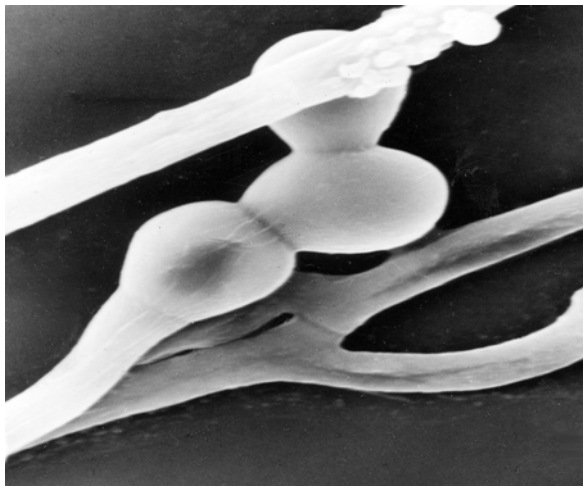

Figure 8: *Fistulina hepatica* (Schaeff.) With. Conidial sporulaion. SEM (× 4000)

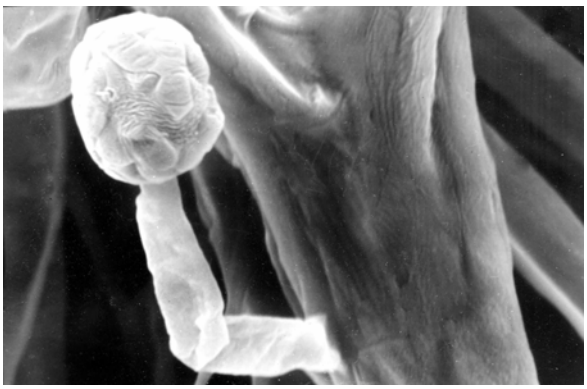

Figure 9: *Coprinus cinereus* (Schaeff.) Gray. Sporangium-like anamorphic structure. SEM (×4000)

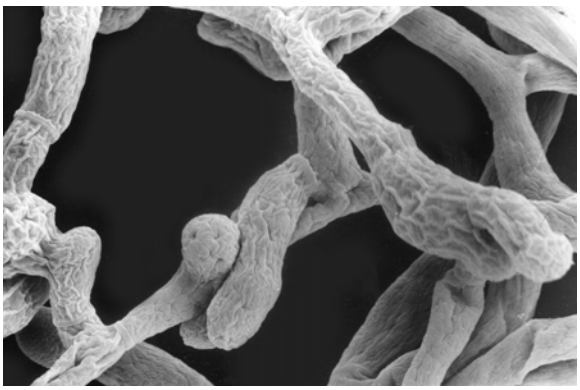

Figure 10 : *Morchella conica* Pers. Budding cells on lacunose hyphae. SEM (× 2000)

Chlamydospores were also discovered in cultures of *Agaricus arvensis*, *A. bisporus* (J.E. Lange) Imbach, *Auricularia auricula-judae*, *A. polytricha, Boletus edulis* Bull., *Calvatia excipuliformis* (Scop.) Perdeck, *Clitocybe gigantea* (Sow.: Fr.) Quél., *Coriolus zonatus*, *Hypsizygus marmoreus*.

In the Ukrainian culture collection of edible and medicinal mushrooms, a diversity of species (about 200), genera (about 100), and strains (about 800) is represented, including over 100 species of medicinal mushrooms. Using scanning electron microscopy about 100 species were studied and the taxinomycal significance of different microstructures is discussed.

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LIGHT-STIMULATIVE EFFECTS ON THE CULTIVATION OF EDIBLE MUSHROOMS BY USING BLUE LED

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ABSTRACT

Fruiting body formation of mushrooms is closely involved with “light”, which seriously affects their productivities in both quality and quantity. Primordium formation in several cultivable mushrooms requires light and seldom occurs under continuous darkness. Light also induces the development of fruiting bodies including stipe elongation and cap formation. LED (light emitting diode) has many advantages over current lightings and has been gradually replaced everywhere in recent years. These prompted us to develop more effective lightings in the cultivation of mushrooms, by using blue LED. The cultivations of popular mushrooms eaten in Japan (*Lentinula edodes*, *Flammulina velutipes*, *Hypsizygus marmoreus*, *Grifola frondosa*, *Pholiota nameko* and *Pleurotus eryngii*) were carried out with mushroom blocks (sawdust substrate media) under several lighting conditions. The exposure to continuous light by blue LED during vegetatively growing mycelial stage in *L. edodes* brought higher productivity and quantity of fruiting bodies than using usual white fluorescent lamps. The characteristic fruiting bodies of *P. nameko* and *P. eryngii* were efficiently obtained by the irradiation with blue LED during fruiting body formation, and high intensity at the primordial stage in *G. frondosa* caused high productivity of fruiting bodies, which were derived from highly induced primordia. Moreover, the surface exfoliation of cultivated mushroom-blocks, a serious issue especially found in *F. velutipes*, was successfully avoided by the exposure to blue LED at primordium formation. We present that several conditions of light environment artificially controlled by LED, a new valuable device, provide us more efficient production of cultivable mushrooms.

Keywords: Light; Blue LED; Fruiting body formation; Cultivation; Edible mushrooms

INTRODUCTION

"Light" is closely involved with fruiting body formation of mushrooms. In particular, the productivities of several cultivable mushrooms have been found to depend on light environment in both quality and quantity. Primordium formation in several cultivable mushrooms such as *Lentinula edodes* (black mushroom, *shiitake*), *Flammulina velutipes* (winter mushroom, *enoki* mushroom), *Hypsizigus marmoreus* (*buna-shimeji*), *Grifola frondosa* (*maitake* mushroom) and *Pleurotus ostreatus* (oyster mushroom) requires light, and seldom occurs under continuous darkness [1-5]. Light also induces the development of fruiting bodies including stipe elongation and cap formation. These phenomena have been also acquired through the continuous processes of trial and error in ordinary mushroom cultivations. An attentive control of the light environment during mushroom cultivation is effective in the improvement of availabilities of valuable products of fruiting bodies.

To perceive environmental light stimuli essential for the initiation of fruiting development in mushrooms, sensory factors and/or machinery such as photoreceptors are believed to be necessary. Our group has analyzed several photoreceptors and photoresponsive factors, which are involved with fruiting body formation in mushrooms. In the basidiomycetous mushroom *Coprinopsis cinerea*, *dst1* and *dst2*, which were evidenced to be involved with photomorphogenesis, were genetically analyzed in detail [6-8]. The PHRA protein homologous to the *C. cinerea dst1* product, a blue-light photoreceptor in *L. edodes*, was identified as a resident protein containing a photo-reactive domain responding to light stimuli essential for fruiting development [9]. The photoreceptor complex of PHRA and PHRB likely regulates the transcription of the tyrosinase gene, whose product makes fruiting bodies turn brown [10]. Two developmental regulators in *L. edodes*, PRIB [11] and Le.CDC5 [12], are photoresponsive transcription factors and those abundant expressions completely coordinate with fruiting body formation in response to blue light [13]. PRIB and Le.CDC5 are suggested to be involved with fruiting development because of their binding activities to specific DNA sequences and the phosphorylation of those by protein kinase A [12,14].

In several cultivation houses for mushrooms, there is a variety of brightness because required artificial lightings are usually set up on the ceiling. Synchronized cultivation through fruiting development, which directly influences productive performances, is inhibited by the uneven luminous intensities under such a condition. To ensure the cultural synchronizations of fruiting development stages (mycelium, primordium, fruiting body, etc.), many cultivators usually give a great care to lighting environment in their cultivation houses.

LED (light emitting diode) has many advantages over current lightings and has been gradually replaced everywhere in recent years. Generally, lighting equipments using LED are compact and it is easy to install desirable lightings on each shelf of mushroom cultivations. The detailed advantages of LED are follows:

(i) Saving electricity: The required electricity emitting the same intensity as usual fluorescent lamps illuminate is low. It can be expected to reduce a waste of electricity.
(ii) Long life: The frequency of replacing bulbs is extremely lower than fluorescent lamps.
(iii) Small and lightweight: As described above, installing lightings on each shelf can diminish places with uneven luminous intensities.
(iv) Low generation of heat: High light-emitting efficiency causes less heat than usual fluorescent lamps. The problems, drying products and media by heat, are also settled.
(v) Single wavelength & its selectivity: Desirable wavelength of light can be selected because LED has a sharp wavelength peak.

(vi) Strong structure: LED bulbs are stronger than fluorescent lamps.

The attempt to adopt LED lightings as the equipment in mushroom cultivation has already progressed. However, there are few analytical reports on optimal or favorable LED usages: improvement of light conditions (light wavelength, strength, timing of irradiations), designs of LED devices, installing LED on shelves, etc.

In this study, we describe the experiments for edible mushrooms cultivated in Japan, *L. edodes*, *F. velutipes*, *H. marmoreus*, *G. frondosa*, *Pholiota nameko* and *Pleurotus eryngii*, using blue LED, whose techniques provide us several valuable merits including further efficient production, change of fruiting body shapes, low electricity in cultivation houses.

MATERIALS AND METHODS

Mushroom strains. The following commercial strains harboring favorable characteristics were used: (i) *L. edodes*: 607 (Hokken Co., Ltd.), XR1 (Mori & Company, Ltd.); (ii) *F. velutipes*: G-5 (Nagano-Nokoken); (iii) *H. marmoreus*: NN-12 (Nagano-Nokoken); (iv) *P. nameko*: KX-N007, KX-N008, KX-N009 (Kinokkusu Corporation); (v) *G. frondosa*: 51 (Mori & Company, Ltd.); (vi) *P. eryngii*: Nara PE2 (Nara prefecture).

Lighting devices. The LEDs used in the experiments are as follows: Red, light emission 580-660 nm, maximum at 631 nm; yellow, light emission 550-630 nm, maximum at 597 nm; Blue, light emission 420-550 nm, maximum at 463 nm; Green, light emission 470-600 nm, maximum at 517 nm. Those LEDs were suitably designed for mushroom cultivation, and the lighting devices were specially constructed with waterproof treatment (Panasonic Corporation). The usual white fluorescent lamp (FL15N, Panasonic Corporation) was also used as the ordinary current lightings. Contained UV wavelength was omitted from white fluorescent lamp by the specified exclusive filters (RuRu Corporation). The intensities of those illuminations were measured with both the LI-190 quantum sensor and the LI-200SA pyranometer, which were connected to the LI-250 light meter (LI-COR, Inc.), and were adjusted by regulating an electrical current in combination with the light diffusion sheet (KIRYU Corporation).

Media and cultivations. The cultivation experiments under the following conditions for individual cultivable mushroom strains were carried out. (i) *L. edodes*: Medium blocks containing sawdust, rice- and wheat-bran were 1kg in weight with appropriate moisture content (62%). After autoclaved, the inoculated blocks were incubated at 21 °C for vegetative mycelium growth and then at 17 °C for fruiting under several lightings conditions; (ii) *F. velutipes*: The inoculated medium bottles were incubated at 16 °C and were irradiated with several lightings (blue LED, white fluorescent lamp or continuous darkness) under various durations of light. The fruiting body formation was carried out at 5 °C with 80 % humidity; (iii) *H. marmoreus*: The durations of light were divided into four terms (0-20, 20-40, 40-64, 60-80 days after inoculation). Cultivations were carried out under several lightings such as blue LED, fluorescent lamp or continuous darkness. The used medium was constituted of the sawdust of Japanese cedar, rice-bran and several nutrients with appropriate moisture content (65%). The treatment for the inducing fruiting body formation was carried out at 15 °C with 100% humidity under various light conditions; (iv) *P. nameko*: The medium blocks constituting of sawdust and several nutrients were bottled and were inoculated after the sterilization by autoclaving. The cultivation was carried out at 20 °C

with appropriate moisture content (65%), under continuous darkness for 30 days after inoculation, and then the medium blocks in bottles were irradiated with blue LED or white fluorescent lamp. After continuous irradiation for 20 days, when the forming of primordia was confirmed, the surfaces of those bottles were divided into two types, with and without scratching (a physical treatment for primordium formation). The formation of fruiting bodies were carried out at 14 °C with at least 90% humidity; (v) *G. frondosa*: Inoculated medium blocks constituting of sawdust and several nutrients were incubated at 22 °C with 65% humidity for 4 weeks under continuous darkness, and then the treatment for fruiting body formation was carried out at 17 °C with 90% humidity under several light conditions. When pores on the bottom side of caps had spread to 2-3 mm, fruiting bodies were harvested. (vi) *P. eryngii*: From 4 days later after inoculation, those medium bottles were incubated at 27 °C under the irradiation with red, yellow, green and blue LED for 1day. The growth rates (mycelial extensions) on agar media under various light conditions were also measured. To test effective light conditions for fruiting body formation, vegetative mycelia in test tubes were irradiated with blue LED or white fluorescent lamp for 15 days after 4weeks of inoculation, and then the treatment for fruiting body formation was carried out. Phenotypes of produced fruiting bodies under several light conditions were compared.

RESULTS AND DISCUSSION

L. edodes. In the cultivation of *L. edodes* using medium blocks, half-opened houses with the combination lighting, both sun and artificial light, are widely used. These cultivation houses, however, usually waste quite a few electricity to keep an optimal air condition due to low insulation, and would be replaced by completely sheltered ones in the near future. Since it is an important issue for such closed rooms to regulate lighting and air conditions, LED, which has the advantages of waterproofing, high durability and low electricity over current lightings, has the potential for improving lighting environment in mushroom cultivation. Our group has been developing the practical application of lighting techniques using by LED in *L. edodes* cultivation [15].

At first, effective lightings by blue LED at vegetative mycelium stage were investigated (Table 1). The higher the intensity of light was, the better the yield of fruiting bodies became in both strains. The irradiation with blue LED was more efficient in the yield of fruiting bodies than white fluorescent lamp. Consequently, the blue LED irradiation during vegetative mycelium growth appears to produce fruiting bodies owing to high promotion of the formation of primordia.

Table 1: Produced fruiting bodies of *L. edodes* cultivated under various light conditions

Type of lighting	Fluorescent	Blue LED		
Intensity of light (μmol/m^2·s)	5.4	1.2	3.6	7.8
Weight (g)	328.6±69.3	331.7±38.7	336.0±24.7	340.2±28.7
The number of fruiting bodies	26.9±9.1	32.7±8.8	34.1±10.2	30.8±11.9
The number of valuable fruiting bodies	9.7±2.3	10.1±2.8	10.3±3.1	11.7±2.0

The irradiation with blue LED 470 nm (peak wavelength) at vegetative mycelium stage increased the productivity of fruiting bodies, whereas the continuous exposure of medium blocks to the same LED during fruiting body formation brought about a slight diminution in the yield of fruiting bodies. The alternative irradiations by 625 nm red or 520 nm green LED had no apparent

effect on the vegetative mycelium growth. The combined irradiation of blue LED at vegetative mycelia stage and usual fluorescent lamp during fruiting body formation succeeded in increasing the yield of valuable fruiting bodies, which are generally favored by the consuming public. In conclusion, the optimal intensity of the irradiation by blue LED during vegetative mycelium growth is estimated at 10.5 μmol/m^2·s.

In the case of the cultivation accompanied with the treatment by high temperature during vegetative mycelium growth, the blue LED irradiation brought about high yield of fruiting bodies.

To investigate the influence by UV, the cultivation under the irradiation with fluorescent lamps, from which UV wavelength was omitted by using the exclusive filters, was carried out. Nevertheless, there was no difference between with and without UV, suggesting that the wavelength around UV has no effect on fruiting body formation of *L. edodes* and that blue LED is sufficiently effective in use for the cultivation of *L. edodes*, even in the absence of UV.

H. marmoreus. For the production of highly valued fruiting bodies of *H. marmoreus*, it is important to adjust the size of cap diameters with hemispheric shape. In this study, we attempted to produce favorable fine arrays of the caps of *H. mormoreus* fruiting bodies by regulation of lightings in cultivation houses.

Generally, the adequate intensity of illumination in cultivation of *H. marmoreus* is estimated at 300-700 lx [16]. As experimental results, the growth of stipes was suppressed by blue light but was facilitated by yellow light. Obvious pigmentations of caps were observed under the irradiation of continuous light for at least 12 hr. The favorable colored caps were obtained effectively under blue light. Although there was no remarkable difference in the yield of fruiting bodies between blue LED and white fluorescent lamp, the duration of exposure to light was related with several characteristics of fruiting bodies such as cap diameters, stem lengths, etc. (Fig. 1).

Figure 1: Effects of the light durations on the shapes of *H. marmoreus* fruiting bodies.
The irradiation with light was done at 40-60 days (A) and 40-70 days (B) after inoculation. The cultivation without light during vegetative mycelium growth was also carried out (C).

By means of the regulation of lightings in this study, the duration till the initiation of fruiting body formation was successfully shortened. The numbers of fruiting bodies were also increased by the devised irradiations with either blue LED or fluorescent lamp; however, the irradiations at early stages of vegetative mycelium growth (at 0 or 20 days after inoculation) showed the tendency to contain unfavorable phenotypes of fruiting bodies (small diameters of caps, bad synchronization of fruiting). Unexpectedly, the yield of fruiting bodies by the irradiation with blue LED at 20 days after inoculation was significantly lower than with either fluorescent lamp or continuous darkness.

The irradiation with light at 40 days after inoculation shortened the duration of overall cultivation. Furthermore, the irradiation with blue LED at the last period of mycelial growth (60 days after inoculation) gave an optimal result in the yield of fruiting bodies. The optimal intensity of blue LED was 12 μmol/m^2·s, as well as the observation in the ordinary cultivation under white fluorescent lamps. UV irradiation had no remarkable effect on the productivity of *H. marmoreus* fruiting bodies.

F. velutipes. For effective cultivation of *F. velutipes* fruiting bodies, the fine-tunings of cap diameter and stipe length of fruiting bodies are generally required. The growth of caps in *F. velutipes* is known to be affected especially by blue light. In this study, the cultivations under various lightings were examined. The optimal intensity of illumination through cultivation was known as 70-150 lx [17]. Indeed, this optimal condition of environmental light gave similar productivities also in our study, even if the durations of light were arbitrarily interrupted. The surface exfoliation of cultivated mushroom-blocks, a serious issue especially found in *F. velutipes*, was successfully avoided by the exposure to blue LED at the primordium formation stage (Fig. 2).

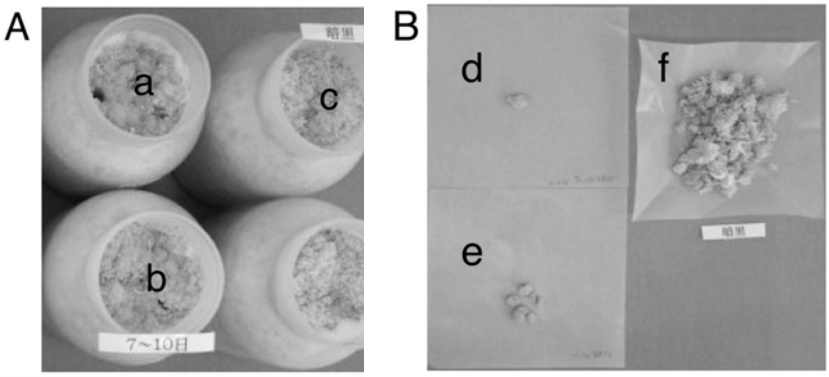

Figure 2: The surface exfoliations of the cultivation bottles of *F. velutipes*.
A: Medium bottles cultivated under blue LED (a), with fluorescent lamp (b) and continuous darkness (c).
B: The exfoliated medium blocks (d), (e), (f) were derived from the bottles (a), (b), (c), respectively.

The adequate irradiations with either blue LED or white fluorescent lamp during vegetative mycelium growth provided good productions and the yield of fruiting bodies. No obvious difference in several favorable characteristics, such as stem length, stem diameter, stem abnormality, cap diameter, and cap thickness, was observed in the harvested fruiting bodies under the examined conditions using various lightings. Consequently, the lighting of blue LED brought approximately the same productivity as usual fluorescent lamp. The longer medium bottles were irradiated with blue LED or white fluorescent lamp in particular during the late stage of vegetative mycelial growth, the higher the yield of *F. velutipes* fruiting bodies became. Our results suggest that light irradiation during the initiation of fruiting body formation has the remarkable effect on avoiding surface exfoliations of medium blocks; however, such irradiations also bring about slightly lower yield of fruiting bodies. UV had no remarkable effect on fruiting body formation of *F. velutipes*, as well as other examined mushrooms.

***P. nameko*.** Most of the cultivations of *P. nameko*, whose glutinous character is appreciated in Japan, are carried out in controlled cultivation houses using medium blocks in bottles. Our group

has been developing effective lightings for *P. nameko* cultivations [18]. Under the slight intensity of light (0.01 lx), the shape of *P. nameko* fruiting bodies revealed an aberrant phenotype similar to the etiolated seedlings of soybean, suggesting that a certain intensity of light is essential for the *P. nemako* cultivation. The increase of light intensity gave dark brownish caps of fruiting bodies, suggesting the relationship between the intensity of environmental light during cultivation and the pigmentation of *P. nameko* fruiting bodies. The optimal intensity of light through cultivation was estimated at 0.2-10 lx, which gave efficient production of favorable and valuable fruiting bodies.

The irradiation with blue LED at the early stage of vegetative mycelium growth brought about higher production of primordia, however, most of those primordia could not grow up to mature fruiting bodies, implying that the timing of the irradiation of light is likely to affect the characteristics of fruiting body development of *P. nameko*. The irradiations with either blue LED or fluorescent lamp during the late stage of vegetative mycelium growth succeeded in the effective induction of primordial formation, and were capable of producing large fruiting bodies for short term (Fig. 3). Our results suggest that blue LED has the potential to control the morphogenesis of fruiting bodies of *P. nameko*. Although the yield of fruiting bodies under the irradiation with blue LED during vegetative mycelium growth was generally higher than usual white fluorescent lamp, the irradiation with blue LED during fruiting body formation had the opposite effect and decreased the yield of fruiting bodies. UV had also no obvious effect on fruiting body formation of *P. nameko*.

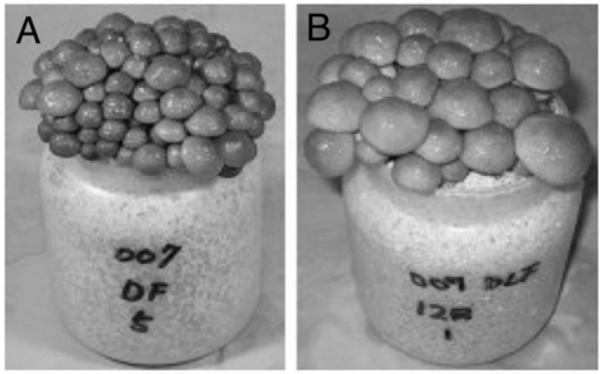

Figure 3: Effects by blue LED on fruiting bodies of *P. nameko*. A: Fruiting bodies without light irradiation during mycelial growth. B: Produced fruiting bodies after the irradiation with blue LED for 12 days at late stage of mycelial growth.

G. frondosa. There are various public demands for colors and shapes of *G. frondosa* fruiting bodies. Light environment through cultivation has been understood to be capable of controlling favorable characteristics of generated fruiting bodies in *G. frondosa*. In this study, several periods of time for the beginning of cultivation, primordial formation, initiation of fruiting body formation and harvest of fruiting bodies, were investigated under various light conditions using blue LED and usual fluorescent lamp. The suitable intensity of light (approximately 13 $\mu mol/m^2 \cdot s$) brought about both the fastest primordium formation and the highest yield of fruiting bodies among all examined conditions. The exceedingly aberrant phenotype of primordia was observed under continuous darkness (Fig. 4). Although those media could generate relatively small primordia subsequently to the cold treatment for initiation of fruiting body formation,

grown-up fruiting bodies derived from those small primordia still retained obviously defect phenotypes with fragile tissues. The light irradiation till primordium formation had no obvious effect to the pigmentation of the upper surface of fruiting bodies. Consequently, light is likely to be indispensable to the primordium formation in *G. frondosa*.

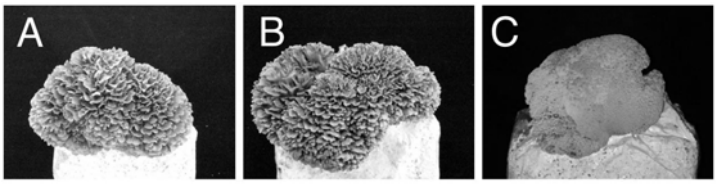

Figure 4: Fruiting bodies of *G. frondosa* developed under various light conditions. A: Using white fluorescent lamp. B: Using blue LED. C: Without light.

The culture of vegetative mycelia under continuous darkness was effective for fruiting body formation, whereas slight intensity of light at the initiation of primordial development was observed to be essential for good production of fruiting bodies. The irradiation with blue LED during fruiting body formation gave higher production of fruiting bodies, however, the timing of harvest of fruiting bodies was later than using white fluorescent lamp. The irradiation with blue LED during vegetative mycelium growth had no effect on the pigmentation of fruiting bodies, while the surface of those fruiting bodies seemed to become fluffy. The irradiation with white fluorescent lamp omitting UV wavelength had no obvious effect on phenotype of fruiting bodies; however, the duration till harvest was longer and the yield of fruiting bodies was higher than using usual fluorescent lamp. It remains to analyze these phenomena.

P. eryngii. Most of cultivated fruiting bodies of *P. eryngii* have small caps and large stipes, because the consumers prefer those unique taste and food texture. To cultivate such fruiting bodies, only a few fruiting bodies per bottle are generally produced by means of thinning out small fruiting bodies [19]. In this study, several light conditions for mycelial growth, primordium formation and fattening up fruiting bodies were investigated, in order to contribute effective cultivation and production of favorable *P. eryngii* fruiting bodies.

Vegetative mycelium growth on agar media was inhibited by irradiation with light, especially with blue light. Moreover, the higher the intensity of light became, the slower mycelia grew, remarkably under either blue or white light. The strong intensity of light (30 μmol/m^2·s) highly inhibited mycelial growth (approximately 40% lower than the mycelial growth rate under usual growth condition). The light wavelength during fruiting body formation significantly affected morphology of fruiting bodies (Fig. 5). Although the spherical primordia under light grew up to mature fruiting bodies with usual stipes and caps, the primordia formed under continuous darkness developed characteristic long stipes. Consequently, both the extension of stipes and the inhibition of cap development were observed under continuous darkness, whereas light irradiation generally appeared to induce an appropriate extension of stipes and the progress of cap development. Light also facilitated the pigmentation of caps.

Figure 5: The phenotypes of *P. eryngii* fruiting bodies developed under various light qualities. The details of used LEDs are described in the Materials and methods. The usual fluorescent lamp (white) was also used.

The light irradiation at vegetative mycelia in bottles had no obvious effect on mycelial growth. However, at the initiation of fruiting body formation, the irradiation with light brought about remarkable differences in fruiting body production. After the light irradiation at primordium formation, the subsequent cultivation under continuous darkness could provide the highest yield of fruiting bodies among all experimental conditions. The later the light irradiation started, the longer the overall duration of cultivation became, under either blue LED or white fluorescent lamp. UV has no obvious effect on fruiting body formation of *P. eryngii*. The low intensity of light produced fruiting bodies with small caps and long stipes. After the formation of primordia, the longer the duration under continuous darkness was, the larger stipes became. The intensity of blue LED significantly influenced the phenotypes of produced fruiting bodies. The stronger the intensity of light was, the larger the obtained fruiting bodies became. The stronger the intensity of light was, the more darkened the pigmentation of fruiting bodies became. In conclusion, phenotypes of produced fruiting bodies of *P. eryngii* are likely able to be modified by means of the regulation of light environment through cultivation.

CONCLUSIONS

For cultivation of edible mushrooms, LED has many useful characters such as low electricity, ecological, etc., and is more effective than the ordinary current lightings such as white fluorescent lamp. The appropriate irradiation with blue LED is effective to increase the yield of fruiting bodies and to improve the productivity of high valued phenotypes, which are favorable to the consuming public. Consequently, UV irradiation has no apparent effect on fruiting body formation of the examined mushrooms. The attentive control of light environment for mushroom cultivation has the potential of further effective productions of fruiting bodies in cultivation houses in the near future.

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EFFECT OF COLOR OF LED (LIGHT EMITTING DIODE) ON DEVELOPMENT OF FRUIT BODY IN *HYPSIZYGUS MARMOREUS*

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ABSTRACT

We aim to elucidate suitable color of light during development of fruit body in *Hypsizygus marmoreus*. The four color of LED (Light Emitting Diode), blue LED (475nm), green LED (525nm), yellowed LED (590nm), and red LED (660nm), were irradiated for formation of fruit body after mycelia growth. As effect of color of LED at all growth stage, the diameter and thickness of pileus and length of stipe in blue LED treatment were similar to control (Fluorescent lamp), and length of stipe was the highest at the red LED and darkness. The commercial yields in blue and green LED treatment were similar to control (Fluorescent lamp). Second, as effect of color of LED by light irradiation time, we could obtain the highest commercial yields at the blue LED 1/2(lighting/lighting-out, hours). And ergosterol was the highest at the blue LED continuous radiation.

Keywords: Ergosterol, LED, Light, Mushroom

INTRODUCTION

The storage of *Hypsizygus marmoreus* excellent vantage points as the export model is equipped with items at Korea. In Korea, there is very little research about the effect on mushroom bottle cultivation by light. Most mushrooms require light to develop properly. Also, light is required for cultivation *of Hypsizygus marmoreus*. And the fluorescent lamps are installed to house a week after planting is often replaced if power outages are occurring frequently. Therefore, we applied to the LED light to the cultivation of edible mushrooms was investigated.

MATERIALS AND METHODS

Experiment mushroom. *Hypsizygus marmoreus(Mangadak 2#)*
Growth environment. Temperature 16±1℃, Relative humidity 90±5%, CO_2 concentration 800~1,200ppm
<Experiment 1>
Light source. Fluorescent lamp(positive control), Darkness(negative control), Blue LED(475nm), Green LED(525nm), Yellow LED(590nm), Red LED(660nm)

<Experiment2>

Light time. Continuous Light(control), 1/1(on/off, hours), 1/2(on/off, hours), 1/3(on/off, hours), 1/4(on/off, hours), 1/5(on/off, hours)
* Experiment Light : LED Light selected at Experiment 1

RESULTS AND DISCUSSION

<Experiment 1>

Fluorescent lamp Darkness BlueLED GreenLED YellowLED RedLED

Figure 1: Primordia photographs under the LED light of *Hypsizygus marmoreus*

Table 1: Effect of LED sources on properties of fruit body in *Hypsizygus marmoreus*

Light source	Diameter of pileus(mm)	Thickness of stipe(mm)	Length of stipe(mm)
Fluorescent lamp	24a[a]	12a	79c
Darkness	16c	10b	105a
BlueLED	22a	12a	68d
GreenLED	19b	10b	89c
YellowLED	14c	10b	98b
RedLED	16c	10b	103a

[a] Values followed by the same letter do not differ significantly at $p>0.05$ according to Duncan's multiple range test.

Table 2: Effect of LED source on yield properties of *Hypsizygus marmoreus*

Light source	No. of available stipes(No./850ml)	Yield (g/850ml)	Commercial yields(g/850ml)	Commercial yields Index[b](%)
Fluorescent lamp	28 ab[a]	148 b	122 ab	82
Darkness	31 a	156 a	114 b	73
BlueLED	29 ab	159 a	135 a	85
GreenLED	35 a	158 a	130 a	82
YellowLED	28 ab	129 c	96 c	74
RedLED	27 b	126 c	77 d	61

[a] Values followed by the same letter do not differ significantly at $p>0.05$ according to Duncan's multiple range test.
[b] Commercial yields / Yield × 100

Fluorescent lamp Darkness BlueLED GreenLED YellowLED RedLED

Fig 1: Morphological properties according to LED of *Hypsizygus marmoreus*

<Experiment2>

Table 3: Effect of blue LED light irradiation time on properties of fruit body in *Hypsizygus marmoreus*

Light irradiation time (lighting/lighting-out, hours)	Diameter of pileus(mm)	Thickness of pileus(mm)	Thickness of stipe(mm)	Length of stipe(mm)
Fluorescent lamp	19 d[a]	7.0 d	11.3 b	82 b
Continuous light irradiation	30 a	9.3 a	11.1 b	73 c
1/1	25 b	9.0 a	11.9 a	73 c
1/2	22 c	7.4 c	10.7 b	84 b
1/3	26 b	8.6 b	12.1 a	82 b
1/4	21 c	7.6 c	10.7 b	85 b
1/5	18 d	6.4 d	10.9 b	93 a

[a] Values followed by the same letter do not differ significantly at $p>0.05$ according to Duncan's multiple range test.

Table 4: Effect of blue LED light irradiation time on yield properties in *Hypsizygus marmoreus*

Light irradiation time (lighting/lighting-out, hours)	No. of available stipes(No./850ml)	Yield (g/850ml)	Commercial yields(g/850ml)
Fluorescent lamp	36 a[a]	140 c	111 c
Continuous light irradiation	22 c	169 a	130 d
1/1	30 b	152 b	128 bc
1/2	38 a	176 a	146 a
1/3	31 b	148 bc	126 bc
1/4	36 a	172 a	130 b
1/5	38 a	177 a	120 c

[a] Values followed by the same letter do not differ significantly at $p>0.05$ according to Duncan's multiple range test.

CONCLUSIONS

<Experiment 1> We selected blue LED that yield, commercial yields and commercial yields index higher than these properties of the other LED treatment.

<Experiment 2> We selected 1/2(lighting/lighting-out, hours) that yield and commercial higher than these properties of the other light irradiation time.

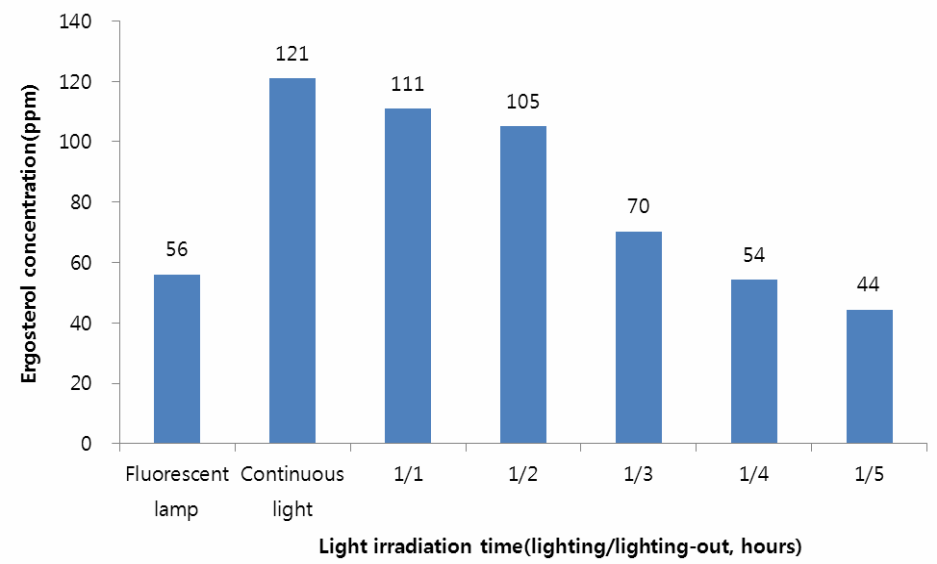

Fig 2: The ergosterol concentration of blue LED light irradiation time.

ACKNOWLEDGEMENT

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A DASH-TYPE CRYPTOCHROME GENE FROM *LENTINULA EDODES*

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ABSTRACT

Fruiting body formation of mushrooms, a characteristically morphological differentiation observed in eukaryotic microorganisms, is affected by several environmental factors such as temperature, light and humidity. Especially, light is known to be one of the most important environmental signals and influences morphogenesis. In *Lentinula edodes* (*Shiitake*), blue light is required for initiation of the fruiting body formation. Therefore, photoreceptors, which are capable of light stimuli, are important for adaptation to immediate environments. Blue light photoreceptors are generally divided into two distinct classes, phototropins and cryptochromes. We previously reported that PHRA/PHRB, a blue-light photoreceptor complex in *L. edodes*, could regulate the transcription of the tyrosinase gene in a light-dependent manner. The PHRA belongs to the phototropin family; no cryptochrome, however, has been identified in basidiomycetes.

In this study, we report the isolation of a cryptochrome-encoding gene in *L. edodes*, designated *Le.cry*. Le.CRY, the expression product of *Le.cry*, contained a DNA photolyase domain and a FAD-binding domain, and was homologous to dash-type cryptochrome proteins in the photolyase/cryptochrome family. Gene expressions of *Le.cry*, *phrA* and *phrB* were analyzed by quantitative RT-PCR during fruiting body formation (primordia, immature and mature fruiting bodies) and parts of fruiting body. The transcription levels of *phrA* and *phrB* were similar during fruiting body formation and in parts of fruiting body, whereas *Le.cry* showed the unique expression pattern. The differential expressions of *Le.cry* and *phrA/phrB* imply that Le.CRY might be involved in a light signaling pathway different from that mediated by PHRA/PHRB.

Keywords: *Lentinula edodes*; Shiitake mushroom; blue-light photoreceptor; cryptochrome dash; fruiting body formation

INTRODUCTION

Light is known to be one of the most important environmental signals for various organisms such as plant, fungi and bacteria, and regulates their developmental and physiological processes [1, 2, 3]. In many basidiomycetous mushrooms, light influence morphogenetic events and cytological events such as initiation of fruiting body formation, pileus development, oidia production, meiosis and mycelial growth. Especially, the wavelength range corresponding to blue light is one of the most important light wavelength regions [4, 5, 6]. *Lentinula edodes*, popularly called *Shiitake* mushroom, also required blue light for the initiation of fruiting body formation

and the pileus development, and complete darkness does not induce fruiting body formation and normal maturation of fruiting body.

Blue-light photoreceptors have been first identified in plant and are divided into two general classes, phototropins and cryptochromes (CRY) [7]. The most well-known blue-light photoreceptor in fungi is the phototropin-like protein White Collar-1 (WC-1) in the ascomycete *Neurospora crassa*, which is an essential component for all known blue-light responses in *N. crassa*, such as biosynthesis of carotenoid in mycelia, formation of vegetative spores and resetting of circadian clock [8]. In several basidiomycetes, WC-1 homologs were also identified in *Cryptococcus neoformans* [9], *Coprinopsis cinerea* [10] and *L. edodes* [11]. In *C. cinerea*, it was shown that a blue-light photoreceptor gene, *dst1*, is involved in fruiting body development [10]. Cryptochromes bearing no LOV domain show high similarities to microbial DNA photolyases and are widely distributed in eubacteria, archaea and eukaryotes [12, 13]. Cryptochromes belonging to the member of the photolyase/cryptochrome family exhibit no conventional photorepair activity but ultraviolet A (UV-A)/blue-light photoreceptor activity [12]. The photolyase/cryptochrome family can be classified into five distinct classes by phylogenetic and functional analyses: class I cyclobutane pyrimidine dimer (CPD) photolyase, class II CPD photolyase, plant CRY, animal CRY including (6–4) photolyases, and CRY-DASH family [13]. In ascomycetous fungi, several cryptochrome homologs ((6-4) photolyase and CRY-DASH family protein) were identified, and those functions were analyzed [14, 15, 16, 17]. However, molecular mechanism basics and signaling pathways mediated by cryptochromes are not well understood. Moreover, no cryptochrome in the basidiomycete has been reported.

This prompted us to isolate a cryptochrome gene from the basidiomycete *L. edodes* (named *Le.cry*) and to elucidate the relationship between cryptochrome and fruiting body formation by analyzing its detailed expressions in vegetatively growing mycelia and developmental tissues in the course of fruiting body formation. In this study, we demonstrate that the *Le.cry* gene play a role for mycelial development and might be involved in other light-signaling pathway during morphological differentiations, which is different from that mediated by both *phrA* and *phrB*, other blue-light photoreceptor genes in *L. edodes.*

MATERIALS AND METHODS

Fungal strains, media and culture conditions. A commercial dikaryotic (binucleate-celled) strain *L. edodes* A567 (Akiyama-Shukin Corporation, Japan) was used in the experiments. The vegetatively growing mycelia were cultured in MYG agar medium (0.4% glucose, 1% malt extract, 0.4% yeast extract and 1.5% agarose, pH 5.6) at 25°C for 40 days. Primordia, immature fruiting bodies and mature fruiting bodies were grown using on sawdust–rice bran medium. Small ball-like and hard clumps (less than 1 cm in diameter) that had not developed stipes and pilei were defined as primordia. Fruiting bodies containing no obvious pileus were defined as immature fruiting body, whereas those harboring completely developed pileus with gills were defined as mature fruiting body. After harvest, all samples were immediately frozen by liquid nitrogen and were subjected to the subsequent analyses.

Cloning of genomic DNA fragments containing the *Le.cry* gene. Genomic DNA was prepared from the *L. edodes* A567 strain according to the previous method [18]. Based on the conserved amino acid (aa) sequences of the DNA photolyase domains and the flavin adenine dinucleotide (FAD) binding domains in fungal cryptochromes, *N. crassa* CRY and *Sclerotinia sclerotiorum*

CRY1, degenerated primer DNAs were designed: 5'- GTIGGIGCIGTITGGATGACI-3' (primer A (forward)) and 5'- ICKCATRTARTCICKCCAIARIARYTC-3' (primer B (reverse)). PCR amplification was done using Blend Taq® (TOYOBO Co., Ltd.) under the standard condition. To obtain the genomic DNA fragment including the complete sequence of the *Le.cry* gene, subsequent inverse PCR was performed using TaKaRa LA Taq® (TAKARA Bio. Inc.). Two primers were designed based on the nucleotide sequences of the amplified PCR fragment: ATCCCAGACCTTCCACTCAATCTTTCCGTCGC (primer 1) and TGTCACCGCGAGACAG GCCAATGCATACATGG (primer 2). Then, the amplified genomic fragment was cloned into pTA2 vector and arbitrarily selected three clones were sequenced by ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

RNA extraction from *L. edodes.* Total RNAs were isolated from vegetatively growing mycelia, primordia, immature fruiting bodies and mature fruiting bodies using RNeasy midi kit (QIAGEN), according to the manufacturer's instructions.

Cloning of full-length cDNA of the *Le.cry* gene. To clarify the structure of the *Le.cry* gene, its cDNA was isolated by reverse transcriptase-PCR (RT-PCR), 5' rapid amplification of cDNA ends (RACE) and 3' RACE. One set of primers was designed based on the genomic sequences of the *Le.cry* gene: CTTCCTCCAAACCAAATCGAAGTATCAGG (primer 3) and TCGTGCGCG CTTGTCTCACC (primer 4). 3' RACE was carried out using Roche Diagnostics GmbH kit (Roche, Basel, Switzerland). Reverse transcription was done using primer C: GACCACGCGTATCGATGTCGAC$(T)_{\sim 20}$. Then, PCR with this reverse transcribed template was done using the following primers: GACCACGCGTATCGATGTCGAC (primer C') and GGATTGAGCGGGCCGTGAAGAAAGAGGGCG (primer 5). 5' RACE was carried out using the 5' RACE System (Invitrogen) according to the manufacturer's protocol. Specific primers of the *Le.cry* gene for 5'RACE were as follows: primer 4 and TGTCCCGAAGTGGTTCAAAGGA TTTTCGGAAGGAAG (primer 6). The 5' RACE and 3' RACE products were subcloned into pTA2 vector and were sequenced.

Phylogenetic analysis. The aa sequences of photolyase/cryptochrome family proteins for phylogenetic analysis were retrieved from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments were carried out using the ClustalW. Phylogenetic tree was constructed using the Neighbor-Joining algorithm in Molecular Evolutionary Genetics Analysis (MEGA) version 4 system.

Table 1. Primers and TaqMan Probes for quantitative RT-PCR

Target gene	Primer/probe	Sequence of oligonucleotide (5'-3')	Amplicon length (bp)	Amplification efficiency (%)
Le.cry	Forward primer	GCCTCCAATACCACCCAAAGT	117	99.9
	Reverse primer	GGTGGTCCGAGTCCACAATC		
	TaqMan MGB probe	FAM-CCTACCCAAGCTGC-MGB		
phrA	Forward primer	TTGTCTGTTCTGGCCCGAGTA	104	93.0
	Reverse primer	TCGTAGGGCCGAAGGTCTAT		
	TaqMan MGB probe	FAM-CTGCTCGACCAGACC-MGB		
phrB	Forward primer	ATCCAAGTCCACCTCCACAAC	127	97.9
	Reverse primer	GCTGGCGTTGTGGTTGAGT		
	TaqMan MGB probe	FAM-CACTTGTTGGGGTTTG-MGB		
β-actin	Forward primer	GTGCTCCTGAGGCCCTCTT	57	97.3
	Reverse primer	GGATTCCGGCACCTTCTAAA		
	TaqMan MGB probe	FAM-CAGCCTGCCTTCCT-MGB		

Analysis of gene expression by real-time PCR. Quality of total RNA was checked by Experion (Bio-Rad Laboratories, Inc.) according to the manufacture's instruction. First-strand cDNA synthesis as templates for real-time PCR was as follows. The same amounts (1 μg each) of total RNA samples were mixed with dNTP mix (each 500 μM) and Oligo $(dT)_{20}$ (500 nM), and volumes of the mixtures were up to 12 μl with distilled RNase-free water. After heated at 65°C for 5 min and quickly chilled on ice, dithiothreitol (10 μM), first-strand synthesis buffer for ReverTra Ace® (TOYOBO Co., Ltd.) and ribonuclease inhibitor (20 units, Wako) were added into the mixtures, and then the mixtures were incubated at 42°C for 2 min. After adding of ReverTra Ace® (100 units), the reaction mixtures (20 μl each) were incubated at 42°C for 60 min and the reactions were inactivated by heating at 98°C for 5 min. Real-time PCR was carried out using TaqMan® assay Fast PCR by StepOnePlus™ Real-Time PCR System (Applied Biosystems). The real-time PCR reaction was conducted in 20 μl consisting of 10 μl TaqMan® universal PCR master mix (Applied Biosystems), 1 μl of above first-strand cDNA mixture, 1 μl of gene-specific primer sets (each 10 μM), 0.5 μl of gene-specific TaqMan probes (each 5 μM) (these sequence was shown in table 1) and 7.5 μl of distilled water. The reactions were performed under the following condition: the pre-denaturing at 95°C for 20 sec (hold stage), the denaturing at 95°C for 1 sec, annealing and extension at 60°C for 20 sec (cycle stage). Expressions of genes were measured in triplicate. Furthermore, the amplified fragments were confirmed by 2.0% agarose gel electrophoresis and were detected as a single band in the predicted size (data not shown).

RESULTS AND DISCUSSION

Cloning of a cryptochrome-encoding gene in *L. edodes*. In the basidomycetous mushrooms, light affects morphological changes such as initiation of fruiting body formation, oidia production, maturation of fruiting body and hyphal aggregation [19, 20, 21, 22, 23, 24, 25, 26]. WC-1 homolog genes, *dst1* in *C. cinerea* and *phrA* in *L. edodes*, were identified as a blue-light photoreceptor-encoding gene in basidiomycetous mushrooms [10, 11], and the involvement of the *dst1* gene in fruiting body development is evidenced by genetical analyses [10]. However, the other photoreceptors such as cryptochromes, phytochromes and opsins have not yet been reported in basidiomycetous fungi. Recently, blue-light photoreceptor genes of the photolyase/cryptochrome family have been reported in several ascomycetous fungi such as *N. crassa* [15], *Aspergillus nidulans* (17), *Cercospora zeae-maydis* [14] and *S. sclerotiorum* [16]. This prompted us to investigate whether *L. edodes* has a cryptochrome-type photoreceptor and to analyze its expression in the course of fruiting-body formation and in parts of fruiting body.

Firstly, we carried out PCR using the degenerate primers and isolated the 740-bp genomic DNA fragment corresponding to the *Le.cry* gene, which encoded characteristic conserved aa sequences similar to the DNA photolyase domains and to the FAD-binding domains of DNA photolyase found in fungal cryptochromes. Based on the nucleotide sequence of the 740-bp DNA fragment, we carried out inverse PCR and identified 5.8-kbp *Bam*HI-*Bam*HI genomic fragment containing the complete structural gene of *Le.cry*. Then, its cDNA was isolated by RT-PCR and 5'/3' RACE. The *Le.cry* gene was found to encode 606 aa and to be interrupted by only one small intron (50 bp).

Comparison of Le.CRY and other fungal cryptochromes. Several domains contained in the expression product of *Le.cry* (Le.CRY) were characterized by Conserved Domain Database of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The deduced aa sequence of the Le.CRY protein contained characteristic domains for DNA photolyase (4-189 aa) and FAD-binding (260-526 aa), which were commonly found in DNA photolyases [27]. Le.CRY was homologous to the cryptochrome dash family proteins in other fungi and plant, e.g. *N. crassa* CRY (Nc.CRY) [15], *S. sclerotiorum* CRY1 (Ss.CRY1) [16] and *Arabidopsis thaliana* (At.CRY3) [28]. As described above, the photolyase/cryptochrome family constitutes five classes, and cryptochromes are further classified into three general groups on the basis of phylogenetic analysis: (1) plant cryptochrome, (2) animal cryptochrome/6-4 photolyase and (3) cryptochrome DASH [12, 27]. Plant and animal cryptochromes including 6-4 photolyase have both C-terminal photolyase-related region and carboxyl-terminal domain, whereas Le.CRY had no obvious carboxyl-terminal domain as well as other DASH-type cryptochromes [27].

To examine the evolutionary divergence between Le.CRY and other photolyase/cryptochrome family proteins, phylogenetic analysis was performed. The resulting phylogram revealed that DASH-type cryptochromes formed a monophyletic clade, which included Le.CRY (Fig. 1). Identities of the entire protein sequences between Le.CRY and Nc.CRY/Ss.CRY1 were 43.9%/48.3%. In the cases of comparing of their respective domains of

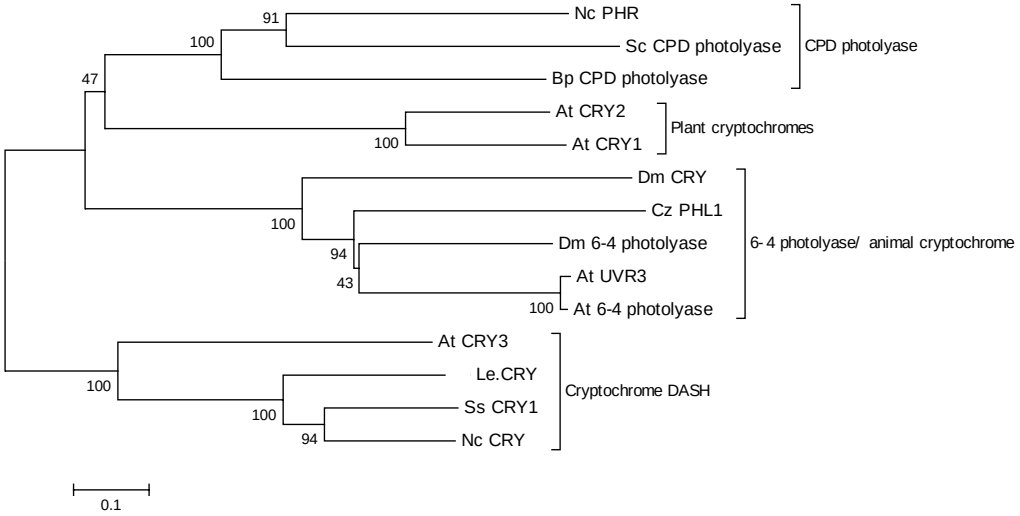

Figure 1: Phylogenetic analysis of Le.CRY and photolyase/cryptochrome families.

Protein sequences of the members in photolyase/cryptochrome family were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). Organisms and accession number of proteins are as follows: Nc PHR, *Neurospora crassa*, P27526; Sc CPD photolyase, *Saccharomyces cerevisiae*, P05066; Bp CPD photolyase, *Bacillus pseudofirmus*, Q04449; At CRY1, *Arabidopsis thaliana*, S66907; At CRY2, U43397; Dm CRY, *Drosophila melanogaster*, NM_169852; Cs PHL1, *Cercospora zeae-maydis*, ACB38886; Dm 6-4, *D. melanogaster*, AY089584; At UVR3, *A. thaliana*, NM_001035626; At 6-4, *A. thaliana*, AB003687; At CRY3, *A. thaliana*, NM_122394; Ss CRY1, *Sclerotinia sclerotiorum*, XP_001593735; Nc CRY, *N. crassa*, XP_965722. A multiple sequence alignment was performed with the ClustalW. The phylogenetic tree was constructed using the Neighbor-Joining algorithm in Molecular Evolutionary Genetics Analysis (MEGA) version 4 system. Neighbor-Joining consensus tree used 1000 bootstrap replicates. The number represents the percentage of bootstrap values.

Le.CRY and Nc.CRY/Ss.CRY1, the identities of aa sequences of the DNA photolyase domain and the FAD-binding domain were 45.7%/49.7% and 55.9%/59.1%, respectively. The characteristic aa residues bound to chromophores (essential for FAD- and methenyltetrahydrofolate (MTHF)-interaction) were highly conserved [29, 30] (data not shown), implying that Le.CRY likely binds to FAD and MTHF and responds to UV-A as well as other DASH-type cryptochromes [15, 31]. In addition, it was reported that the DASH-type cryptochrome in *A. thaliana* interacts with DNA through specific six aa residues [32, 33]. Also in Le.CRY, the corresponding aa residues were completely conserved, strongly suggesting the possibility that Le.CRY has DNA-binding ability. Although Nc.CRY and Ss. CRY1, DASH-type cryptochromes in ascomycetous fungi, have glycine-rich region in the C-termini, Le.CRY lacked the corresponding region. The glycine-rich region has been found to function as protein-protein interaction domain [34, 35], protein-RNA interaction domain [35, 36] and processing signal [37]. These data suggest that the light signal transduction mechanism mediated by Le.CRY might differ from those by Nc.CRY and Ss.CRY1.

Transcription analysis of *Le.cry*, *phrA* and *phrB* during fruiting body formation. In basidiomycetous mushrooms, near UV-A wavelength or blue-light positively contributes to the initiation of fruiting body formation and the development of fruiting body [20, 21, 38]. In addition, it has been reported that the formation of sclerotia, hyphal knots and chlamydospores is inhibited in *C. cinerea* cultivated under continuous UV-A or blue light [22]. Both the blind mutant of *dst1* (the WC-1 homolog gene in *C. cinerea)* and the WC-2 homolog disruptant of *C. cinerea* revealed the aberrant development of fruiting body and the abolishment of the repression of oidia production by light [10, 20]. However, genes responsible for other photomorphogenesis such as formation of sclerotia, hyphal knots, and chlamydospores have not been identified. In addition, the action spectrum study revealed that hyphal aggregation in the basidiomycetous mushroom *Pleurotus ostreatus* is likely to be regulated by a cryptochrome-like photoreceptor [24]. These reports suggest the possible existence of a light signaling pathway different from that mediated by WC-1 and WC-2 homologs. In *L. edodes*, *phrA* and *phrB* have been isolated as the WC-1 and the WC-2 homologous genes [11, 39]. It was revealed that PHRB has sequence-specific DNA-binding activity and forms a complex with PHRA [39]. In this study, the Le.CRY protein was identified as another blue-light photoreceptor in *L. edodes*. To compare the detailed

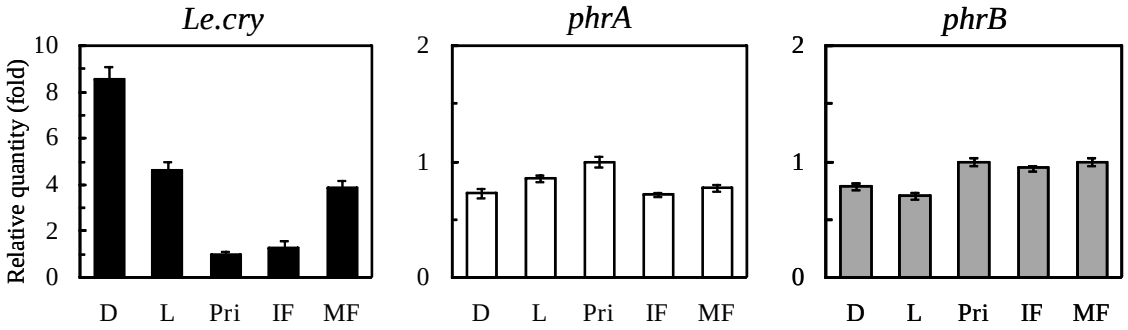

Figure 2: Quantitative RT-PCR of *Le.cry*, *phrA* and *phrB* during fruiting body formation.
D, vegetatively growing mycelia grown under continuous darkness; L, vegetatively growing mycelia grown under continuous light; Pri, primordia; IF, immature fruiting bodies; MF, mature fruiting bodies. Error bars represent standard deviations. The *actin* gene was used as an internal control.

difference of the expression of the *Le.cry*, *phrA* and *phrB* in *L. edodes* during fruiting body formation, we performed quantitative RT-PCR. The analysis was performed using a variety of *L. edodes* tissue samples, vegetatively growing mycelia cultivated under continuous light (L) or continuous darkness (D), primordia (P), immature fruiting bodies (IF) and mature fruiting bodies (MF). The *actin* gene was used as an internal control. The results revealed that the *phrA* gene and the *phrB* gene were constitutively expressed in all developmental stages. By contrast, the exceedingly highest expression of *Le.cry* was detected in vegetatively growing mycelia cultivated under continuous darkness (Fig. 2). The expression of *Le.cry* was significantly weakened during fruiting body formation, but gradually increased along with the developmental process of fruiting body. These results suggest that Le.CRY might be involved in a light signaling pathway different from that mediated by PHRA/PHRB and play a role mainly in vegetatively growing mycelium cells of *L. edodes*. In addition, it has been reported that the transcription level of the *Ss.cry1* gene in *S. sclerotiorum* is weakened in sclerotial stage, but is strengthened in vegetatively growing mycelia and the late stage of apothecia development [16]. Similarly, the *cryA* gene in *A. nidulans* (*An.cryA*) is highly expressed before sexual development and at the late stage of sexual development, and the transcription level of it decreases in the early stage of sexual development [17]. These data imply that Le.CRY might have a similar role to An.CryA and Ss.Cry1, in *L. edodes*.

Gene expressions of *Le.cry*, *phrA* and *phrB* in parts of fruiting body. The *Le.cry* gene was most abundantly expressed in mycelial stage, whereas mature fruiting bodies also contained relatively large amounts of the *Le.cry* transcript. Next, we performed quantitative RT-PCR to investigate whether the expression of *Le.cry* is different in parts of fruiting body. The fruiting body was separated into stipe (S), pileus without hymenophore (gills) (P) and hymenophore (H) (Fig. 3). Despite the fact that hymenophores contained slightly low amounts of the *phrB* transcipts, no significant difference of the expressions of *phrA* and *phrB* was detected in the investigated tissues. In contrast with *phrA* and *phrB*, the transcript of the *Le.cry* gene was more abundantly accumulated in pilei than in stipes and hymenophores. The result implies that *Le.cry* is likely to work predominantly in pilei.

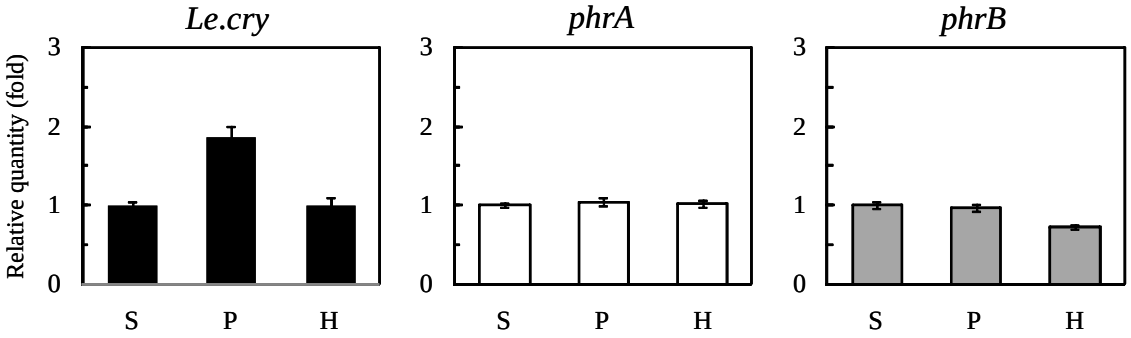

Figure 3: Transcript accumulation of the *Le.cry* gene, the *phrA* gene and the *phrB* gene in parts of fruiting body. S, stipes; P, pilei without hymenophores (gills); H, hymenophores. Error bars represent standard deviations. The *actin* gene was used as an internal control.

CONCLUSIONS

In conclusion, we identified a new blue-light receptor gene, which encodes a DASH-type cryptochrome, and analyzed transcripts of the *Le.cry* gene during fruiting body formation and in

parts of fruiting body. The mycelial stages, especially vegetatively growing mycelia cultivated under continuous darkness, contained large amounts of the transcript of the *Le.cry* gene. In addition, it was revealed that the *Le.cry* transcription appears to differ obviously from the transcriptions of *phrA* and *phrB* during fruiting body formation and in parts of fruiting body. The detail of the molecular mechanisms of light signaling pathway mediated by Le.CRY may enhance our understanding of the mechanism underlying *L. edodes* photomorphogenesis.

ACKNOWLEDGEMENT

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PROTEINS EXPRESSED DURING HYPHAL AGGREGATION FOR FRUITING BODY FORMATION IN BASIDIOMYCETES

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ABSTRACT

The first visible step in fruiting body development in basidiomycetes is the formation of small hyphal knots by localized intense branching of hyphae of restricted length accompanied by hyphal aggregation. In *Coprinopsis cinerea*, the first not yet fruiting-specific step of hyphal branching occurs in the dark, the second step requires a light signal. Hyphal aggregation implies cell-cell contacts and protein interactions on the outer cell walls are anticipated. Few protein candidates were identified and discussed in the past for such function. Amongst were the galectins of *C. cinerea* and the Aa-Pri1 protein (aegerolysin) of *Agrocybe aegerita* that are specifically expressed during the step of hyphal aggregation as well as during subsequent primordia development. In this study, we follow up the distribution of genes for proteins with lectin and/or hemolysin function in the steadily growing number of available genomes of basidiomycetes. Neither galectin genes nor genes for other lectins nor *Aa-pri1*-like genes nor other hemolysin genes are present in all mushroom species, making an essential role for such functions in hyphal aggregation unlikely.

Keywords: Lectin, hemolysin, mushroom formation, hyphal knots, predator defence

INTRODUCTION

Vegetative mycelial growth of filamentous fungi basically consists of tip growth of leading hyphae with sporadic subterminal initiation of a sidebranch that then also undergoes tip elongation for further growth [1]. Such simple mycelial growth can thus locally be considered as just two-dimensional. Fruiting body development in contrast is a complex process which changes from simple two-dimensional vegetative growth of the mycelium to formation of a compact three-dimensional aggregated structure in which differentiation of specific cap and stipe tissues takes place [2]. The first visible structure is the hyphal knot generated by intense localized formation of stunted, growth-restricted sidebranches that interweave and eventually aggregate with each other. In *Coprinopsis cinerea*, we distinguish primary from secondary hyphal knots (Figs. 1, 2) which form within dark and subsequently upon (blue) light illumination, respectively [3, 4]. Initiation of hyphal aggregation is controlled by the mating type genes [5, 6] and environmental factors – which in addition to light are temperature, nutrients, humidity, and aeration [2, 7] –, but little is yet known on the cellular processes leading to aggregation. Different proteins have however been implicated in basidiomycetes in functioning in hyphal aggregation, based on observations of coincidental expression of their genes with initiation of fruiting and subsequent primordia development [7, 8].

Many different types of sugar moieties-binding lectins are known to occur in mushrooms as candidate proteins for mediating cellular aggregation [8, 9]. Galectins are β-

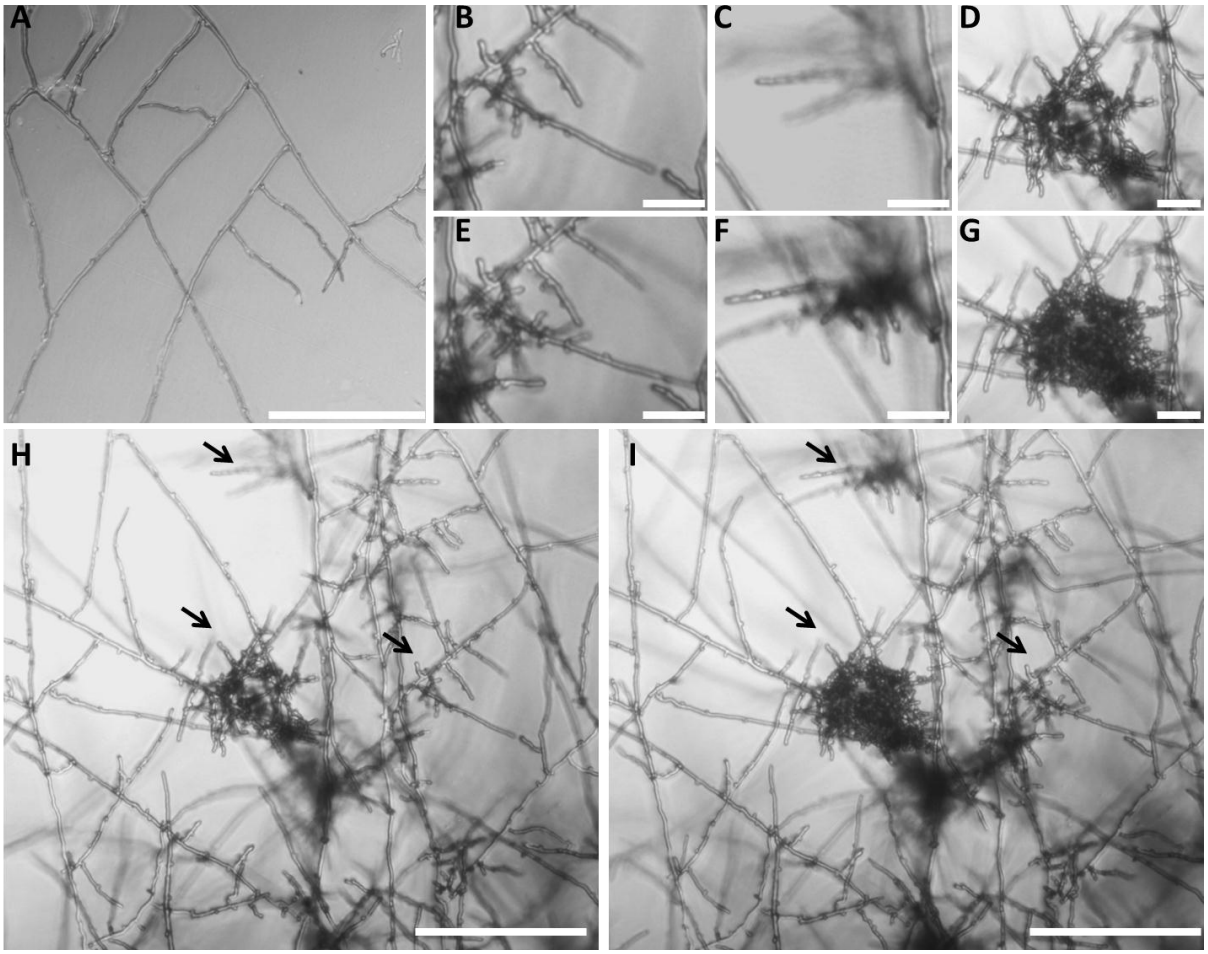

Figure 1: Primary hyphal knot formation of *C. cinerea* homokaryon AmutBmut in an YMG/T agar culture: Mycelial lattice after 24 h of growth (A). Primary hyphal knots within the lattice after 40 h (B-D,H) and 60 h of growth (E-G,I). Arrows in I and H point to structures shown enlarged in B-D and E-G, respectively. Size bar = 20 µm (B-G), = 100 µm (A,H,I).

galactoside binding lectins characterized by a specific sugar-binding domain [10]. In *C. cinerea*, expression of galectins (CGL1, CGL2) and a galectin-related lectin (CGL3) starts at the stages of primary, respectively secondary hyphal knot formation and continues throughout primordia formation. Galectins are secreted and localize to cell walls and the extracellular matrix (ECM) of mushroom tissues. Highest expression is found in the outer cap and outer stipe tissues [4, 11-13]. Although a function in cell-cell aggregation had been postulated [4], more recent studies showed that the proteins are not essential and point to a role in protection against grazing arthropods and nematodes [14-16]. Related mushroom-specific galectins exist in *Agrocybe aegerita* (syn. *cylindracea*) [17, 18], *Heterobasidion annosum* (*irregulare*) [8], *Laccaria bicolor* and *Laccaria amethystina* [12, 19]. Application of isolated *A. aegerita* galectin AAL in fresh cultures reduced mycelial growth rates and induced mycelial cord formation. Most interestingly, application on own and on also foreign (*Auricularia polytricha*) established mycelium resulted in formation of aggregates and primordia differentiation [20, 21]. Also *Agrocybe* lectins have anti-nematode activities [22].

Members of another family of β-galactoside binding lectins [FB (fungal fruit body) lectin super-family] occur in *Athelia* (*Sclerotium*) *rolfsii* (SRL; SLR-like), *Agaricus bisporus* (ABL), *Xerocomus chrysenteron* (XCL), *Pleurotus cornucopiae* (PCL-M, PCL-F), *Boletus edulis* (BCL) and *Paxillus involutus* [23-30]. Functions in aggregation in sclerotia formation and in inhibition of sclerotia germination have been reported for SRL in *A. rolfsii* [31].

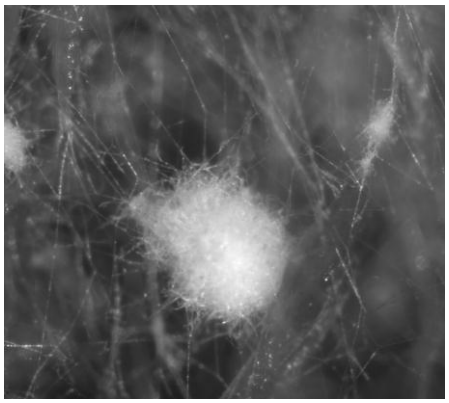

Figure 2: View on an about 100 µm sized secondary hyphal knot (center) grown on horse dung and neighbouring primary hyphal knots to the left and right.

PCL-F is envisaged to contribute to aggregation during fruiting although lectin-deficient *P. cornucopiae* mushrooms of normal shape appear to exist [32, 33], SRL, SLR-like and XCL showed anti-nematodal and insecticidal activities [24,26,34]. Structurally, ABL and XCL resemble actinoporins [27, 35], a family of membrane-integrating pore-forming toxins that act hemolytic [36]. LSLa is one of three closely related lectins (LSLa to LSLc) from *Laetiporus sulphureus* and represents another small characterised mushroom protein with combined lectin and pore-forming activities. This hemolysin divides into an N-terminal lectin-domain and a C-terminal porin domain of the haemolytic aerolysin protein family [37]. Further mushroom lectins are represented by ricin B-type proteins from *Clitocybe nebularis* [38], *Pleurotus squamosus* [39], *Pleurocybella porrigens* [40], and *Marasmius oreades* [41], and the immunomodulatory lectin FIP-fve from *Flammulina velutipes* [42]. Lectin PVL from *Psathyrella* (*Lacrymaria*) *velutina* is an integrin-like protein with seven internal repeats expressed both in mycelium and in mushrooms. There is a homolog in *C. cinerea* [43].

Aegerolysins (for which *A. aegerita* Aa-Pri1 = aegerolysin was name-giving) belong to another family of pore-forming hemolysins. Lectin-like interactions are not described but these proteins interact with lipid rafts in cellular membranes [44]. *Pleurotus ostreatus* ostreolysin and pleurotolysin A (with pleurotolysin B from a two-component system), *Pleurotus eryngii* erylysin A (interacting with erylysin B) and possibly *Pleurotus nebrodensis* nebrodeolysin are other closely related members of this family [45-48]. Postulated to be aggregation factors [49, 50], application of ostreolysin to *P. ostreatus* mycelium was found to be fruiting inducing [45]. In coincidence, aegerolysins in *Agrocybe* and *Pleurotus* species are expressed at initiation of fruiting and during fruiting body development [49, 50]. Also in *Moniliophthora perniciosa*, aegerolysin genes expressed along with fruiting body formation have been described [51]. Flammutoxin from *F. velutipes* is another type of pore-forming hemolysin specifically expressed during fruiting [52].

MATERIALS AND METHODS

Strain, culture conditions and microscopy. The self-compatible *C. cinerea* homokaryon AmutBmut able to form fruiting bodies due to mutations in both mating type loci [4] was cultivated on YMG/T complete medium or on horse dung as described [53]. For microscopy of hyphal development, observation windows were made within fully grown fungal YMG/T cultures by cutting out agar pieces of about 1 cm^2. Cultures were further incubated at 28^oC in the dark for about 50 h and hyphal growth within the windows was monitored at intervals of 3 to 4

hours, using an inverse Axiovert (Zeiss, Göttingen, Germany) microscope in a dark room with a yellow filter placed into the light beam of the microscope.

Sequence analysis. *L. velutina* PVL (GenBank ABB17278), *C. cinerea* CGL1 (AAB04141; CCG1_05003), *F. velutipes* FIP-fve (ADB24832), ricin B-like lectins of *C. nebularis* (ACK56062), *P. squamosus* (BAC87876), and *L. sulphureus* (LSLa; 1W3A_A), FB lectins of *A. bisporus* (ABL; Q00022), *P. cornucopiae* (PCL-F1; AB056470), and *X. chrysenteron* (XCL; AAL73235), *A. aegerita* aegerolysin (AAC02265), *P. eryngii* erylysin A (BAI45247) and B (BAI45248), and *F. velutipes* flammutoxin (BAA76510) were used in tblastn searches (expect 1.0E-0; word size 3; no filter) of basidiomycete genomes available in July 2011. From the MycoCosm page (http://genome.jgi-psf.org/programs/fungi/index.jsf) of the Joint Genome Institute (JGI) were used Pucciniomycotina *Melampsora laricis-populina* v1.0, *Puccinia graminis*, *Rhodotorula graminis* strain WP1 v1.1, and *Sporobolomyces roseus* v1.0, Ustilagomycotina *Malassezia globosa* and *Ustilago maydis*, Agaricomycotina *A. bisporus* var. *bisporus* (H97) v2.0, *A. bisporus* var. *burnettii* JB137-S8, *Auricularia delicata* SS-5 v1.0, *Ceriporiopsis subvermispora* B, *Coniophora puteana* v1.0, *C. cinerea* Okayama 7, *Cryptococcus neoformans* var. *grubii* H99, *Dacryopinax* sp. DJM 731 SSP-1 v1.0, *Dichomitus squalens* v1.0, *Fomitiporia mediterranea* v1.0, *Fomitopsis pinicola* SS1 v1.0, *Ganoderma* sp. 10597 SS1 v1.0, *Gloeophyllum trabeum* v1.0, *H. annosum* (*irregulare*) v2.0, *L. bicolor* v2.0, *Phanerochaete carnosa* v1.0, *Phanerochaete chrysosporium* v2.0, *Phlebia brevispora* HHB-7030 SS6 v1.0, *P. ostreatus* PC9 v1.0 and PC15 v2.0, *Postia placenta* MAD-698, *Punctularia strigosozonata* v1.0, *Schizophyllum commune* v1.0, *Serpula lacrymans* S7.3 v2.0 and S7.9 v1.0, *Stereum hirsutum* FP-91666 SS1 v1.0, *Trametes versicolor* v1.0, *Tremella mesenterica* Fries v1.0, and *Wolfiporia cocos* MD-104 SS10 v1.0, from the fungal site (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=fungi) of the National Centre for Biotechnology Information (NCBI) *Microbotryum violaceum* p1A1 Lamole, *Puccinia triticina* 1-1 BBBD Race 1, *Mixia osmundae* IAM 14324, *Melassezia restricta* CBS 7877, *Cryptococcus gattii* R265 and WM276, and *M. perniciosa* FA553, and from the Munich Information Center for Protein Sequences (MIPS) *Sporisorium reilianum* (http://mips.helmholtz-muenchen.de/genre/proj/sporisorium/). Where required, computer-defined gene coordinates were manually corrected and genes annotated on respective pages at JGI (except *S. lacrymans* 1627212: delete N-terminal 54 aa). *M. perniciosa* ABRE01005301 (NCBI) was also amended: join 834-1117,1172-1310. For other accession numbers see figures. Using ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) and GeneDoc version 2.6.002 (http://www.psc.edu/biomed/genedoc/), sequences were aligned Phylogenetic trees were calculated by neighbour joining by MEGA version 4.0 [54].

RESULTS AND DISCUSSION

Hyphal knot formation and early hyphal aggregation. Fig. 1A shows at first two-dimensional vegetative mycelial growth (known as lattice formation [3]) of *C. cinerea* homokaryon AmutBmut when entering a new surface. About 20-30 hours later, hyphal knots of different developmental stages can be found at distinct places within the mycelium (Fig. 1B-D, H). Clearly, intense initiation of formation of sidebranches occurs highly localized over a restricted length (about 50 µm) at one leading hypha or, in closest distance, at two or three neighbouring hyphae. Although the sidebranches stop growing after a few µm (up to about 30 µm as estimated from Fig. 1B-G), the close vicinity of areas of intense branching at neighbouring hyphae allow sidebranches from different leading hyphae to intermingle (Fig. 1D,E).

Table 1: Potential lectins and/or hemolysins in mushroom forming Agaricomycotina

Species	Lectins					Pore-forming lectins			Pore-forming hemolysins		
	***F. velutipes* FIP-fve**	***L. velutina* PVL**	***C. cinerea* CGL1**	***C. nebularis* ricin B-like lectin**	***P. squamosus* ricin B-like lectin**	***L. sulphureus* LSLa**	***D. squalens* 158296 *G. trabeum* 129557**	**FB lectins ABL, PCL-F1, XCL**	***A. aegerita* aegerolysin, *P. eryngii* erylysin A**	***P. eryngii* erylysin B**	***F. velutipes* flammutoxin**
A. bisporus var. *bisporus*	-	-	-	-	-	-	-	75698 194888 194894	-	-	-
A. bisporus var. *burnettii*	-	-	-	-	-	-	-	114704	-	-	-
A. delicata	57009	-	-	-	-	-	-	67476 85006 115017 115035 117944	-	-	199645
C. subvermispora	172151[§] 172155[§] 172156[§]	-	-	-	117217 117225 125532	172144[+]	-	-	172089	-	-
C. puteana	-	-	-	-	-	-	-	-	-	-	-
C. cinerea	-	CC1G _03091*	CC1G _00723 _05003 _05505	CC1G _10077 _10083	CC1G _10075 _10077 _10083	CC1G _08369[+] _10318 _11805	CC1G _08369[+] _10318 _11805	-	-	-	-
Dacryopinax sp.	-	-	-	-	-	-	-	-	-	-	-
D. squalens	53695 101883 125142	-	-	-	148933 172700 172722 172724 172726 172744 201382 201385 201389[Δ]	158296	158296	-	69680	-	90817
F. mediterranea	-	-	-	-	-	-	-	-	-	-	160286 160275 187205 187206
F. pinicola	-	-	-	-	82284 82401 82402 82405 82406 82407 82435 82645 89059	124282 281656[+]	124282	-	-	-	82673 95762
Ganoderma sp.	-	-	-	-	-	-	-	-	-	-	-
G. trabeum	-	-	-	-	-	129557	129557	-	-	-	-
H. irregulare	-	-	58543	-	-	-	-	-	148469	38497	65755
L. bicolor	-	692684	236913 312069 723752	-	-	576524[+] 461940	461940	185716	-	-	-
M. perniciosa	-	-	-	-	EEB99847[Δ] EEB94816[Δ]	ABRE01 005301*	-	-	EEB90416 EEB92328[Δ] EEB93043[Δ] EEB93315[Δ] EEB95579[Δ] EEB96271	EBB89936 ABRE01 017070[§]	-
P. carnosa	192435	-	-	-	211794 261044	-	-	257886 263561	-	-	-
P. chrysosporium	-		-	-	-	-	-	6917 140897	-	-	-
P. brevispora	79844 117676	-	-	-	-	-	-	71190	-	-	-
P. ostreatus PC9	-	-	-	-	122379	-	-	107763	72745	133806	67050 117864
P. ostreatus PC15	-	-	-	-	1119533[+]	-	-	1044138	1090164	1090161	168572 1091975
P. placenta[#]	135177 135180	-	-	92379 135173	92379 135173 135175 135176[Δ] 135181 135182 135188 135191	135167 135168	135167 135168	46158 46169 57081 135165	-	-	135146 135148

* model corrected; [+] model from gene with frameshift/deletion/early stop codon; [§] gene remnant?; [#] contains hits from alleles; [Δ] incomplete gene; [¶] putative family 5 glycoside hydrolase with two N-terminal ricin B-like motifs

Table 1: Potential lectins and/or hemolysins in mushroom forming Agaricomycotina (continued)

Species	Lectins					Pore-forming lectins			Pore-forming hemolysins		
	***F. velutipes* FIP-fve**	***L. velutina* PVL**	***C. cinerea* CGL1**	***C. nebularis* ricin B-like lectin**	***P. squamosus* ricin B-like lectin**	***L. sulphureus* LSL a**	***D. squalens* 158296 *G. trabeum* 129557**	**FB lectins ABL, PCL-F1, XCL**	***A. aegerita* aegerolysin *P. eryngii* erylysin A**	***P. eryngii* erylysin B**	***F. velutipes* flammutoxin**
P. strigosozonata	-	-	-	-	-	-	-	134444 143781 154836 154837$^{\Delta}$	101965	-	-
S. commune	-	-	-	-	103548	-	-	-	-	-	74780
S. lacrymans S7.3	-	-	-	-	162712* 173380 187490	-	-	-	-	-	-
S. lacrymans S7.9	-	-	-	-	442012 457021 477093	-	-	-	-	-	-
S. hirsutum	-	-	-	-	-	-	-	153353 182934	-	-	-
T. versicolor	121721 184741	-	-	-	-	-	-	-	52920	52921	-
T. mesenterica	-	-	-	-	73633¶	-	-	-	-	-	-
W. cocos	-	-	-	-	-	-	-	81600	-	-	-
Total	**12**	**2**	**7**	**4**	**46**	**13**	**9**	**28**	**13**	6	**16**

Formation of further primary and also higher order side branches occurs so that within the developing primary hyphal knot first hyphal aggregation becomes possible (Fig. 1D, E, G-I). When this happens, it becomes difficult to follow up further processes of development by simple light microscopy since the three-dimensional structures are more and more impervious to light. How the step from primary hyphal knot to the secondary hyphal knot and compact aggregation happen is thus still to be clarified. Fig. 2 shows an impression of a secondary hyphal knot with primary hyphal knots growing in the neighbourhood.

Candidate proteins for hyphal aggregation. Proteins proposed to act in hyphal aggregation for mushroom formation (see Introduction) were used to search the genomes of in total 40 different species (7 Pucciniomycotina; 4 Ustilagomycotina; 29 Agaricomycotina). Allelic genomes of two different monokaryons were available for analysis of *P. ostreatus* (PC9, PC15) and *S. lacrymans* (S7.3, S7.9) and, due to dikaryon sequencing, also for *P. placenta* [55]. Strikingly, none of the tested proteins detected candidate genes in the tblastn searches with any of the Pucciniomycotina, the Ustilagomycotina, and the yeast-like Agaricomycotina, suggesting that these proteins are specific to the mushroom-forming Agaricomycotina (see results in Table 1). Also remarkable, none of the species had genes for all types of proteins but most had one or more genes for lectins and/or hemolysins. Species from different orders share types of proteins and closely related species in contrast do not. The gene distribution limited to always only a few and often even unrelated species does not argue for an essential function of any of the tested proteins in hyphal aggregation and fruiting body formation.

FIP-fve-like lectins and integrin-like proteins. Genes for FIP-fve-like lectins were found in seven wood-rotting species from the Auriculariales, Polyporales and Corticiales (Table 1). In contrast, genes for lectins with integrin-like repeats such as PVL of the saprotroph *L. velutina* were only detected in the dung fungus *C. cinerea* and the ectomycorrhizal *L. bicolor* from the Agaricales (Table 1, Fig. 3). PVL binds N-acetylglucosamine and N-acetylneuromic acid in dependence of calcium and this may help in defence of bacteria [43]. However, it might not be by accident that this type of lectin is not present in any of the many wood-rotting species analysed, raising the question whether occurrence of this type of lectin is restricted to saprotrophic and mycorrhizal species for example to help, as suggested [43], in colonisation of humic soil containing pectins and polygalacturonic acid from decomposing plant material.

Galectins and FB lectins. Genes for galectins were only found in the Agaricales *C. cinerea* and *L. bicolor* and in *H. irregulare* from the Russulales (Table 1), all of which were reported before [8,13]. In contrast, FB lectins are wider distributed and genes were found in Agaricales (4 of 7 species), Auriculariales (1 of 1 species), Corticiales (4 of 4 species), Polyporales (2 of 7 species) and Russulales (1 of 2 species), irrespectively of whether *A. bisporus* ABL, *P. cornucopiae* PCL-F1 or *X. chrysenteron* XCL were used in tblastn searches (Table 1). The β-galactoside binding galectins and FB lectins are distantly related [8]. Galectins have a carbohydrate recognition domain (CRD) consensus of H-3x-R-(7-11)x-N-(6-7)x-W-2x-E-x-R. The *C. cinerea* galectin-like CGL3 (CC1G_00723) contains an R instead of the sugar-recognizing W. Due to this R, CGL3 binds chitooligosaccharides but, unlike galectins, not lactose [13]. Of the members of the fungal galectin family, only LBG3 has also this residue while others possess the classical galectin CRD motif or have 1-2 changes at other positions (Fig. 4). The W residue of the conserved amino acids in the CRD of the galectins is also found in the FB lectins [8] but other amino acids make contact with the sugars (Fig. 4, [57]). The sugar-binding residues (Fig. 4) are highly conserved in all proteins of the FB lectin super-family analysed in this study, although only the residue G was found in all 28 of them and only 8 of them had a perfect central HNY-4x-D-I/V/L-x-T motif (Fig. 4; not further shown).

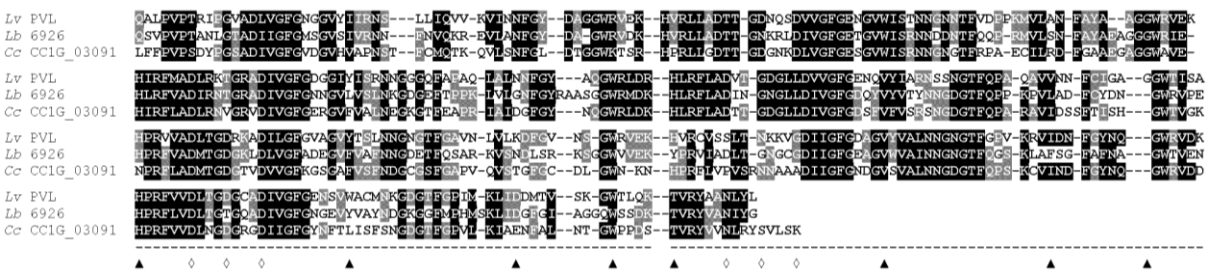

Figure 3: Integrin-like repeats (underlined, 2x per line) from *L. velutina* (*Lv*), *C. cinerea* (*Cc*) and *L. bicolor* (*Lb*) lectins. N-termini of proteins are not shown due to ambiguities in the protein models. Amino acids of sugar and calcium binding are indicated by▲ and ◊, respectively [43].

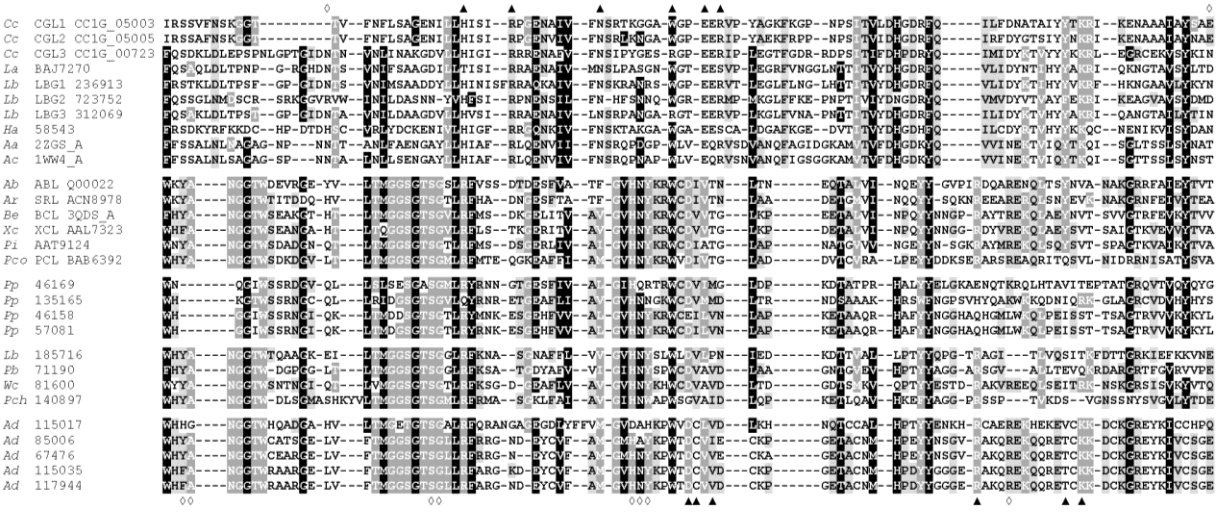

Figure 4: CRD motifs of fungal galectins and galectin-like proteins and of selected FB lectins from four different phylogenetic clades (see Fig. 5). ▲marks primary sugar binding sites for galectins and FB lectins, ◊ extra sites for CGL3 and secondary sites for FB lectins [13, 56].

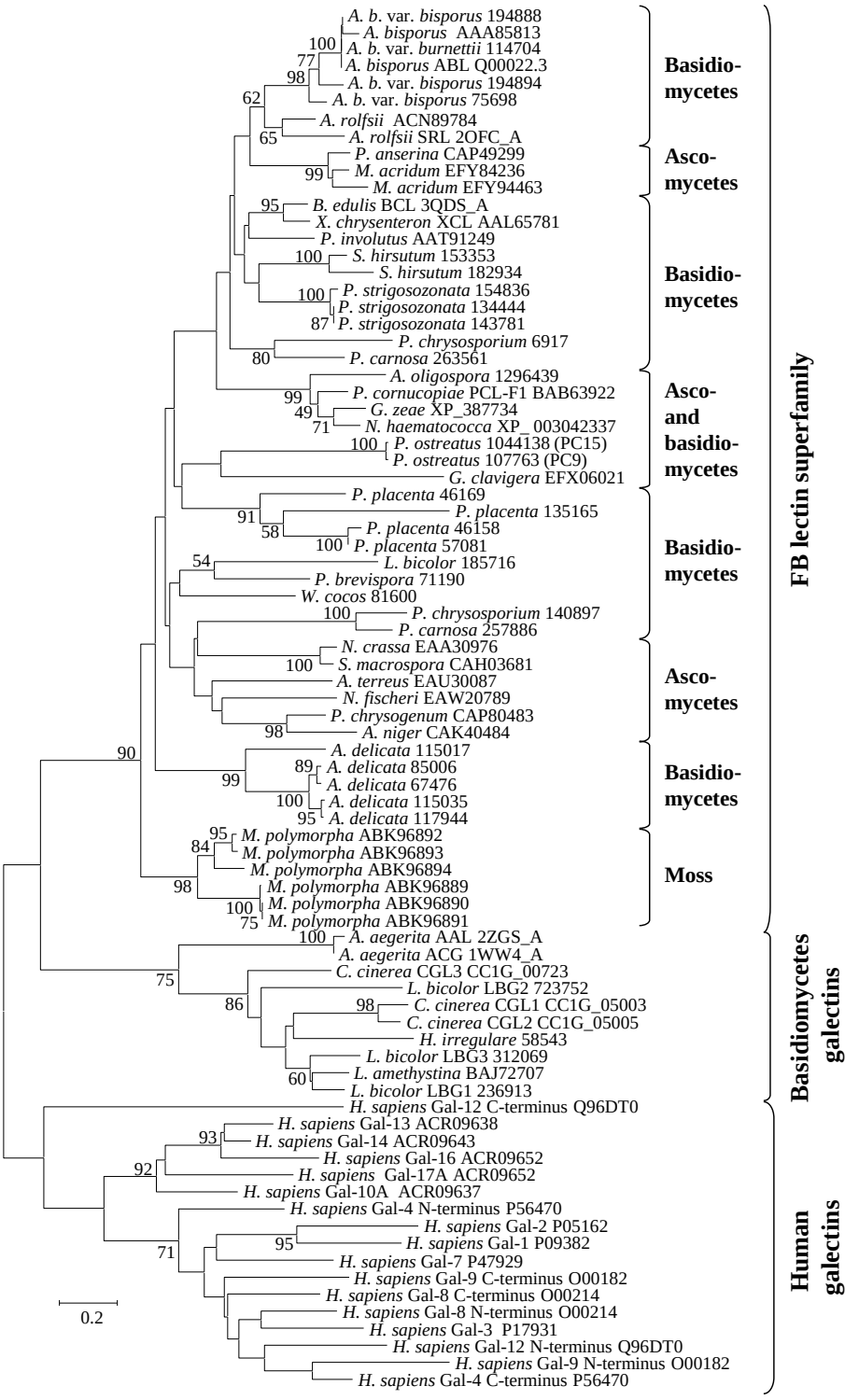

Figure 5: Phylogenetic tree of fungal FB lectins, galectins and galectin-related lectins, FB lectins from the liverwort *Marchantia polymorpha* and human galectins (where present duplicated CRDs were included as N- and C-terminal domain). Fungal species other than those with genomes analysed in study were from the basidiomycetes *A. aegerita*, *A. rolfsii*, *Boletus edulis*, *L. amethystina*, *P. involutus*, *P. cornucopiae*, and *X. chrysenteron*, and from the ascomycetes *Arthrobotrys oligospora*, *Aspergillus niger*, *Aspergillus terreus*, *Gibberella zeae*, *Grosmannia clavigera*, *Metarhizium acridum*, *Metarhizium anisopliae*, *Nectria haematococca*, *Neosatorya fischeri*, *Neurospora crassa*, *Podospora anserina*, and *Sordaria macrospora*. JGI protein IDs and GenBank accession numbers are given in the figure. Bootstrapping values (500 replications) above 50 are shown at tree branchings. Scale bar = number of nucleotide substitutions per site.

A phylogenetic tree was produced from all fungal galectins and FB lectins, using the human galectins and FB lectins found recently in a liverwort as foreign proteins (Fig. 5). The tree suggests that FB lectins might have been evolved from ancestors common to the fungal galectins. The position of the group of moss FB lectins is interesting since it might point to a split of galectins and FB lectins prior to the split of plants and fungi. In support of this, there are also genes for galectin-like proteins in plants [10]. Interesting is further to note that ascomycete and basidiomycete FB lectins intermingle with each other. Duplications of FB lectins happened frequently late in evolution close to speciation. There are four major clades of fungal proteins within the FB super-family (Fig. 5) which correspond largely to differences in the sugar-interacting residues in the CRD motifs (Fig. 4 and not shown). Whether these go along with alterations in sugar binding (efficiencies or sugar types) remains to be elucidated.

Ricin B-like lectins. Of the known fungal ricin B-like lectins, two were used in this study in genome searches. Hits to genes were rare for the *C. nebularis* protein unlike the *P. squamosus* lectin that obtained wider distributed hits, including multiple genes in some of the species and the hits by the *C. nebularis* protein. Genes were found in 4 of 7 Agaricales, 1 of 2 Boletales, 1 of 4 Corticiales, 4 of 9 Polyporales, and in the Tremellales species (Table 1). The products divide in four subgroups of simple ricin B-like lectins plus three other ricin B proteins. Subgroups of the ricin B super-family may have little amino acid identity but they share a β-trefoil structure and contain a conserved Q/NxW motif for sugar binding [38, 40]. All 46 proteins listed in Table 1 had this motif in one or more copies. Of the putative simple lectins, 2 had 1, 14 had 2, 3 had 3, 13 had 4, and 1 had 6 copies [proteins of subgroups I (132 to 182 aa), II and III (256 to 336 aa)] and 6 had 6 to 8 copies [proteins of subgroups III [774 to 838 aa)], respectively. Searching the JGI pages with the keyword ricin revealed many more potential genes for ricin B-like lectins in the fungi than found in our current tblastn searches (also in species with so far no detected gene of interest; Table 1). It apparently will be a major but also a fascinating task to resolve the complete ricin B super-family in the basidiomycetes.

Hemolytic LSL-like lectins. *L. sulphureum* LSLa is special by its N-terminal lectin and C-terminal aerolysin domains [37]. Evidence for such dual proteins was found in 8 different species of the Agaricales and Polyporales (Table 1). These proteins divide into four different groups with highly conserved aerolysin domains and less conserved N-terminal domains (Fig. 6). The N-terminal domain in LSLa adopts a β-trefoil structure exposing specific sugar- contacting residues [37]. These residues are not much conserved between the proteins except *F. pinicola* 124282 (Fig. 6), suggesting that the N-termini of the new proteins have either no lectin function or that they developed novel sugar-binding sites. The N-terminal domains of *L. bicolor* 461940, *C. cinerea* CC1G_11805, and *P. placenta* 135167 were individually used in tblastn searches. Neither gave any further meaningful hits, implying unique evolutionary developments for these domains.

Aegerolysins and flammutoxins. Like for the analysed types of lectins, genes for different types of hemolysins were only found in some species (Table 1). In *D. squalens*, *H. irregulare*, and *P. ostreatus*, genes for different types of hemolysins were detected; other species had only one type in one or more gene copies or no gene for any of the tested kinds of hemolysins. The distribution was independent of the fungal order. Aegerolysin genes occurred in Agaricales, Corticiales, Polyporales, and Russulales and orthologues for flammutoxin in Auriculariales, Agaricales, Hymeochaetales, Polyporales, and Russulales (Table 1).

The closely related *A. aegerita* aegerolysin and the *P. eryngii* erylysin A detected the same set of putative aegerolysin genes (Table 1). Some species contained aegerolysin genes and, in addition, genes for erylysin B-like proteins (Table 1). The latter type of gene never occurred

without an aegerolysin gene. Moreover, where both present, the genes come together in divergently transcribed pairs (not shown), emphasizing a common functional role such as has been suggested in *P. eryngii* and *P. ostreatus* by the experimental finding of dimerization of their products [46, 47]. In *D. squalens*, an erylysin B-like gene was missing but there was a footprint of a former gene upstream to the aegerolysin gene 69680 (not shown).

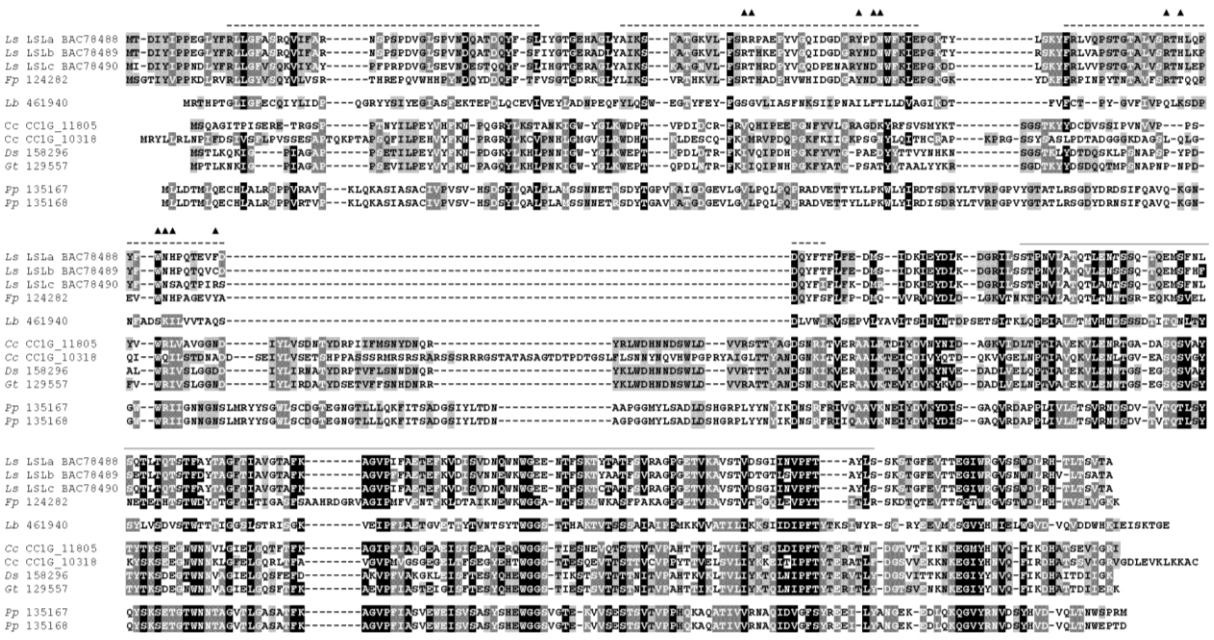

Figure 6: Alignment of putative lectins with aerolysin domains. The β-trefoil module and the sugar-binding residues at the N-terminus of *L. sulphureum* LSLa are marked above the sequence by dashed lines and ▲ and the aerolysin region is indicated by a solid line [37]. Note that from Table 1 only proteins from complete and from intact genes are shown.

CONCLUSIONS

Different lectins and hemolysins have been forwarded as candidate proteins to act in hyphal aggregation, often evidenced by their expression correlating to fruiting (see Introduction). Genome analysis in this study revealed that genes for the tested types of proteins were specific to the mushroom forming species. However, genes for none of the various specific types of proteins occur in all mushroom forming species, although presence of one or more types of lectins and of hemolysins was not rare. The results imply that either these proteins may not be essential for fruiting body initiation and hyphal aggregation or that in evolution different routes have in parallel been developed for the course of events to initiate and continue the fruiting process. Since fruiting body development stands as an essential step at the beginning of sexual spore formation, it appears unlikely that a process central to initiation has been independently invented several times. Experimental evidences we have so far from studies in the literature suggest neither for the different lectins nor for the pore-forming hemolysins a direct function in aggregation.

Pore-forming hemolysins may influence membrane signalling in specific interaction with lipid rafts [44] and, by this, they may indirectly influence the hyphal aggregation process by for example modifying the frequency of fruiting or the environmental conditions under which fruiting occurs. Such effect could explain the observation of induction of fruiting upon application of ostreolysin (in excess?) to vegetative *P. ostreatus* mycelium [45]. Is this a true biological function or an experimental artefact by just exceeding the normal threshold of the

protein? What might be another function of fungal hemolysins? Do they possibly (also) act in defence? Lectins linked to fruiting body development have been seen to act toxic against small putative predators [15, 16, 22, 24, 26, 34]. This may reflect an adoption of a secondary function of these proteins, if there is any in hyphal aggregation. *F. velutipes* LSL-type lectins have an extra pore-forming domain for membrane interaction [37]. FB lectins resemble in structure the bacterial pore-forming haemolytic actinoporins [27, 35]. Lectins may thus interact with membranes [57], possibly via binding to glycolipids [12, 31], thereby effecting signalling [57]. Application of fungal galectins to vegetative mycelium resulting in initiation of fruiting [20,21] might reflect such effect possibly mediated by unnatural high protein concentrations. The importance of membranes in fruiting of Agaricomycotina is supported by the fruiting-inducing effects of various membrane-interacting substances and surfactants when added to vegetative mycelium [2,7] and by the finding of a gene for a cyclopropane fatty-acid synthase being essential for fruiting body initiation in *C. cinerea* [58].

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EFFECT OF EXOGENOUS SPHINGOLIPIDS ON GROWTH AND METABOLISM IN SURFACE CULTURES OF BASIDIAL FUNGI

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ABSTRACT

Sphingolipids (sphingoid bases, ceramides and glycosylceramides) being components of signal cascades and/or lipid rafts were shown to regulate diverse processes in fungi. Particularly as it was demonstrated, glycosylceramides (GlCer) are involved in the regulation of fungal cell growth and differentiation. In this study we analyzed a range of modifications including changes in cell growth, morphology and metabolic pathways following the supplementation of medium by sphingolipids (GlCer and sphingoid bases) extracted from different organisms. Our findings suggest that effects of exogenous sphingolipids on growth rate and metabolic profiles of *Flammulina velutipes* can vary depending on the structure of sphingoid base and acyl group. The treatment of *F. velutipes* cultures with the exogenous GlCer from animal, plant and mushroom sources reveals growth-inhibitory and fruiting-stimulatory activity of these lipids.

Keywords: Basidial Fungi; *Flammulina velutipes*; Sphingolipids; Membrane Lipids; Growth Regulation

INTRODUCTION

Fungal growth, differentiation and morphogenesis are modulated by a number of universal and specific regulatory systems including adenylate cyclases, MAP kinases, G-proteins, as well as cascades mediated by lipid molecules. One of the most intriguing group of bioactive lipids is complex sphingolipids, which consist of amino-alcohol backbones (sphingoid bases) N-acylated by fatty acids and O-glycosylated by one or more sugar residues, mainly glucose and/or galactose.

Previously considered to play only a structural role in cell membranes, sphingolipids together with their precursors and the breakdown products are now also recognized as the important part of the signaling systems that regulate cell functions. Particularly, GlCer with one sugar residue (monohexosylceramides) has attracted increasing attention, because they were found to be highly bioactive molecules mediating growth, proliferation and apoptosis. In fungi they exhibit some specific activities participating in cell recognition, yeast-mycelium phase transition, budding, spore germination and fruiting [1, 2, 3, 4]. However, the study of GlCer functions in the regulation of fungal growth is in its infancy.

Recently it has been shown that functional activity of GlCer strongly depends on their structural features: length of carbon chains, number and position of double bonds in sphingoid bases and fatty acids [2, 5].

There are some differences in GlCer structures among plants, animals and fungi. Whereas animals have GlCer derived from sphingosine (d18:1^4) and phytosphingosine (t18:0), plants predominantly accumulate GlCer with 4,8-sphingadienine (d18:2^{4,8}) as a sphingoid base. Fungal GlCer have a number of structural features, including 9-methyl group branching of the sphingoid base.

This work was carried out to investigate morphological and metabolic effects of exogenous sphingoid bases and GlCer (monohexosylceramides) from animal, plant and fungi sources on the basidiomycete *Flammulina velutipes*. Poviding data about the cell biochemical status, the metabolom analysis is an excellent approach for revealing the sphingolipid functions.

MATERIALS AND METHODS

Fungi Material and Growth Conditions. The culture of basidial fungus *Flammulina velutipes* (Curt.:Fr.)Sing. (strain 1483 from the collection of Komarov Botanical Institute RAS) was used as a model. The mycelium was spotted on the ale-wort agar medium in the center of a Petri dish and it was grown at 25ºC as a surface culture for 8 days in darkness.

Experimental Conditions. To study the effects of exogenous sphingolipids the medium was supplemented by animal GlCer (from bovine brain, Sigma, Germany), mushroom GlCer (extracted from fruit bodies of basidiomycete *Pholiota nameko*), plant GlCer (extracted from wheat roots) and synthetic sphingosine (Sigma, Germany) in the concentrations of 0.04 mg/ml of the medium. All the sphingolipids were previously dissolved in ethanol (the final concentration of ethanol was 0.4 μl/ml, including control samples).

Bioassay of Growth and Fruiting Activity. Two days after the inoculation of *F. velutipes* mycelium on a Petri dish, 4 paper discs (1 cm in diameter), each charged with 10 mkl of solvent-dissolved sample, were dried and placed at the margin of the plate together with a control disc. The concentrations of each sphingolipid applied by this technique were 0, 10, 50 and 100 μg per disc. The plates with discs were further incubated for 4 weeks under the light (2000 lux) in the growth incubator (Sanyo MLR-351H).

Micromorphology of the mycelium was observed by means of light microscope Carl Zeiss Axio Scope A1 (differential interference contrast microscopy (DIC)).

Metabolome Analysis. Soluble metabolites (sugars, sugar alcohols, amino acids, organic acids) were extracted with methanol and chemically derivatized by silylation reagent BSTFA. The samples were analyzed by GC-MS. Peaks obtained were normalized using the amount of the sample dry weight and internal standard (hydrocarbon C23). The identification of analytes was carried out by the mass spectral comparison with the custom mass spectral libraries of genuine compounds. The targeted analysis of lipids (phospholipids, GlCer) extracted with isopropanol-chlorophorm (1:1) at 70ºC by the method of Nichols [6] was carried out by HPTLC [7]. Lipid classes were quantified densitometrically.

Individual molecular species of GlCer were identified by means of the electrospray ionization mass spectrometry (ESI$^+$-MS) and the low energy tandem collision-induced dissociation mass spectrometry.

Statistical Analysis of the data was performed with the Origin 7.5 software. The statistically significant changes are only discussed in the article. Data are presented as mean±se (n=3-4).

RESULTS AND DISCUSSION

ESI-MS/MS analysis of exogenous GlCer (monohexosylceramides) used in the study revealed significant differences in their structure. Whereas mushroom GlCer contained predominantly one molecular species d18:2^{9Met}/16:0-OH, animal and plant GlCer were presented by the mixture of molecules with sphingosine (d18:1), phytosphingosine (t18:0) and sphingadienine (d18:2) as a sphingoid base acylated by different fatty acids (Table 1, Figure 2). In addition, animal GlCer contained more short and middle-chain (14–18 carbons atoms) fatty acyls, than plant GlCer which comprised 60% of long-chain fatty acyls in the composition of GlCer. The fatty acids with the chain shorter than 24 atoms and/or having a 2-hydroxy group were shown to have the stronger activity for fruiting regulation [8, 9].

Table 1. Molecular composition* of GlCer used to supplement the medium

mushroom GlCer (fungal fruit bodies)	**plant GlCer** (wheat roots)	**animal GlCer** (bovine brain)
97% d18:2^{9Met}/ 16:0-OH	10% d18:1 / 16:0(OH)	37% d18:1/ 14:0(OH) 18:0(OH) 18:0
	30% d18:2 / 16:0(OH) 18:0(OH)	39% d18:1 / 24:1(OH) 20:1(OH) 24:1
	25% d18:2 / 24:1(OH) 24:0(OH) 20:1(OH)	24% t18:0 / 14:0(OH) 18:0(OH)
	35% t18:1 / 24:0(OH) 24:1(OH)	

* Structure of sphingoid base of GlCer (before slash) and fatty acyl (after the slash) of GlCer.
Symbols: the number of carbon atoms: the number of double bonds, **d** - dihydroxy-, **t** - trihydroxy-.

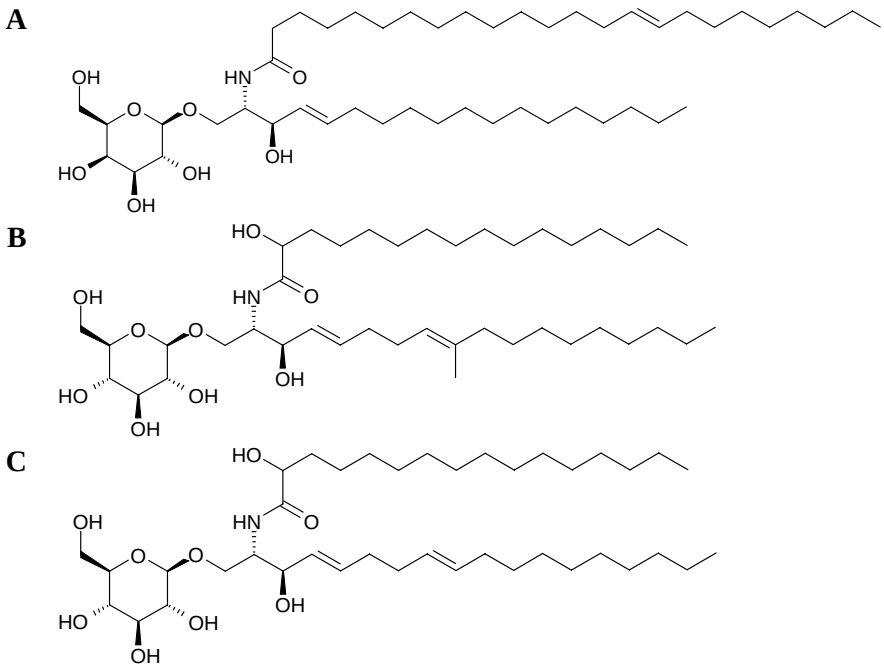

Figure 1: Structure of predominant GlCer of animal (A), mushrooms (B) and plants (C)

Growth and Fruiting Activity. Treatment of *F. velutipes* cultures with exogenous GlCer spotted on paper discs showed its growth-inhibitory effect in dose dependent manner. The growth intensity of a fungal colony was radically reduced by the highest concentrations (50 and 100µg per disc) of mushroom and plant GlCer, in a less degree by animal GlCer. These tendencies were reproduced in experiment when GlCer have been dissolved in growth medium (Figure 2).

Control Animal GlCer Mushroom GlCer Plant GlCer Sphingosine

Figure 2: Effect of GlCer on growth of *Flammulina velutipes*, the 8th day of cultivation in darkness

Morphological observations revealed that at optimal conditions the culture of *F. velutipes* was presented mainly by undifferentiated thin vegetative hyphae as well as conidiogenic ones. On the later stages of its development the part of highly differentiated cells such as thick-wall hyphae or the hyphae with granular content or curved hyphae increased. Plant GlCer induced the formation of spiral hyphae at an early stage of the cultivation (the 8th day).

Treatment of *F. velutipes* cultures with sphingosine (d18:1) also resulted in growth inhibition. Besides, this effect was accompanied by the production of vacuolated and thick-wall empty hyphae. This observation is in agreement with already described growth-inhibitory [10] and apoptosis-inducing [11] activities of sphingoid bases in fungal cells.

Bioassay with paper discs charged with GlCer was used for the study of their fruiting-inducing activity. In the case of mushroom GlCer, fruiting bodies were observed around the test discs in a dose-dependent manner. Other GlCer didn't affect fruiting. This fact is in accordance with the earlier observation that the fruiting-inducing activities of some plant GlCer were considerably lower than the ones from fungi [9]. So methyl group seems to be essential for the high fruiting-inducing activity.

According to the results obtained by means of ESI-MS/MS, GlCer of *F. velutipes* were presented by glucosylceramides containing long chain base d18:2^{9Met} and different fatty acid residues, mainly 16:0-OH (Fig. 1B). The treatment of cultures with exogenous GlCer didn't change the proportion of molecular species of GlCer.

Metabolomics. From the approximately 50 metabolites that were identified by GC-MS, the amount of more than 35 of them were significantly changed in response to supplementation by at least one of the exogenous sphingolipids. These metabolites included amino acids, sugar and sugar alcohols, organic acids, free fatty acids, phospholipids.

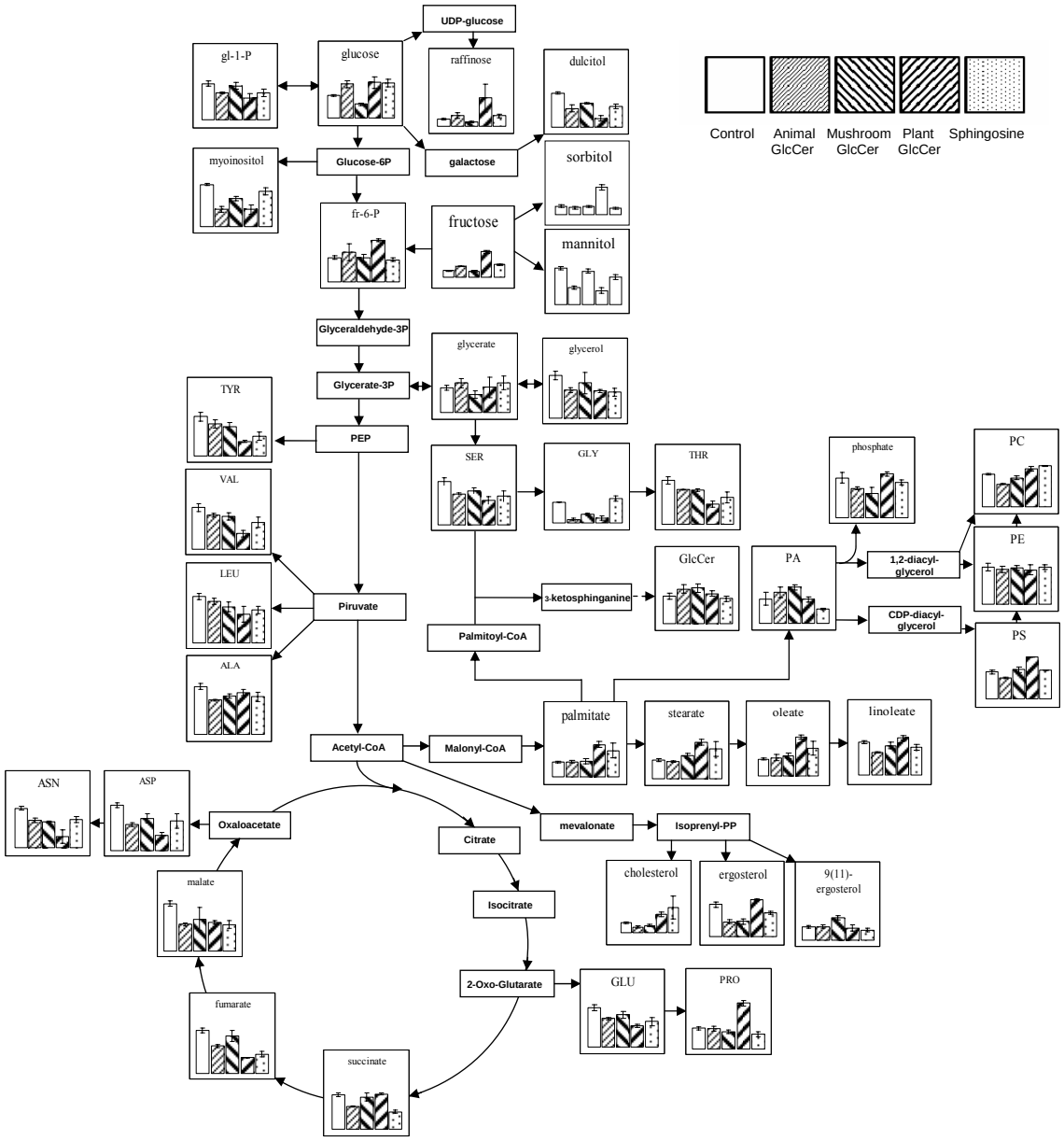

Figure 3: Shift of metabolic pathways in response to exogenous sphingolipids.
PEP, phosphoenolpyruvate; gl-1-P, glucose-1-phosphate; fr-1-P, fructose-1-phosphate; PA, phosphatidate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine. Amino acids are abbreviated with their common three-letter code. Data are mean+se (n = 3–4).

Most of the metabolites demonstrated different responses to diverse molecular species of GlCer. However plant GlCer had the most significant effects on metabolom. Almost all analyzed amino acids were run down into the cultures, grown on medium supplemented by plant GlCer – TYR, VAL, LEU, ASP, ASN, GLU, SER, THR, except PRO, which amount strongly increased. Among sugars and sugar alcohols the quantity of fructose, fructose-6-phosphate, sorbitol, raffinose increased. Besides, free fatty acids (palmitate, stearate, oleate) and PS were accumulated.

General response of metabolom to exogenous GlCer independent of their source was shown by minor sugars myoinositol, dulcitol, as well as SER, GLY, Thr.

In addition, exogenous GlCer affected the PC/PE ratio that usually correlates with the stage of fungal development. Animal GlCer increased a proportion of PE that is typical for differentiated

state on the later stages of *F. velutipes* development. Plant GlCer, as well as sphingosine, by the contrary, reduced proportion of PE. Mushroom GlCer had no effect on PC/PE ratio.

Sphingosine induced some changes in sugar exchange (dulcitol, mannitol) and decreased amount of some amino acids (ASN, GLU, LEU, SER, THR, TYR) and organic acids (cuccinate, fumarate, malate). The effect of sphingosine on phospholipid metabolic pathways was unexpected. Phosphatidic acid metabolism is known to be an important target for sphingoid base action [12]. Sphingosine inhibits the activity of phosphatidic acid phosphohydrolase [13,14], the enzyme which degrades phosphatidic acid to diacylglycerol. However in present study the sphingosine treatment resulted in a depleted amount of phosphatidic acid and glycerol, but increased amount of phosphatidylcholine.

It is interesting to note that exogenous GlCer also influenced on the sterol composition. Animal and mushroom GlCer decreased by twice the amount of ergosterol and cholesterol, however under the treatment with plant GlCer the quantity of ergosterol stayed constant, but cholesterol and Δ9(11)ergosterol were accumulated. It is known that sterols and sphingolipids interact specifically in biological membranes, increasing the lipid order and creating membrane microdomains, which are shown to be signal platforms. Besides, the composition of sterols in membranes, particularly in lipid rafts, differs from kingdom to kingdom: animals contain the high amount of cholesterol; plants have sitosterol, campesterol and stigmasterol, whereas fungi have mainly ergosterol. Our data are likely to show the existence of some mechanisms that allow fungi to respond to the presence of specific sphingolipid structures in their membranes and to adjust their sterol composition accordingly. The comparable situation (changes in sphingolipid metabolism in response to specific sterol composition) has recently been shown in yeast [15].

Reduction of pool of some primary metabolite levels (fumarate, malate, SER and THR) in the combination with the growth suppression can indicate the depression of metabolic activity under the treatment by all the sphingolipids used in the work.

CONCLUSIONS

In this study, we used a combination of morphological and metabolomics approaches to determine putative functions of GlCer in basidiomycetes. We demonstrated that bioactive properties of GlCer depend on their structure.

The treatment of *F. velutipes* cultures by exogenous sphingolipids allow us to conclude the following:

1) Exogenous sphingolipids affect the growth and differentiation of fungal mycelium. 2) Regulatory effects of GlCer depend on the structure of their hydrophobic part: length and hydroxylation of fatty acyls, unsaturation and methylation of sphingoid base. 3) Exogenous sphingolipids affect phospholipid metabolism. Animal GlCer increase proportion of PE that is typical of differentiated state of fungal colony, whereas plant GlCer and sphingosine, by contrast, decrease PE. 4) Among diverse changes in metabolite profile in response to exogenous GlCer two principal tendencies can be separated: unspecific decrease in the levels of some primary intermediates (fumarate, malate, SER, GLY and THR) and specific changes in amino acid, phospholipid and sterol metabolism.

ACKNOWLEDGEMENT

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HEALTH FROM FOREST – ANTIOXIDATIVE PROPERTIES OF ENDOPHYTIC FUNGI FROM SCOTS PINE ROOTS

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ABSTRACT

Endophytic fungi are a diverse group of micro-organisms living inside the tissues of host plants. Although the ecological significance of these fungi is not fully understood, they are known to increase plant's fitness and stress tolerance in harsh environmental conditions. They are also a potential source of chemically novel, bioactive compounds for e.g. various medical purposes.

In this study, we monitored antioxidative properties of endophytic fungi living in association with Scots pine roots. On regeneration areas of drained peatlands pine saplings are often subjected to oxidative stress due to extreme temperatures and changing ground water level. It may be expected that the survival of these trees is assisted by endophytic fungi producing antioxidative, protecting compounds.

Altogether 26 mycorrhizal or endophytic fungal strains were isolated from surface sterilized roots of pines and cultivated as pure cultures for four weeks on solid or liquid modified Melin-Norkrans or Hagem medium at room temperature. The fungal tissues were ground with liquid nitrogen and the water soluble compounds were extracted thereafter with water or PBS buffer in a hot water bath. Antioxidative potential of the extracts was screened by determining the ferric reducing ability/antioxidant power (FRAP-test) and the total phenolic content with modified Folin-Ciocalteu's phenol test using microplate applications.

The total phenolic contents of the water-base fungal extracts after protein removal were up to 24±10 mg/g as gallic acid equivalences (GAE) per dry weight of fungus, and the FRAP-values up to 197±11 µmol/g µmol/g as FeSO4 equivalences per dry weight of fungus. The results showed relatively high variation between the fungal isolates in their antioxidant potential. On the other hand, the ferric reducing abilities of the fungal extracts were quite strongly associated with the total phenol test results.

The results show, that the studied endophytic fungal species living in pine roots have antioxidative properties. Additionally, the antioxidative potential of these fungi determined by their ferric reducing ability correlates positively with the production of phenolic compounds.

Keywords: Endophytes; Fungi; Antioxidants

INTRODUCTION

Endophytes are organisms that, during some period of their life, live symptomlessly in the internal tissues of their host plants. In nature, all higher plants are hosts to one or even hundreds of endophytic organisms, which may be fungi, bacteria or actinomycetes. [1, 2]. It has been noticed in laboratory tests, that plants with endophytes are healthier and have more resistance against biotic and abiotic stress than plants without endophytes [3]. The endophytic micro-organisms e.g. produce secondary metabolites to deter herbivores and pathogens, improve resistance to insect pests, enhance thermotolerance of the host plant, or its tolerance to drought, heavy metal presence, low pH or high salinity. They also may produce plant growth promoting substances (phytohormones, cytokines, vitamins, etc.), enhance the host's absorption of nutritional elements, such as N and P, and regulate nutritional qualities, such as the C/N-ratio. A

wide variety of secondary metabolites of endophytic organisms have been reported including alkaloids, steroids, terpenoids, isocoumarin derivatives, quinones, phenylpropanoids and lignans, phenols and phenolic acids, aliphatic compounds, lactones, *etc*. There are also plenties of novel chemical compounds that could be utilized in medical or agricultural purposes such as antibiotics, antioxidant agents or antipathogens [1, 2].

In this study, we monitored the antioxidative potential of a group of endophytic fungi from Scotch pine roots using Folin-Ciocalteu's phenol test and FRAP testing.

MATERIALS AND METHODS

The fungal strains were isolated from the roots of eight year old Scots pines growing on a drained peatland.

Pure cultures of 25 endophytic fungi were isolated from surface sterilized roots onto modified Melin-Norkrans medium (MMN2) [4, 5], or Hagem agar medium [6]. Ectomycorrhizal fungus, *Paxillus involutus* (P.inv.), known to be rich in phenolics, was included among the tested species.

Identification of the fungi isolated from roots was performed by sequencing the amplified ITS-region of the fungal DNA according to White *et al* [7], Gardes and Bruns [8], and Korkama *et al* [9], and comparing the nucleotide sequences to the information in the GenBank.

The fungal cultures were grown for 4 weeks on solid or liquid MMN2 or Hagem medium at room temperature. Agar (15 g/l) was used for solidification of the culture media. There was also a sterilized cellophane membrane on top of the agar medium to prevent the fungus from growing into the agar medium (P 400, Visella Oy, Valkeakoski, Finland). One or three plugs, each 5 mm in diameter and cut from the margin of a 1-month-old fungal colony were then placed on the cellophane membrane on the petri dishes (diameter 9 or 14 cm) and cultivated in the dark at room temperature. Liquid medium, in turn, was used on petri dishes (diameter 9 or 14 cm, liquid volume 25 or 80 ml per dish, respectively) or in bottles (150 ml per bottle). Culturing bottles were stirred 50-100 rpm for aeration.

The fungal mass was collected using a scalpel (solid medium) or by filtering (liquid medium), stored at -20 ºC, and ground with liquid nitrogen in a mortar. About 1 g of each fungal sample was weighed on a petri dish and dried at +45 ºC to determine the percentage of moisture of the samples. The fungal pulps were extracted in sealed polypropylene test tubes (BD Falcon™ or SuperClear™, VWR Collection) with PBS buffer or deionized water in water bath of +95-+100 ºC. One litre of PBS buffer contained 8.0 g NaCl, 0.2 g KCl, 1.42 g $Na_2HPO_4 \cdot 2H_2O$, and 0.23 g KH_2PO_4 in deionized water (reagents from Merck KgaA). Extractions were performed in 2-3 cycles using extractant volumes of 1-15 ml per gram of fresh fungus. Between each cycle the extraction tubes were centrifuged at 6000-7000 g for 10-20 minutes (Eppendorf Centrifuge 5804R, Hamburg, Germany) and the supernatants were collected. In the end the supernatants were mixed and filtered through nylon syringe filters (0.2 µm, Cronus Filter from SMI-LabHut Ltd, Maisemore Gloucester GL2 8EY, UK or 25mm Syringe filter from VWR International, USA). Aliquots of water and PBS buffer without fungal material were extracted simultaneously for control samples.

Antioxidative potential of the extracts was screened by determining the total phenolic content and the ferric reducing ability of the samples with microplate applications of Folin-Ciocalteu's phenol test and FRAP-test, as illustrated below. These tests were performed for original extracts and for extracts, from which proteins were removed by precipitating them (one part) with methanol (four parts) [10]. Protein contents of the samples were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) on microplates based on the Bradford dye-binding procedure [11].

The statistical analyses were performed using SigmaPlot version 11.9 (Systat Software Inc.) and Pearson product moment correlation test (correlation coefficient r).

Phenol testing procedure. The fungal extracts were analyzed for total phenolics using the Folin-Ciocalteu procedure [12] modified for use on 96-well microplates (BD Labware, Franklin Lakes, NJ. USA), as follows.
Gallic acid (Sigma-Aldrich) dilutions of 500, 250, 100 and 25 mg/l in deionized water were prepared freshly each day for standard solutions from a stock solution made by dissolving 500 mg gallic acid in 10 ml of ethanol and diluting with deionized water to 100 ml.

Predilutions of 1/50 from standards and fungal samples were made freshly each day in deionized water. A volume of 160 µl of these dilutions was placed in quadruplicate on a 96-well microplate. One of the quadruplicates was for sample blanking and the three others for color reaction. Additionally, samples of deionized water were placed on each plate in quadruplicate for blanking the color reagent. The final values were thus corrected for background absorbances caused by either the sample or the reagents. As testing samples treated with methanol for protein removal, the standard dilutions and the water blank for the phenol test were treated with methanol similarly.

For the sample blanks, 40 µl of 20% Na_2CO_3 (Merck KGaA) solution was added. Then, 10 µl of the Folin-Ciocalteu's phenol reagent (Merck KGaA) was added for the color reaction samples and the microplate was shaken for one minute. After five minutes, 30 µl of 20% Na_2CO_3 solution was added to the color reaction samples, and the plate was shaken again for one minute. The absorbance at 750 nm was monitored by a microplate reader (Multiskan RC, Labsystems, Finland) after one hour of incubation at room temperature from addition of the Na_2CO_3 solution. The plate was shaken just before the measurement for one minute. A lag time of 15 seconds was used between the measurements of each plate columns. The phenol test results are expressed as gallic acid equivalences, mg/g dry weight of fungus (GAE).

FRAP testing procedure. FRAP assay of fungal samples was performed using a modified version of the method described by Firuzi *et al* [13], as follows.

Dilutions of 1000, 500, 200, and 50 µM of ferrous sulphate for standard solutions were prepared in deionized water freshly each day from a stock solution of 10 mM. The stock solution was also prepared freshly for each day by dissolving 0.1390 g of $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich) in 50 ml of deionized water.

For FRAP color reagent, acetate buffer (300 mM, pH 3.6, reagents from Merck KgaA), ferric chloride (20 mM, $FeCl_3 \cdot 6H_2O$ from Sigma-Aldrich), and TPTZ-solution of 10 mM [2,4,6-Tris(2-pyridyl)-s-triazine from Sigma-Aldrich] were mixed in proportion of 4:1:1. The mixture was made freshly for each day.

For acetate buffer of 300 mM (pH 3.6), 1.68 g of anhydrous sodium acetate was dissolved in 800 ml of deionized water, and 16 ml of acetic acid was added. The total volume was added to 1000 ml with deionized water. The pH was corrected with acetic acid or with 0.1 N NaOH, as needed.

Ferric chloride of 20 mM was made by dissolving 270 mg FeCl3·6H2O to 50 ml of deionized water, and for TPTZ-solution of 10 mM, 160 mg of TPTZ was dissolved in 50 ml of 40 mM HCl.

A volume of 25 µl of standard dilutions and fungal samples was added on a 96-well microplate (BD Labware, Franklin Lakes, NJ. USA) in quadruplicates. One of these aliquots was for sample blanking and the three others for color reaction. Additionally, samples of deionized water were placed on each plate in quadruplicate for blanking the color reagent. The final values were thus corrected for background absorbances caused by either the sample or the reagents. As

testing samples treated with methanol for protein removal, the standard dilutions and the water blank for the FRAP test were treated with methanol similarly.

Thereafter, 50 µl of acetate buffer (300 mM) was added to each well. For the sample blanks, an additional 100 µl of the same acetate buffer was added. For standards diluted in water and for water extracted fungal samples, 25 µl PBS buffer was added. For the fungal samples extracted with PBS buffer, in turn, 25 µl of deionized water was added to equalize the matrix effects in the reaction mixtures.

Finally, 100 µl of the FRAP reagent was added to the color reaction wells of the plate, and the plate was shaken for 30 seconds. The absorbances were read after 4 minutes at 590 nm using a lag time of 7 seconds between the plate columns.

RESULTS AND DISCUSSION

DNA sequences of 16 fungus isolates of the 26 (Table 1) showed matching with Genbank database on species or family level. Antioxidant potential showed relatively high variation between the fungal isolates. Also the type of growth medium used for culturing, as well as the extractant affected the results markedly (Fig. 1A-D). The highest total phenolic contents [24 ± 10 mg/g gallic acid equivalences (GAE) per dry weight] and FRAP values [197 ± 11 µmol/g as $FeSO_4$ equivalences ($FeSO_4$ eq.) per dry weight] were measured from PBS buffer extract (without proteins) of *Articulospora* sp. (code N) (Fig. 1B).

The protein contents correlated positively with the phenolic test results of the original extracts, but not with the ones after protein removal (Fig. 2A). On the other hand, the FRAP values of extracts before and after protein removal correlated positively with protein contents (Fig. 2B). Methanol used for protein precipitation seemed to affect the phenol and the FRAP test results by increasing the values as seen in the Fig. 2A and Fig. 2B, which is in line with earlier reports e.g. by Firuzi *et al* [13]. Also the Folin-Ciocalteu's phenol test is known to be interfered by several factors [12].

The ferric reducing abilities of the fungal extracts were strongly associated with the phenol test results. This was the case both in the samples including proteins (data not shown, $r = 0.688$, $p < 0.001$, $n = 22$) and in the samples after protein removal (Fig. 3, $r = 0.652$, $p < 0.01$, $n = 22$). Similar correlation between phenol contents and FRAP values has been shown to exist also e.g. in medicinal plant extracts [14].

Table 1: The species or the family names of the fungi under investigation according to the information of the GenBank.

Fungus code	Species or family name	Fungus code	Species or family name
A	Acephala applanata	S15	Ascomycete clones
B	unknown	S16	unknown
C	unknown	S17	unknown
D	Phialophora lignicola	S18	Meliniomyces variabilis
E	unknown	S19	Pezizomycotina
F	unknown	S20	Pezizomycotina
G	Dermea sp.	S21	unknown
H	unknown	S22	unknown
L	Ascomycete clones	S23	Phialocephala sp.
M	Umbelopsis sp.	S24	Ascomycete clones
N	Articulospora sp.	S25	Phialocephala fortinii
R	Phialocephala sp.	S26	unknown
		S27	Penicillium sp.
		P. Inv	Paxillus involutus (Ectomycorrhizal fungus)

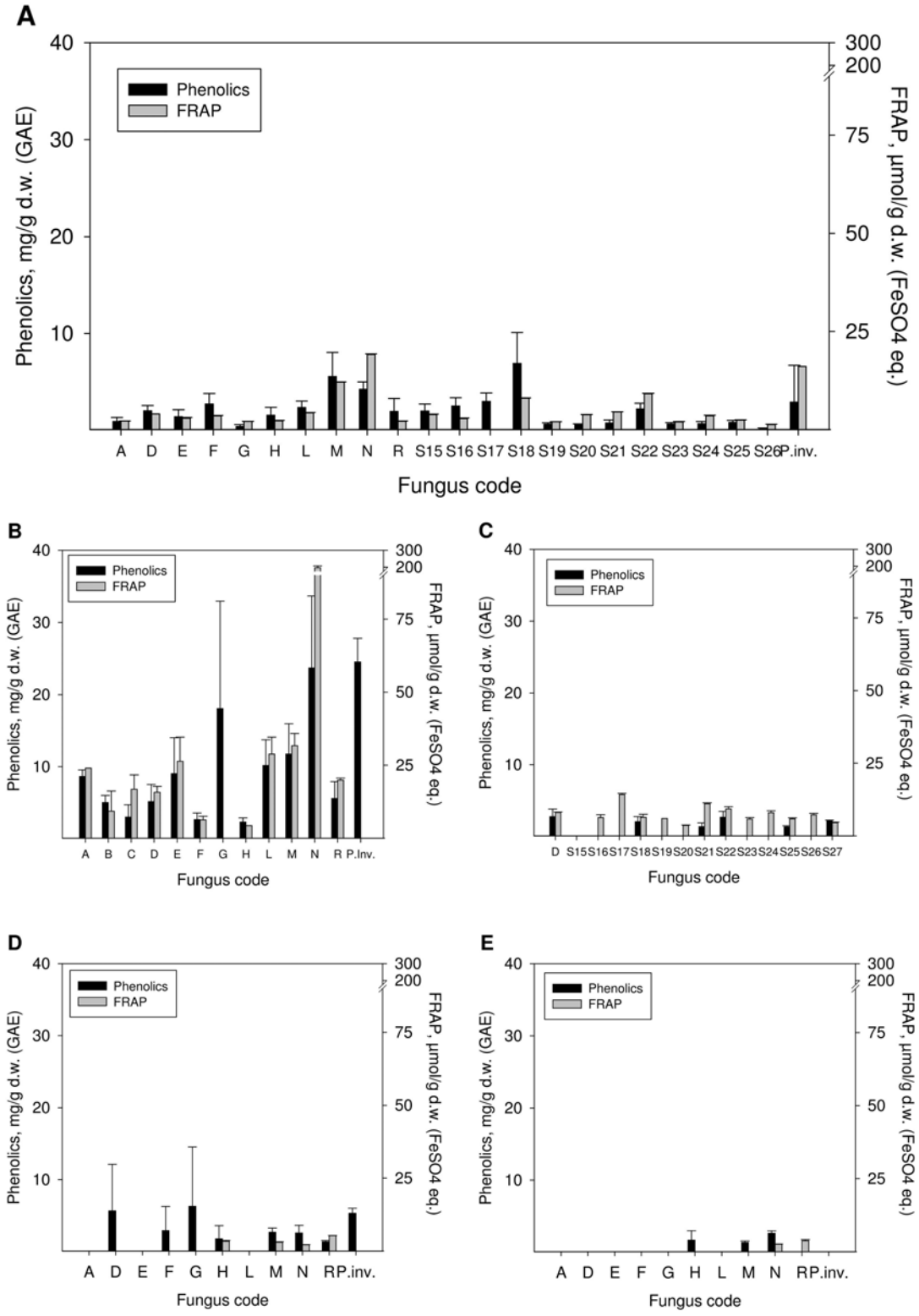

Figure 1: The phenolic contents (mg GAE/g dry weight) and FRAP values (µmol $FeSO_4$ eq./g dry weight) of fungal extracts without protein (methanol precipitation). (A) Fungi from solid Hagem plates, water extraction. (B) Fungi from solid MMN2 plates, PBS buffer extraction. (C) Fungi from solid MMN2 plates, water extraction. (D) Fungi from liquid Hagem plates, water extraction. (E) Fungi from liquid MMN2 plates, water extraction. The results are expressed as mean of triplicate measurements ± SD.

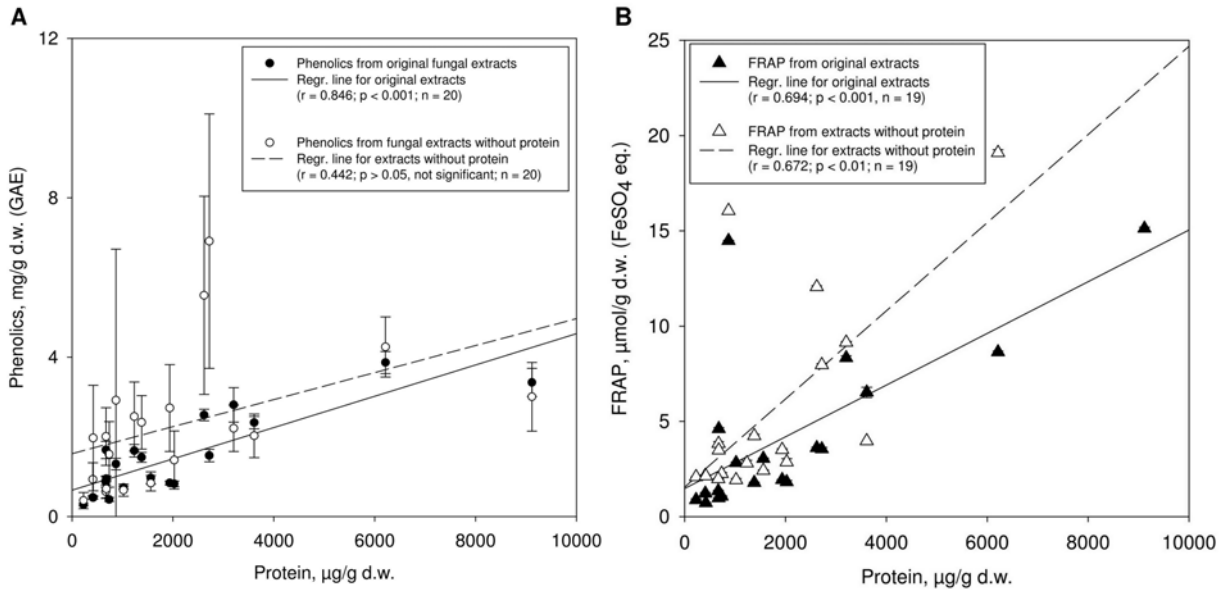

Figure 2: The mean protein contents of the original fungal water extracts (fungi from solid Hagem plates) versus (A) the phenolic contents of the original extracts and of the extracts precipitated with methanol to remove protein with the corresponding regression lines, and (B) the FRAP values of the original extracts and of the extracts precipitated with methanol to remove protein and the corresponding regression lines. The Pearson coefficients for correlation (r), significances (p) and number of samples (n) are found in the figure legends. The results of y-axels are expressed as mean of triplicate measurements ± SD.

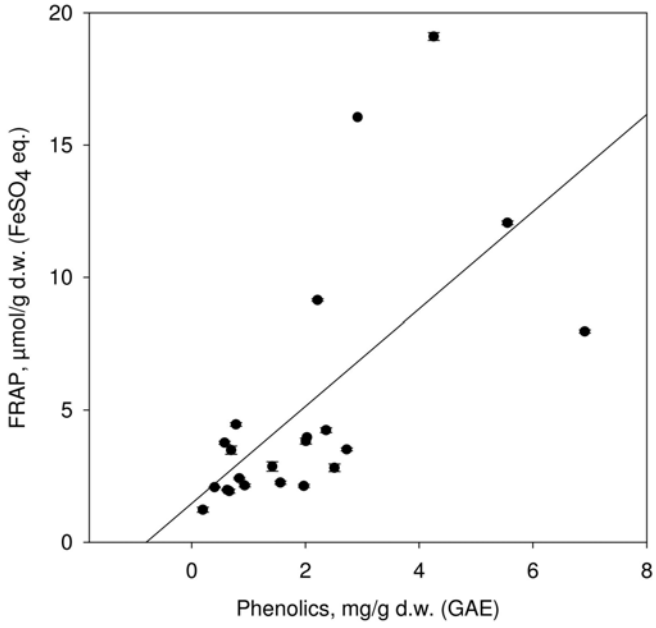

Figure 3: The mean phenolic contents versus the FRAP values with the corresponding regression line of the fungal water extracts (fungi from solid Hagem plates) precipitated with methanol to remove protein (r= 0.652, p < 0.01, n = 22). The FRAP results are expressed as mean of triplicate measurements ± SD.

CONCLUSIONS

The results show, that the fungal endophytes living in pine roots have antioxidative properties. Additionally, the antioxidative potential of these fungi related to their ferric reducing ability, appears to correlate with the production of phenolic compounds. This suggests that phenolics

may function as antioxidants in the studied endophytic fungi. The correlations between ferric reducing abilities of the fungi with the amount of proteins show that protein structures may be linked to the mechanisms of antioxidative properties, but not necessarily as functional components.

ACKNOWLEDGEMENTS
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EDIBLE MUSHROOMS AS POTENTIAL SOURCES OF NEW HYPOCHOLESTEROLEMIC COMPOUNDS

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ABSTRACT

Coronary heart disease (CHD) is the leading cause of death in the Western world after cancer according to World Health Organization. Many studies have established that high total-cholesterol levels and low-density lipoprotein (LDL) cholesterol levels are risk factors for CHD and mortality.

Many investigations have been carried out to explore the possibility of increasing components which have hypocholesterolemic effects in the diet. Two particular groups of substances phytosterols and β-glucans gained much attention in the last decade and there are already commercialized functional food products supplemented with plant sterols and/or derivatives (sterol-esters, stanols, etc) and specific polysaccharides mainly obtained from cereals which are able to inhibit the absorption of exogenous cholesterol.

Edible mushrooms are good sources of phytosterol-like structures such as ergosterol, fungisterol and other derivatives since they were present in all mushrooms because they are constitutive compounds of the hyphae membranes. On the other hand, edible mushrooms contained polysaccharides and depending on the specie, they showed high levels of β-glucans. Apart from these compounds, some mushroom species included certain molecules that were different than lovastatin (since statins were not detected) able to impair the synthesis of endogenous cholesterol by inhibiting the HMGCoA reductase (3-hydroxy-3-methyl-glutaryl CoA reductase) the key enzyme in the cholesterol metabolism.

Keywords: cholesterol, statins, glucans, sterols, HMGCoA reductase

INTRODUCTION

Maintenance of cholesterol homeostasis is one of the major issues in the human body since it is a key constituent of the cell membranes. Thus, if the molecule is not obtained from diet liver synthesizes it by a specific metabolic pathway. When cholesterol enters the lumen of the small intestine it is coming from 3 different sources: diet, bile and intestinal epithelial sloughing. Nowadays in industrialized countries, the average daily intake is approximately 300 – 500 mg. Bile provides 800 mg – 1200 mg cholesterol per day to the intraluminal pool. The turnover of intestinal mucosal epithelium is approximately 300 mg cholesterol per day. The synthesized cholesterol can reach ca. 1000-1600 mg per day. When an excess of exogenous cholesterol is absorbed and reach the liver, it induces several regulation effects such as inhibition of cholesterol biosynthetic pathway and of the LDL-R (low density lipoproteins-receptor) gene expression [1, 2].

Nutritionists have given wide range of dietary recommendations (fruit, vegetables, fish etc.) but with limited success. Only the novel foods products (yogurt, breakfast cereals etc.) offered in the market claiming hypocholesterolemic effects have got a little higher acceptance by consumers. At present there are two types of functional foods recognized by EFSA as able to reduce the risk of CVD because of their ability to reduce cholesterol absorption [3, 4]. One is those products including plant sterols (phytosterols) or derivatives (sitostanol esters etc.). Apparently, intaking of 1.5 g/day of these compounds reduced LDL-cholesterol in hypercholesterolaemic patients by 10 and 15% within 3 - 4 weeks [5]. The other type of functional foods is those containing β-glucans, mainly obtained from cereal products and able to reach 15% cholesterol reduction [6].

Both functional products are able to lower cholesterol in serum by reducing its absorption. However, it has been shown that in subjects who were administered β-glucan, the cholesterol biosynthetic pathway was stimulated compared with control subjects [6]. Thus, in order to increase treatment efficiency, it could be necessary to combine inhibitors of the cholesterol absorption with inhibitors of the cholesterol synthesis.

Edible mushrooms are good sources of phytosterol-like structures such as ergosterol, fungisterol and many other derivatives. The major fungal sterol, ergosterol (9.61-1.28 mg/g dw), is abundant in all mushrooms species since it is a constitutive compound of the hyphae membranes and it is known as a vitamin D_2 (ergocalciferol) precursor [7 - 9]. These molecules might act as plant phytosterols and reduce cholesterol absorption by displacement of the molecule from the dietary mixed micelles formed during intestinal digestion.

Beside oat bran, pectin, guar gum etc. edible mushrooms also contains β-glucans such as lentinan from shiitake, schizophyllan from splitgill, grifolan from maitake mushrooms, α- and β-glucans from sun, reishi and oyster mushrooms, glucuronoxylomannans from tree-ear and white jelly-leaf mushroom etc. [10] and some of them were able to effectively lower serum cholesterol levels [11 – 13]. Apparently, the viscous and gel-forming properties of these compounds could lower the cholesterol absorption by inhibiting the formation of micelles in the small intestine and perhaps they might also interact with the bile acids similarly as explained for bran β-glucans, leading to an increase in faecal bile acids excretion and increasing of hepatic conversion of cholesterol into bile acids [12].

However, if cholesterol is not obtained from the diet, it enhances the *de novo* synthesis in the liver. Statins or vastatins are the most potent drugs available for reducing plasma low density lipoproteins (LDL)-cholesterol concentrations [14]. According to previous reports, several oyster mushroom strains showed lovastatin (mevinolin) a compound able to lower cholesterol levels by inhibiting the 3-hydroxy-3-methyl-glutaryl CoA reductase (HMGCoA-red), a key-enzyme in the cholesterol metabolism [15]. However, other compounds such as lanosteroids, ganoderols etc., obtained from other mushrooms are also able to perform such an inhibition [11].

Thus, in this work a preliminary screening of these potentially active compounds is carried out as an attempt to design new functional foods based on mushrooms extracts able to effectively reduce the cholesterol levels in serum by impairing both the synthesis and the absorption of cholesterol.

MATERIAL AND METHODS

Biological material and samples preparation. Mushroom strains used in this investigation were *Cantharellus cibarius* (Fr.), *Agaricus bisporus* L. (Imbach), *Pleurotus ostreatus* (Jacq.Ex Fr.) Kummer, *Lentinus edodes* S. (Berkeley), *Boletus edulis* (Bull. Ex Fr.), *Amanita caesarea (*Scop. Ex Fri.) Pers. Ex Schw., *Lactarius deliciosus* (Fr.), *Lyophyllum Shimeji* (Kawam.), *Agrocybe aegerita* (Briganti) Singer, *Ganoderma lucidum* (Curtis) P.Karst., *Craterellus*

cornucopioides (L. Ex Fr.) Pers, *Marasmius oreades* (Bolt. Ex Fr.) Fr., *Pleurotus eryngii* (D.C. Ex Fr.) Quel, *Lepiota procera* (Scop. Ex Fr.) Singer, *Agaricus blazei* Murill ss. (Heinem), *Amanita ponderosa* Malençon & R. Heim, *Grifola frondosa* (Dicks.) Gray and *Flammulina velutipes* (Curt. Ex Fr.) Singer.
Fruiting bodies from wild mushrooms were purchased on season from the local market in Madrid (Spain) and the cultivated strains were harvested from the cultivation rooms at CTICH (Autol, Spain) facilities. Fruiting bodies were dehydrated and ground into fine powder as described by Ramirez-Anguiano *et al*. (2007) [16]. Dried mushroom powders were stored at -20ºC until further use.

Reagents. Ascorbic acid and KOH were obtained from Sigma-Aldrich. Ethanol was purchased from Panreac, 2,6 di-tert-butyl-p-cresol (BHT) from Fluka, ergosterol from Alfa Aesar GmbH & Co KG (Germany), pravastatin, lovastatin, simvastatin and atorvastatin from Cinfa (Spain).

Determination of sterols levels in mushrooms. Freeze-dried mushroom powders (0.2 g) and 0.3 g of ascorbic acid were mixed with 15 ml of 11.5% KOH in ethanol:water (55:45 v/v) and vigorously stirred for 15 min at 80ºC. Afterwards, the mixture was cooled down and 10 ml of 0.01% (w/v) BHT in hexane were added. The mixture was shacked during 2 min and left at room temperature (5 min) until complete separation of the phases. Organic fraction was collected and 5 ml BHT solution were added to the aqueous fraction for a second extraction. Both organic phases were pooled together in a round bottom flask and evaporated on a rotary vacuum extractor at 30ºC until dryness. Dry extracts were dissolved in a $ClCH_3$: MeOH (2:1 v/v) solution including hexadecane as internal standard and submitted to GC analysis.

Mushroom extracts were injected into a GC column (Zebron ZB-5 30m x 0.25 mm, 0.25 μm, Phenomenex, France) from a CP-3380 gas chromatography (Varian, Spain) with a flame ionization detector (FID). Split was set 1:10. The injector was set at 260ºC, the detector at 350ºC and the oven temperature was maintained at 60ºC for 1 min, then increased with a rate of 40ºC/min until a final temperature (310ºC) that was maintained during 30 min (modification of the method proposed by Teichman *et al.* (2007)[8]).

A standard curve of ergosterol was used to develop and validate the GC method (linearity, LOD, LOQ, precision and reproducibility were determined using standardized protocols) and to quantify ergosterol and derivatives.

Determination of β-glucans levels in mushrooms. The *beta*-glucan content of the selected mushroom powders (50 mg) was determined according to the protocol described at the user´s manual of the Megazyme assay for mushroom and yeast β-glucan determination (Megazyme, Barcelona).

Determination of HMGCoA-red inhibitors in mushrooms. Mushroom powders (50 mg/ml) were dissolved in methanol, water or combined mixtures. Suspensions were shacked in a Vortex for 1 min and centrifuged at 12000 g 2 min. Supernatants (10 μl) were applied into a 96 wells-plate according to the user´s manual of the HMG-CoA Reductase Assay Kit (Sigma, Madrid). Absorbance at 340 nm was monitored at 37ºC using a microplate reader (Tecan Group Lt, Switzerland).

Several extraction methods were tested to isolate statins such as mixture of 200 mg mushroom powder with methanol: water (1:1 v/v), methanol or water and 20 mM phosphate buffer (pH 7.7): acetonitrile (1:1 v/v) following the procedures before described for lovastatin determination [15, 17, 18]. Depending on experiment, mushroom extracts and lovastatin solutions were centrifuged (12000 g 2 min) and the obtained supernatants filtered through 0.45 μm filters (Millipore). Filtrates were injected into a Sep-Pak® C18 cartridge (Waters)

(preconditioned with acetonitrile and washed with water) and eluted using acetonitrile. Acetonitrile fraction was vacuum concentrated and injected into an HPLC system. Lovastatine and sample preparations were also treated according to Yang & Hwang (2006) [19] to transform lovastatin-lactone into its hydroxy acid form.

Mushroom extracts showing higher HMGCoA-red inhibitory activity (20 µl) and statins were injected in an HPLC system (Agilent) equipped with a column (Zorbax SB-C18 0.3x150mm, 5µm particle size, Agilent) and developed using an isocratic mixture of CH_3CN: 0.5% CH_3COOH (60:40) and a at a flow of 1 ml/min. Peaks were detected with a Diode array detector and identified comparing the spectra and retention times with those of a few statins.
The same samples and lovastatin solutions (20µL) were also injected in an LC-MS (Agilent 6410A Triple Quadrupole LC/MS system coupled with an Agilent 1200 Series Rapid Resolution (RRLC) system) with a C18 column (ACE 3 C18-AR, 150 x 4.6mm particle size 3µm) and developed on isocratic conditions using CH_3CN: 0.5% CH_3COOH (60:40) as mobile phase and a flow rate of 0.5 ml/min. The column eluent was introduced into the electrospray ionization source. The nebulizing gas flow-rate was 9mL/min, drying gas temperature was 350 °C and the capillary voltage was 3500 V. The samples responses from the column were monitored in the positive as well as in the negative ionization mode with full scan from (m/Z: 90-1200).

RESULTS AND DISCUSSION

Ergosterol-derivatives in mushrooms. Ergosterol (ergosta-5,7,22-trien-3β-ol) and its derivatives are compounds structurally similar to plant phytosterols however, only a few reports studied their influence on the cholesterol metabolism. Ergosterol was pointed as a potent agonist for liver X receptor (a factor involved in the regulation of cholesterol homeostasis) and as inducer of ABC-transporters expression (promoters of the active efflux of cholesterol and plant sterols from the enterocyte into the intestinal lumen for excretion). It was also described as a potent C24-reductase inhibitor, an enzyme which catalyzes the reduction of the double bond at C-24 in the cholesterol-biosynthesis pathway [20].

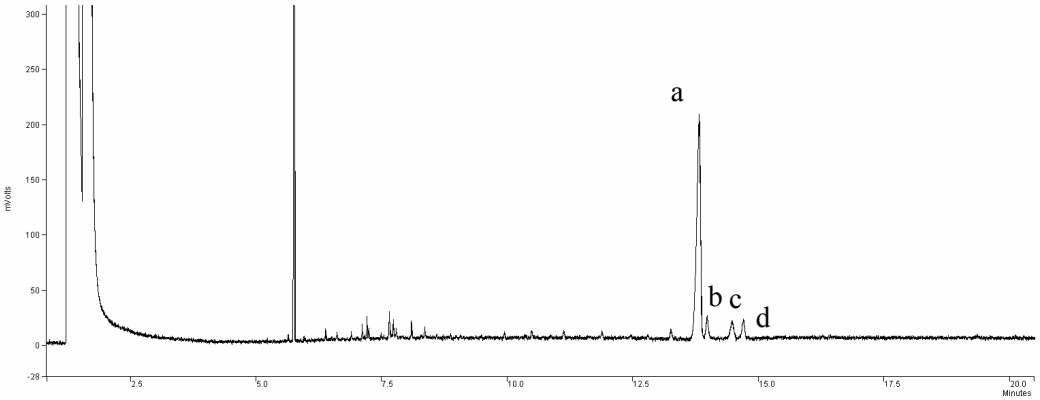

Figure 1: GC chromatogram of the unsaponifiable fraction of *Amanita caesarea*. (a) ergosterol, (b) ergosta-7,22-dienol, (c) ergosta-5,7-dienol, (d) fungisterol.

Ergosterol was the major sterol found in all the analyzed samples (Figure 1) except for *G. lucidum* which showed similar concentrations of ergosterol and ergosta-7,22-dienol. The distribution of ergosterol derivatives was strain dependent (Table 1). Some mushroom species such as *C. cibarius* and *C. cornucopioides* showed almost exclusively ergosterol, other species showed more ergosta-5,7-dienol than fungisterol (ergosta-7-enol) (*L. shimeji, P. ostreatus*), other lacked one or two of the derivatives or presented similar concentrations of the three identified ergosterol-derivatives.

Table 1: Sterols concentration (mg/g dw) in several mushroom species.

Mushroom specie	Ergosta-5,7,22-trien-3-ol (ergosterol)	Ergosta-7,22-dienol	Ergosta-5,7-dienol	Ergosta-7-enol (fungisterol)	Total
Cantharellus cibarius	2,61	ND	ND	ND	2,61
Agaricus bisporus	3,06	0,65	0,67	0,67	5,05
Pleurotus ostreatus	3,75	0,79	0,98	0,51	6,04
Lentinula edodes	5,51	0,40	ND	0,43	6,34
Boletus edulis	5,69	1,21	0,93	0,87	8,71
Amanita caesarea	3,81	1,05	1,15	1,09	7,09
Lactarius deliciosus	1,60	0,36	ND	ND	1,96
Lyophyllum shimeji	4,64	ND	1,55	0,68	6,87
Agrocybe aegerita	5,11	ND	1,01	ND	6,12
Ganoderma lucidum	0,69	0,59	0,17	0,25	1,70
Craterellus cornucopioides	0,79	ND	ND	ND	0,79
Marasmius oreades	3,85	0,54	0,59	0,57	5,55
Pleurotus eryngii	1,40	0,20	0,25	0,22	2,06
Lepiota procera	2,57	0,67	ND	0,64	3,88
Agaricus blazeii	1,73	1,06	0,60	0,75	4,13
Amanita ponderosa	1,65	ND	0,60	0,62	2,87
Grifola frondosa	3,24	0,57	ND	0,61	4,42

ND: Not detectable

Ergosterol concentrations in the selected strains ranged from 0.7 up to 5.7 mg/g dw being *B. edulis* the specie with the highest levels and *C. cornucopioides* the specie with the lowest concentration of total sterols. These results were in concordance with values previously reported for some of the strains for instance, *B. edulis* was also pointed as the mushroom with the largest ergosterol content with concentrations estimated between 9.61-4.89 mg/g depending on authors, *C. cibarius* showed 2.78-3.04 mg/g, *A. bisporus*, *P. ostreatus* and *L. edodes* 7.8-4.4 mg/g. Other mushroom species such as *Suillus granulatus* showed 7.02 mg/g ergosterol and 0.8 mg/g fungisterol (ergosta-7-enol), *Russula cyanoxantha* and *Clitocybe nebularis* contained 1.28 and 1.04 mg/g fungisterol too [8, 9, 21, 22]. However, no previous information concerning the ergosterol content was found for other mushroom species indicated in this study such as for instance *L. procera, M. oreades, A. aegerita, L. shimeji* etc.

***β*-Glucans in mushrooms.** At present, fungal polysaccharides are the subject of several studies because their specific carbohydrate composition and structure appears to confer many important biological activities as antitumour, antioxidant, antiviral, immumodulatory activities etc [10]. Many of the polysaccharides responsible for those activities are β-glucans including hypocholesterolemic activities [6, 13, 23].

β-Glucans were also quantified in the same mushroom species than above described and similarly, their β-glucan content was strain dependent (Figure 2). Some of them showed a high β-glucan concentration such as for instance *G. Lucidum, P. ostreatus, L. edodes, A. aegerita* and *L. deliciosus*. However, mushrooms such as *L. procera, A. blazeii* and *A. bisporus* showed low levels compared to the others.

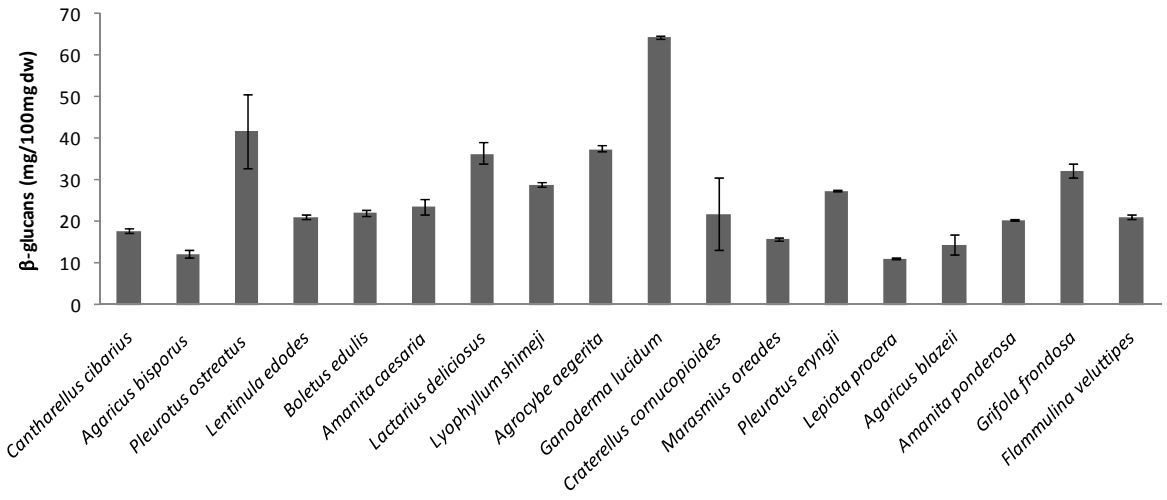

Figure 2: β-Glucan concentration in several mushroom species.

These values were higher than those previously reported by few publications from the the same research group [24 – 26] since the described β-glucan contents ranged from 0.14 to 0.53 mg/100 mg in similar strains such as *Pleurotus pulmonarius, P. ostreatus, P. eryngii, B. edulis, A. aegerita* and *L. edodes*. Differences between results could be due to values expression since in their first publication they express their results on ´dry matter´ basis and later for similar values they indicate that they were referred to their ´edible portion´ which for raw mushrooms could be considered as ´fresh weight´. Moreover, in their β-glucan extraction procedure fresh fruiting bodies were utilized while dry mushroom powder was used to determine the β-glucan content of the samples presented in Figure 2.

A more recent publication estimating the total β-glucan levels by a completely different method indicated concentrations between 2.6 - 13.4 mg/100mg dw for *A. bisporus, F. velutipes, L. edodes, P. ostreatus* and *P. eryngii* [27] and Lee *et al.* [28] ranged the β-glucan contents from 3.2 mg/100mg for *F. velutipes* to 33.5 mg/100mg for *G. frondosa* with other intermediate values for other species including *Pholliota nameko*. Those values are more in concordance with the presented results.

HMGCoA-red inhibitors in Oyster mushrooms. Nowadays, there are many *in vivo* evidences indicating the capability of *Pleurotus* spp. fruiting bodies to lower cholesterol levels in serum [29, 30]. Apparently, this activity could be partially due to the presence of lovastatin, a statin detected in mycelia culture broths as well as in mushroom fruiting bodies in all the developmental stages and tissues [15, 17, 18].

A few *P. ostreatus* strains were screened for HMGCoA-red inhibitors using different extraction procedures such as a mixture of methanol:water (1:1 v/v), water or methanol with not or overnight incubation at 30ºC and applying different extract concentrations.

According to previous publications, overnight incubation (30ºC) of the fruiting bodies with methanol:water (1:1) was the best method to extract lovastatine from fresh Oyster mushrooms [15, 17, 18]. When the dry mushroom powders were tested, the three selected *P. ostreatus* strains showed significant HMGCoA-red inhibitory activity (54.4, 37.7 and 18.8%) and their inhibitory activity was increasing with increasing extracts concentrations (Fig. 3). However, no significant differences were found when the extracts were freshly prepared and applied or after overnight incubation.

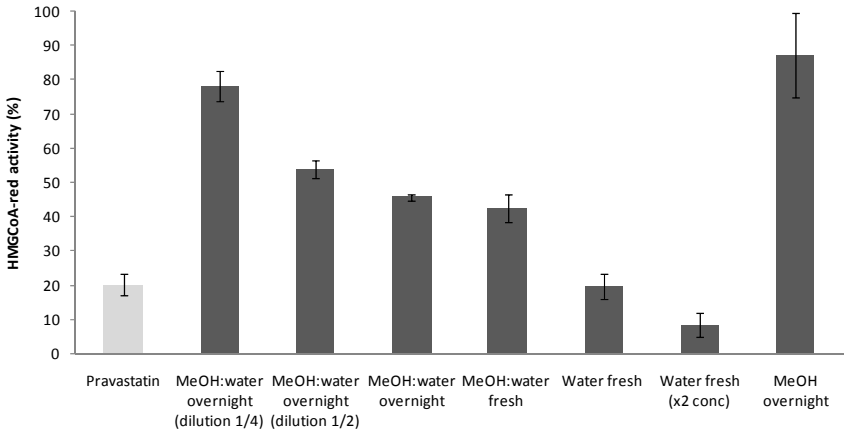

Figure 3: HMGCoA-red activity in the presence of several *Pleurotus ostreatus* extracts prepared under different conditions. Pravastatin was used as positive inhibitory activity.

When 100% methanol or water were tested as solvents no significant inhibitory activity was found in the methanol extracts but for some strains, the water extracts showed a remarkable HMGCoA-red inhibitory activity (Fig. 3). Extraction in 20 mM phosphate buffer (pH 7.7) showed slightly higher HMGCoA-red values than water and its mixture with acetonitrile did not change the value indicating that water was the best solvent to extract HMGCoA-red inhibitors.

Mushroom samples and statin standards were injected in HPLC-DAD and developed with two different mobile phases, the one reported by Gunde-Cimerman & Cimerman [15] and one including acetonitrile and 0.5% acetic acid. The later method showed narrower peaks than the previously reported and proper separation of the 4 selected statins and their hydroxy acid forms therefore it was further utilized to detect and quantify statins in mushrooms (Figure 4).

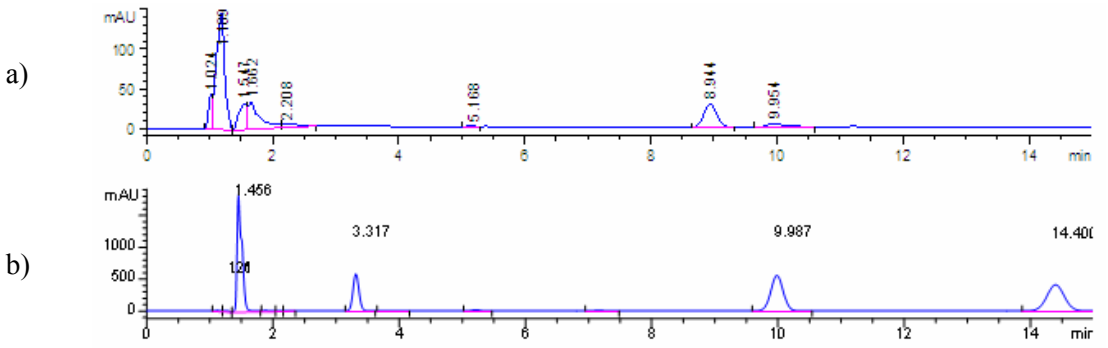

Figure 4: HPLC chromatograms at 240 nm of a) *Pleurotus ostreatus* concentrated extract and b) a mixture of statins including pravastatin (R.T. 1.4 min), atorvastatin (R.T. 3.3 min), lovastatin (R.T. 9.9 min) and simvastatin (R.T. 14.4 min).

Pleurotus ostreatus water, methanol:water (with positive HMGCoA-red inhibitory activity) or methanol extracts yielded no detectable peak at the retention time of lovastatin. Neither compatible peak was detected when the samples were treated to generate lovastatin-hydroxy acid form. Only when the samples were concentrated using the SPE (Solid phase extraction) column, two peaks were observed at 8.9 and 9.9 min with similar spectra than lovastatin since they both showed a maximum at 240 nm (Figure 5).

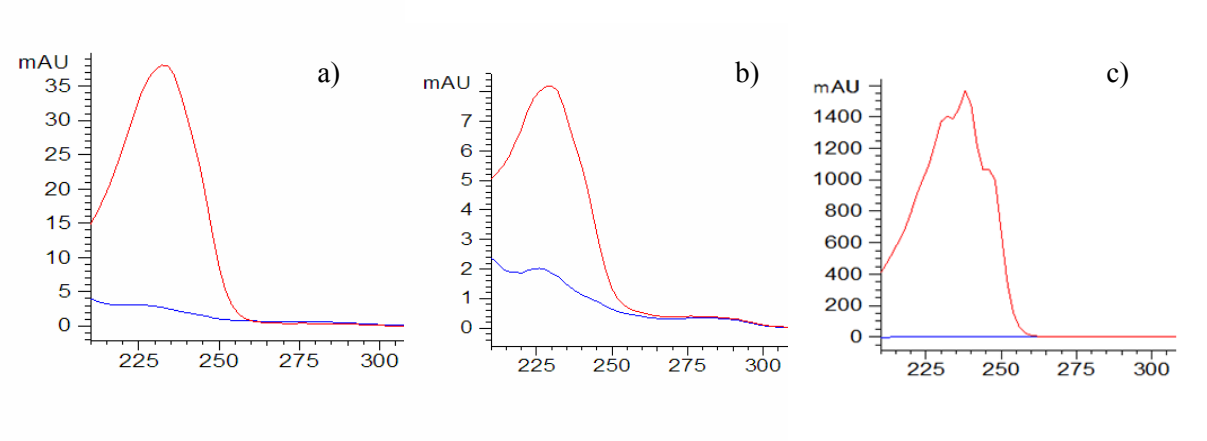

Figure 5: UV-VIS spectra of a) peak eluting at 8.9 min and b) 9.9 min from the *P. ostreatus* extract and c) lovastatin spectrum (9.9 min).

Nevertheless, since many different compounds could also show similar UV-VIS spectra at that maximum, a standard of lovastatin and a *P. ostreatus* extract were injected into a LC-MS in order to determine and compare their masses.

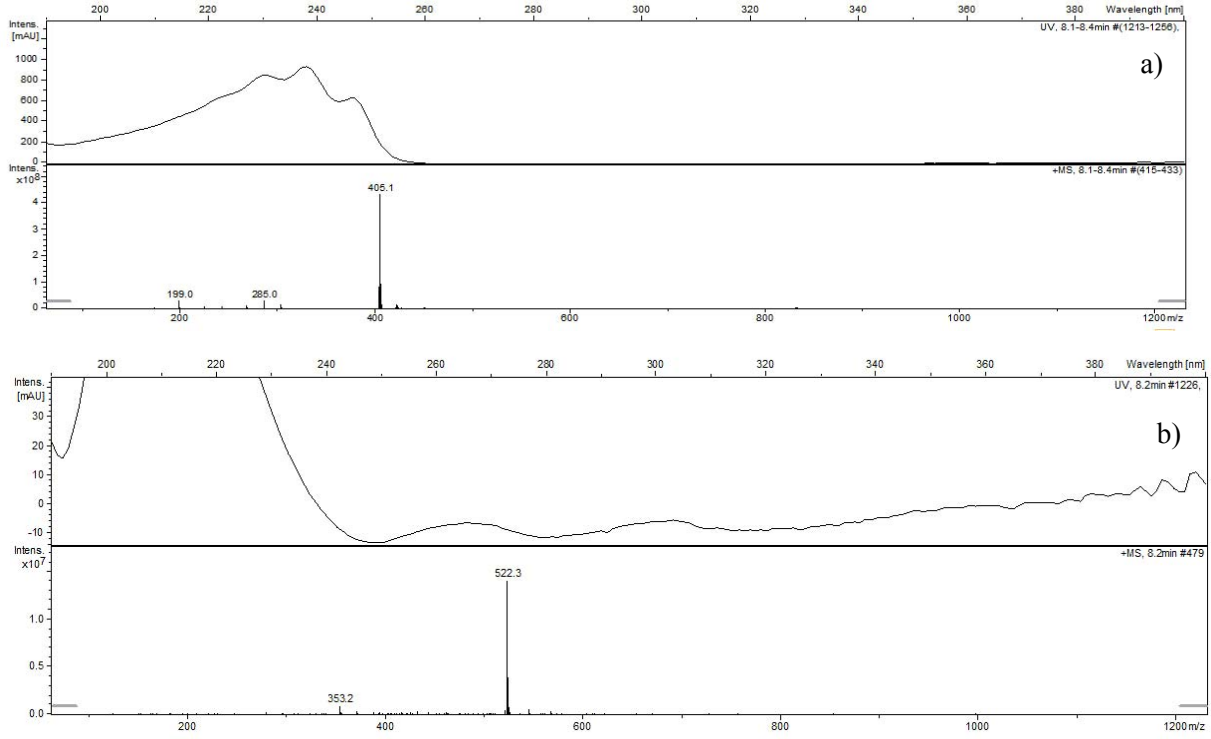

Figure 6: UV-VIS and mass spectra of a) lovastatin (eluting at 8.2 min) and b) of *P. ostreatus* (peak with R.T. 8.2 min).

Lovastatin injected in an LC-MS showed a single peak with a retention time of 8.2 min and a single mass peak of 405.1 undoubtedly corresponding to the 404.55 gram/mol assigned by the literature (Figure 6a). However, the peak from the *P. ostreatus* extract eluting at similar R.T. showed a mass of 522.3 indicating that they were different compounds. Moreover, none of the other detectable peaks showed a mass similar to neither lovastatin nor other statins.

CONCLUSION

Mushrooms fruiting bodies or their extracts might be considered as a new source of compounds with potential hypocholesterolemic activity because they are rich in ergosterol-derivatives, β-glucans and HMGCoA-red inhibitors. Since not a single mushroom strain showed the highest levels of the three type of compounds, a mixture of a few of them could be used to prepare bioactive supplements to functionalize foods potentially able to reduce levels of cholesterol in serum.

Although lovastatin was not detected in the studied oyster mushrooms, water extracts showed remarkable HMGCoA-red inhibitory activity. Therefore, further investigations are at the present being carried out to optimize these supplements and to identify the compounds responsible for the HMGCoA-red inhibitory capacity observed.

ACKNOWLEDGEMENTS

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SCREENING OF FILAMENTOUS TROPICAL FUNGI FOR THEIR NUTRITIONAL POTENTIAL AS SOURCES OF MINERALS AND NUTRITIONALLY IMPORTANT FATTY ACIDS

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ABSTRACT

Seven edible mushrooms cultivated in Brazil were analyzed for calcium, magnesium and zinc contents as well as for their fatty acids profiles. Salmon Hiratake presented major levels of magnesium and zinc (763 and 71 mg/100 g fw, respectively) while calcium was more available from Champignon de Paris (330 mg/100 g fw). Some omega 3 fatty acids (C18:3-*n*3, C20:3-*n*3, C20:5-*n*3 and C22:6-*n*3) were present in most species, with a highlight for Salmon Hiratake that showed to be constituted by 21.97% of C18:3-*n*3 (α-linolenic acid). Linoleic acid, an omega 6 fatty acid (C18:2-*n*6) was present in relatively high percentages in Champignon Portobello (15.17%), Salmon Hiratake (20.21%) and Champignon de Paris (41.67%). EPA and DHA were founded in all mushrooms studied. Shiitake presented 3.8% of EPA from the total fatty acid quantified and black Shimeji presented 5.6% of DHA.

Keywords: mushrooms, calcium, magnesium, fatty acids, α-linolenic acid

INTRODUCTION

Mushrooms are widely consumed worldwide, especially because of the peculiar tastes and aromas that vary from species to species. Many cultures consume mushrooms due their nutritional and medicinal value, however their use as a functional foods is more notable in oriental cultures [1]. Around 25 species are widely accepted as food but only a few reached a production at a commercial level [2]. In Brazil, the species most commonly cultivated and consumed are *Agaricus bisporus, Lentinula edodes* and *Pleurotus* spp. [1].

FAO data (2004) [3] point out that 2.4 million tons of mushrooms were produced in 2002, mostly by China (708 thousand tons), USA (390 thousand tons), Netherlands and Japan. The two later, along with Germany and China are the biggest consumers. Latin America produces only 1.3% of the total of cultivated mushrooms worldwide. Mexico (58.6%), Chile (17.6%) and Brazil (10.6%) head this production [4].

The nutritional value of edible mushrooms, cultivated or wild, has been extensively studied [5-9]. In general, mushrooms are good sources of proteins, carbohydrates, fibers and, at the same time, poor in fat and energy contents [10].

Literature points out that mushrooms contents are severely affected by seasonal variations. The aim of the present work was to evaluate the fatty acids profiles and quantify levels

of calcium, magnesium and zinc present in some mushrooms cultivated in Brazil, and to compare the results with reported data found in the literature for the same species, grown in other countries.

MATERIALS AND METHODS

Materials. Samples of *Agaricus bisporus*, *Lentinula edodes*, *Hypsizygus marmoreus, Pleurotus ostreatus* and *Pleurotus* spp., were acquired in the city of Belo Horizonte (MG, Brazil) and maintained frozen prior to the analysis. For GC analysis, a standard containing a mixture of 37 fatty acids methyl esters (FAME) from C4 to C24 was used (Supelco, Bellefonte, USA) together with an internal standard of methyl-nonadecanoate (C19:0) (AccuStandard, New Haven, USA). Reagent used for methylation reaction was BF_3/methanol (14% p/v) (Sigma, St Louis, USA) while purified water used in the reactions was obtained from a Milli-Q apparatus (TGI Pure Water Systems).

Analysis of Ca, Mg. Zn and Fe contents. For mushrooms mineralization, 200 mg of each sample were individually heated (450 ^{o}C) on an oven [11]. After 6 h, 2 mL of nitric acid were added to each sample and further heating was carried out on the oven for additional 15 min. The material was then removed from heat and 1 mL of HCl was added. Samples were again heated by 5 min on a hot plate (120 ^{o}C) and quantitatively transferred to 5 mL volumetric flasks completing the volume with deionized water. Levels of Calcium (Ca), magnesium (Mg), zinc (Zn) and iron (Fe) were determined using atomic absorption spectrometry on a Hitachi-Z8200 spectrometer coupled to a graphite Hitachi oven (Japan).

Fatty acid methyl esters analyses . Sliced mushrooms were extracted with distilled hexane (300 mL) for 24 hours and then ultra sonicated by 10 min. Extraction was repeated three times, after which the extracts were combined and the solvent was vacuum-removed. Then, samples were hydrolyzed and methylated using BF_3/methanol (14% p/v) solution at 80 °C. 100μL of a solution (2 mg/mL) containing methyl-nonadecanoate (C19:0) were added to each fatty acid ethyl esters (FAME) sample before injection to set an internal standard. In the same way, a Supelco 37 FAME mix was used to identify the FAME present in the mushroom samples. A Gas Chromatograph Varian 3380, equipped with flame ionization detector coupled with a capillary column DB-WAX (J&W Scientific, 30m x 0.25mm internal diameter x 0.25 μm) was used. Column temperature was 100 ºC by 1 min, increasing to 240 ºC by a rate of 7 ºC/min and maintained by 10 min. Detector and injector (splitless 1:100) were kept at 260 °C. Volume injected was 2 μL. Hydrogen was used as carrier gas. Data were acquired and treated by the Software Star 5.52 (Varian).

RESULTS AND DISCUSSION

Nutritional potential of mushrooms Champignon Portobello, Champignon Paris, Salmon Hiratake, White Hiratake, Black Shimeji, White Shimeji and Shiitake was evaluated based on the analysis of minerals and fatty acids contents. The scientific names of the mushrooms studied, as well as the percentages of the major saturated and unsaturated fatty acids (detected as their respective FAME) found are compiled in Table 1.

Table 1. Scientific and popular names and composition of major saturated and unsaturated fatty acids (%), detected as the respective methyl esters (FAME) of the studied edible mushrooms.

Mushroom commom name	Mushroom scientific name	Major saturated FAME (%)	Major unsaturated FAME (%)
Black Shimeji[12]	*Hypsizygus marmoreus*	C16:0 (9.84)	C18:2n6 (6.00)
Champignon Paris [13]	*Agaricus bisporus*	C16:0 (17.58)	C18:2n6 (41.67)
Champignon Portobello [14]	*Agaricus bisporus*	C16:0 (24.53)	C16:1 (24.70)
Hiratake white [15]	*Pleurotus ostreatus*	C16:0 (8.81)	C18:2n6 (7.27)
Salmon Hiratake [16]	*Pleurotus ostreatus*	C16:0 (20.55)	C18:3n3 (21.97)
Shiitake [17]	*Lentinula edodes*	C16:0 (17.69)	C18:2n6 (11.34)
White Shimeji [18]	*Hypsizygus marmoreus*	C16:0 (26.78)	C18:1n9 (18.46)

According to the fatty acids profile determined by GC present by the studied mushrooms (Table 1), all of them presented palmitic acid (C16:0) as the major FAME. Black Shimeji and White Hiratake presented the lower levels of palmitic acid (9.84 and 8.81, respectively) while the better sources of this fatty acid were Champignon Portobello (24.53%) and White Shimeji (26.78%). Presence of miristic (C14:0), pentadecanoic (C15:0) and stearic (C16:0) acids were also detected in the samples as minor constituents. Among the unsaturated fatty acids, distribution was not the same in the mushrooms. Four mushrooms (Black Shimeji, Champignon Paris, White Hiratake and Shiitake) presented linoleic acid (C18:2n6) as the principal unsaturated fatty acid but in very different concentrations (6.00, 41.67, 7.27 and 11.34, respectively). Linoleic acid was reported as the major fatty acid present in two *Pleurotus* species studied by Pedneault *et al.* [19]. Champignon Portobello was richer in the C16:1 (palmitoleic acid), while Salmon Hiratake showed linolenic acid as the principal unsaturated components. Unusual FAME C20:3n3, C20:5n3 and C22:6n3 were also detected in some samples, especially in White Hiratake and Black Shimeji, however in lower levels.

Contents on saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids were calculated for the analyzed mushrooms (Figure 1). PUFA contents were almost the half of the FAME detected in Salmon Hiratake and Champignon Paris. In White Hiratake, levels of PUFA were higher than other type of fatty acids. Salmon Hiratake presented nearly equivalent amounts (~21%) of α-linolenic acid and linoleic acids.

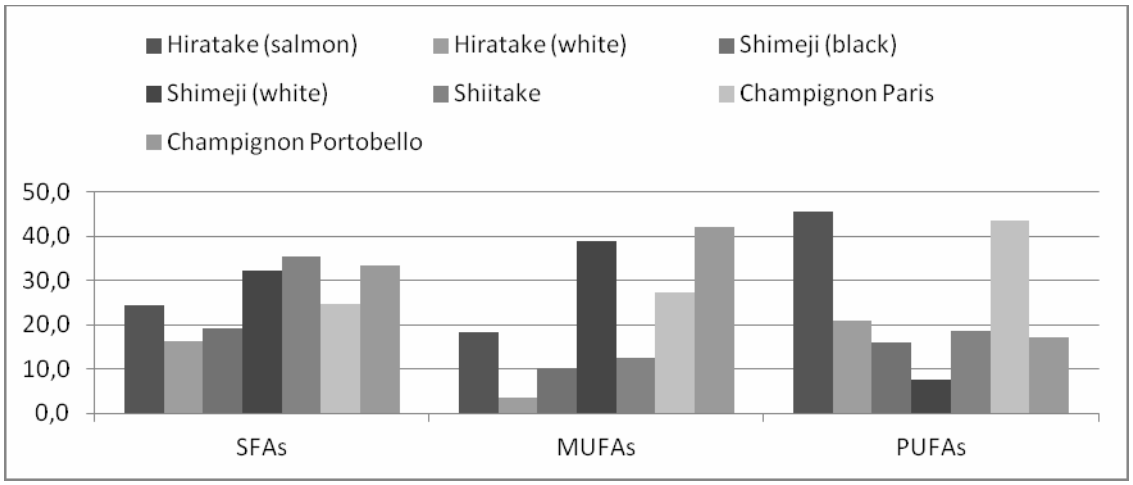

Figure1. Total SFA, MUFA and PUFA in edible mushrooms.

White Shimeji and Champignon Portobello species showed to possess high levels of MUFA-type fatty acids, with the proportion of SFA:MUFA:PUFA of 33:42:18 and 32:39:9 %, respectively. Both presented high levels of palmitoleic (C16:1 *n*–7) and oleic (C18:1 *n*–9) acids (Table 3). Shiitake presented SFA contents (35.4 %) higher than the total amount of PUFA (22%) and MUFA (13%).

Levels of Ca, Mg, Zn and Fe were determined by atomic absorption (Table 2), that showed the most abundant one to be magnesium, followed by calcium and zinc in the species studied. The values determined are important because there is a great variation of values reported, that may be due to seasonal or related variations [20] as exemplified by the works of Mattila *et al.* [21] and Çağlarırmak [16]. In the present work Calcium levels reached 329 mg and 210 mg/100g of fresh Champignon Paris and Champignon Portobello, respectively, while magnesium and zinc levels reached not more than 762 and 71 mg/100 g fw, as detected for Salmon Hiratake sample. It is worth to observe that mushroom species studied in the present work have higher Zn levels than the minimum required daily consuming amount [22]. The Zn values found for Brazilian cultivated mushrooms are higher than values reported by Gençcelep (2009) [23] and Mattila *et al.* [21].

Table 2. Levels of calcium, magnesium and zinc in edible mushrooms in fresh weight (fw).

Common name	Ca (mg/100g)	Mg (mg/100g)	Zn (mg/100g)
Champignon Portobello	210.0	426.4	14.0
Champignon Paris	329.5	464.4	19.8
Salmon Hiratake	73.0	762.5	70.9
White Hiratake	56.7	626.2	24.8
Black Shimeji	23.3	659.0	28.8
White Shimeji	49.6	520.4	21.9
Shiitake	39.4	548.1	32.1

CONCLUSIONS

This comparative analysis of the fatty acids and Mg, Ca, Fe and Zn contents present in mushrooms commercialized and consumed in Brazil showed different values from those reported for the same species cultivated in other countries and even in different regions of the country. Some species showed to be rich in calcium, magnesium and even zinc, being able to supply the daily recommended consumption amount. Presence of the essential fatty acid linoleic acid, as major components in Champignon Paris and Hiratake salmon reinforces the potential of these species as health foods, especially because omega-3 and omega-6 fatty acids were remarkably detected in all studied species. This work contributes for the knowledge of the regional variation of minerals and fatty acids contents of mushrooms aiming at stimulating their consuming as rich nutritional sources in countries like Brazil and other tropical countries where mushrooms are still most often regarded only as delicacy foods.

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ZINC ACCUMULATION AND DIFFERENT WAYS TO SEQUESTRATION OF INTRACELLULAR ZINC IN FRUIT-BODIES OF ECTOMYCORRHIZAL FUNGI *RUSSULA* SPP. AND *HEBELOMA* SPP.

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ABSTRACT

Mycorrhizal fungi, including ectomycorrhizal (EM) species, play an important role in the environmental cycling of elements and protection of their host plants against various stress factors such as toxic levels of heavy metals. The Zn content of *Hebeloma* spp. sporocarps grown in pristine environments was found within the range of 50 to 150 mg kg^{-1}, which is typical for most EM fungi, including the majority of *Russula* spp. We identified four Zn-accumulating species, *R. atropurpurea*, *R. ochraleuca*, *R. pumila* and *R. viscida*, which clade together and show common fruit-body Zn concentrations of 300 to 1100 mg kg^{-1}. Three analyzed *Hebeloma* species showed virtually all Zn of cell-free extract complexed with glutathione (GSH). In contrast, 70 to 80% of extracted cellular Zn of Zn-accumulating species of *Russula* spp. was sequestered by 6-kDa MT-like peptides, while the remaining Zn was associated with GSH. We detected Zn sequestration contributed by MT-like peptides also in 25 species of poorer Zn accumulators of *Russula* spp., thereby indicating that the capacity to produce Zn-MT is independent of phylogenetic relation and Zn-accumulation phenotype.

Keywords: Zinc uptake; Metal ligands; Metallothionein; Glutathione; Metal tolerance

INTRODUCTION

Fungi are ubiquitous components of soil communities with a substantial role in biogeochemical cycling of the elements, including heavy metal species [1]. While the capacity of macrofungi to accumulate heavy metals in large quantities has been reported since the middle of the last century, deciphering of the molecular mechanisms underlying this phenotype is being challenged only recently. Especially interesting are fungi forming the mutualistic partnerships with land plants (mycorrhizas), which appear to be of functional importance for plant nutrition and healthy growth [2]. Several studies have shown that metal-resistant arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) fungi can contribute to the metal tolerance of associated higher plants [3-8] which might be also exploited for biotechnological application such as phytoremediation [1, 9]. The fungal mechanisms of toxic metal tolerance the plants may benefit from include metal immobilization on the soils (e.g. by biosorption on hyphae cell-walls, extracellular precipitation of secondary mycogenic minerals, binding by extracellular metabolites) and intracellular sequestration (e.g. by specialized peptidaceous cytosolic ligands, compartmentalization in vacuoles) [10-14].

Zinc is an essential divalent metal ion in all life forms, where it serves catalytic and structural roles. As a borderline Lewis acid, Zn^{2+} can accept electrons from residues of several

amino acids including cysteine, histidine, aspartate, glutamate, serine, threonine, and tyrosine, which form stable coordination spheres in binding centers of Zn-metalloproteins [15]. Like with the other essential metals, the healthy growth is impaired if an organism is unable to acquire sufficient Zn; however, Zn can be detrimental to organisms when present in excess [16]. Therefore, the Zn homeostasis must be maintained by uptake and efflux of Zn transporters and the coordinated management of the Zn pool involving compartmentalization within the cell, intracellular complex formation and deposition of Zn^{2+} at target metalloproteins via specialized metallochaperone proteins [17].

In animals and plants the majority of the Zn cytoplasmic storage pool is sequestered by metallothioneins (MTs) [18, 19]. MTs are a gene-encoded group of cysteine-rich peptides of distinct lengths (e.g. 61 to 65 aminoacids in mammals, 42 to 84 in plants), capable of high affinity coordination of heavy metal ions via cysteine residues shared along the peptide sequence in Cys-X-Cys or Cys-Cys motifs. While the role of plant MTs is generally attributed to the homeostasis of essential heavy metals such as Zn and Cu [19], in mammals MTs are also associated with protection against heavy metals (especially Cd) and oxidant damage [18]. Differential transcription of MT genes during mycorrhiza development in the EM *Pisolithus tinctorius* [20] and the AM *Gigaspora margarita* [21] suggests that the respective MT peptides might be involved in regulation of the metal homeostasis. Efforts have been made towards understanding the role which MTs of AM and EM fungi play in enhanced tolerance against heavy metal stress. Unlike mammalian and plant MTs, showing sequence conservancy, fungal MTs produced in a response to stress conditions encompass a quite diverse group of peptides. Exposure to Cu (and oxidative stress) induced transcription of the *GintMT1* gene encoding 71-amino acid residue (AA) GintMT1 in the AM fungus *Glomus intraradices* [22]. A gene encoding 34-AA PiMT involved in sequestration of Cd in *P. involutus* was isolated and shown to confer higher Cu-tolerance to transgenic EM fungus *Hebeloma cylindrosporum* [23, 24]. We recently described 34-AA AsMT1a, AsMT1b, and AsMT1c (unrelated to PiMT) from Ag-hyperaccumulating *Amanita strobiliformis* [25, 26], which share 82% identity, providing the first evidence of the presence of MT isoforms in EM fungi. Only the Ag-AsMT1a complex was detected in a *A. strobiliformis* fruit body in which As*MT1a* was the prevailing transcript. In *H. cylindrosporum*, Cu induces expression of indigenous Hc*MT1* and Hc*MT2* encoding 59-AA and 57-AA MTs, which share only 40% identity [27]. While transcription of Hc*MT2* is also induced by Cd, neither Hc*MT1* nor Hc*MT2* were induced by Zn.

Besides MTs, glutathione (GSH) appears an indispensable component of heavy metal and redox homeostasis. As a fundamental antioxidant molecule, GSH directly eliminates reactive oxygen radicals induced by heavy metal ions in cells [28], and provides reducing equivalents in the ascorbate-glutathione antioxidation cycle [29]. It also appears likely that in some EM fungi, including *P. involutus*, GSH is involved in cellular detoxication of Cd^{2+} dependent upon exclusion of the metal into vacuoles [13]. The vacuolar sequestration of Cd was detailed in the yeast *Saccharomyces cerevisiae*, in which the uptake to vacuoles is accomplished by the ATP-dependent ABC-type YCF1 transporter effective on the bis(glutathionato)Cd complex [30]. Studies in the same yeast have revealed that vacuolar sequestration of Zn depends on the cation diffusion facilitator (CDF) family transporters ZRC1, mediating transport via a Zn/H^+ antiport mechanism, and COT1 [17]. Both ZRC1 and COT1 appear to be required for homeostasis sequestration of zinc for later use under zinc-limiting conditions as well as for detoxication of excess Zn. Recently Blaudez & Chalot [31] described the CDF transporter ZnT1 of *H. cylindrosporum* capable to complement *zrc1*Δ mutation in *S. cerevisiae*.

In the present paper we described the analysis of intracellular speciation of Zn in fruit-bodies of two EM genera, *Hebeloma* spp. and *Russula* spp. We showed that while the sequestration of excess Zn in *Russula* spp. is dominated by MTs, these were not detected as Zn-

ligands in analyzed *Hebeloma* spp. Our data suggested that the whole Zn storage pool of *Hebeloma* spp. is bound to GSH and/or compartmentalized.

MATERIALS AND METHODS

Collection and characterization of fruit-bodies. The young fruit-bodies of *Hebeloma* spp. and *Russula* spp. (Fig. 1) were collected from their natural habitat in pristine areas of the Czech Republic. The fruit-bodies were cleared of substrate debris, washed with sterile distilled water and stored at -80 °C. The total metal content was determined by INAA as described previously [33]. The molecular phylogeny was inspected by sequencing of ITS regions of rDNA amplified from the total chromosomal DNA in polymerase chain reaction. Total DNA was extracted from frozen tissue using the Power Soil DNA Isolation Kit (MoBio Laboratories) according to the manufacturer's instructions.

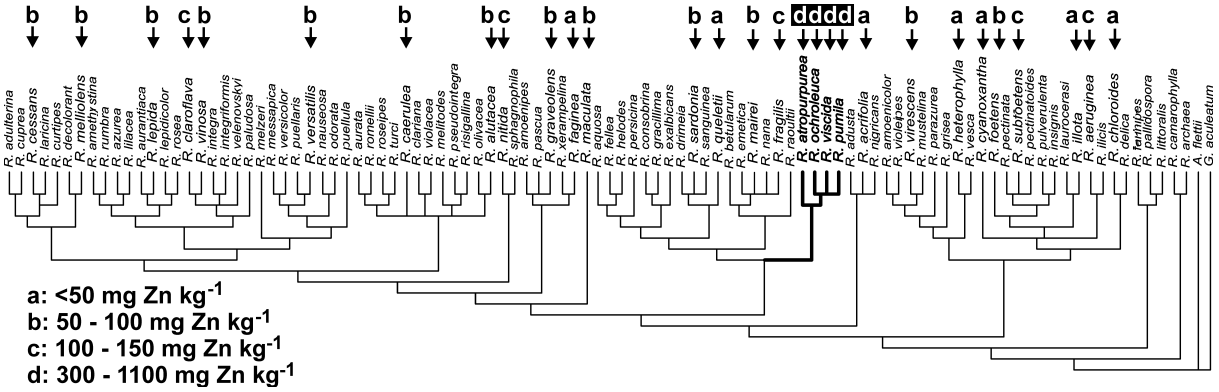

Figure 1: *Russula* species used in this study (indicated by arrows) and their phylogenetic position according to Miller & Buyck [32]. Concentration of Zn in fruit-bodies was determined by INAA and the range particular fruit-body fell in is indicated.

Isolation of metal complexes. The fungal tissue extracts were obtained from 3 g of fruit-bodies (stipes and caps in natural proportion). These were ground in liquid N_2 with mortar and pestle and the disintegrated tissue was extracted with 2.5 ml of 25 mM HEPES (pH 7.0). Cell debris was removed by centrifugation at 20 000×*g* for 10 min at 4 °C. To fractionate the extract by using size exclusion chromatography (SEC), 2 mL of the extract was loaded onto a Superdex Peptide 10/300 GL column (GE Healthcare). Separation was performed with a BioLogic DuoFlow FPLC system (BioRad) and 50 mM HEPES, 25 mM KNO_3 (pH 7.0) as a mobile phase at a flow rate of 0.5 mL min^{-1}. Ribonuclease A (GE Healthcare), ubiquitin (Sigma), synthetic 2.1-kDa peptide and glutathione (Merck) were used as molecular weight (MW) standards. Metal contents in aliquots of 0.5 ml fractions from SEC were determined by atomic absorption spectrometry (AAS; model Spectr AA300, Varian, Inc.).

Reverse phase chromatography and electrophoresis of the ligands. SEC fractions containing complexes of MW ≥5-kDa were pooled and brought to a final volume of 50 to 80 µL by ultrafiltration with Microcon YM-3 (Millipore). Pooled fractions containing the low-molecular weight Zn-complexes were freeze-dried and the lyophilizate was dissolved in 50 µL of distilled water. The sulfhydryl containing molecules of isolated complexes were fluorescent labeled in reaction with 7-fluorobenzofurazan-4-sulfonic acid (SBD-F) and resolved by reversed-phase high-performance liquid chromatography (RP-HPLC) or sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously [26, 34]. The RP-HPLC analyses were performed with an Agilent 1200 HPLC with PEEK capillaries, an Agilent G1321A

spectrofluorimetric detector (Agilent Technologies, Inc.; excitation at 385 nm, emission at 515 nm) and an analytical 250 mm column (4 mm id) packed with Separon SGX C8 (5 μm; Tessek, Ltd.).

The acetonitrile proportion in water (both with 0.1 % [v/v] trifluoroacetic acid) during elution was 5 to 25% (v/v) linear gradient from 0 to 20 min, 25 to 70% from 20 to 25 min, 70% from 25 to 33 min and 70 to 5% from 33 to 35 min. The electrophoretic separation of ligands was conducted in the presence of SDS and under reducing conditions in 16% polyacrylamide gels (SDS-PAGE) in a Tris-Tricine buffer system with 6M urea as described previously [26, 34]. The fluorescent electrophoreograms were recorded with a ≥605 nm filter after suboptimal excitation at 312 nm. Glutathione (Merck), γ-glutamylcysteine and cysteine (Sigma), (γ-glutamylcysteine)$_n$glycine (phytochelatin; n=2 and 3; Vidia, Ltd.) were used as standards.

RESULTS AND DISCUSSION

The fruit-bodies of *H. mesophaemum*, *H. sacchariolens*, *H.* cf. *crustiliniforme* analyzed here were obtained from pristine soil with common Zn background levels and contained 164, 121 and 61 mg Zn kg^{-1} of dry weight. To inspect the cellular Zn-deposition form, the disintegrated fruit-body tissues were extracted under mild conditions of neutral pH. The disintegration allowed extraction of nearly 80% of the total accumulated Zn. The size exclusion chromatography (SEC) of extracts from all three species showed that the majority of Zn (81 to 83%) was contained in low-MW fractions of apparent MW of ≤1 kDa (Fig. 2A shows speciation of Zn in extracts from *H. sacchariolens* as an example). Remaining Zn portions associated mainly with proteins of MW ≥ 20kDa (column size exclusion limit) could be attributed to physiological Zn-containing metalloproteins of the fungus. The size of the major Zn-complex suggested that GSH or short phytochelatin (PC) molecules could be considered likely ligands. PCs are small peptides of general structure (γ-Glu-Cys)$_n$X (PCn; n = 2-11; X represents Gly, Ser, β-Ala, Glu, Gln or no residue) enzymatically synthesized from GSH or its analogues in a metal-dependent manner. They have pivotal role in the detoxication of various heavy metals (especially Cd) in plants and certain yeasts [35, 36] and PC2 and PC3 were also reported as dominant Cd-binding ligands in the EM *Boletus edulis* [37]. Although PCs have the capacity to bind Zn and PC synthesis is seemingly induced in plant cultures by Zn present in media [38], it is unlikely that they play a role in the Zn homeostasis and detoxication in plants and yeasts [35, 36].

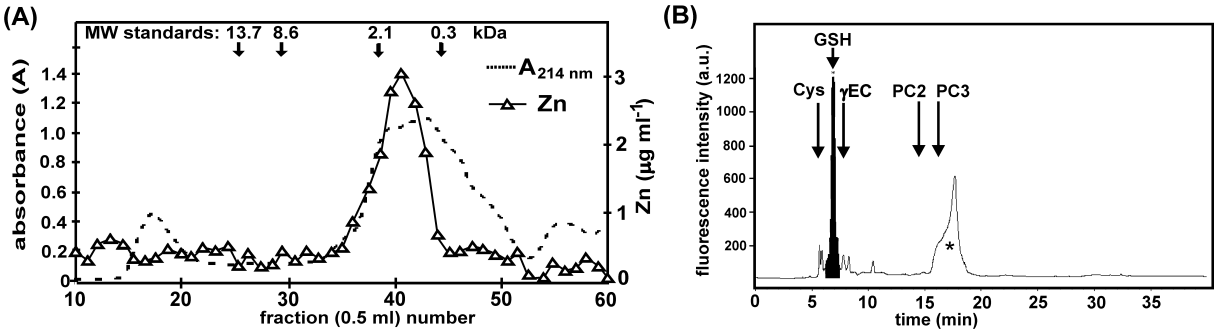

Figure 2: Speciation of Zn in fruit-body of *H. sacchariolens* and characterization of thiol-containing peptides from ≤1 kDa Zn-complex. **(A)** SEC fractionation of the fruit-body extract. The elution maxima of the MW standards are indicated by arrows. **(B)** RP-HPLC of peptides from *H. sacchariolens* labeled with SBD-F. The retention times of GSH, γ-glytamylcysteine (γEC), cysteine (Cys) and phytochelatins PC2 and PC3 are indicated by arrows. Asterisk indicates a peak observed also in a blank sample prepared with water instead of the SEC fraction aliquot.

To characterize Zn-ligands of the low-MW Zn complexes of *Hebeloma* spp. cell-free extracts, the corresponding SEC fractions were pooled, concentrated by lyophilization and thiol-containing compounds were labeled with a sulfhydryl-specific SBD-F probe. Separation of

labeled compounds by RP-HPLC revealed the presence of a high intensity GSH peak in fractions from all analyzed species (Fig. 2B shows *H. sacchariolens* as an example), thereby suggesting the presence of a glutathionatoZn complex in the cell-free extracts. It also showed that PC2 and PC3 were absent from the complex (Fig. 2B). It should be noted that our approach does not allow us to conclude that GSH represents the major Zn-ligand sequestering the metal *in vivo*. As the cytosolic GSH levels lay generally within a range of 2 to 3 mM [39], the formation of a secondary glutathionatoZn complex from Zn^{2+} released from cellular compartments in the extract could not be excluded. In plants and yeasts subcellular compartmentalization of Zn depends on transporters of the CDF family, which mediate secondary active transport of Zn^{2+} ions into organelles [17, 30]. The CDF transporter ZnT1 present in the endoplasmatic reticulum membrane and the presence of a Zn pool in small cellular vesicles (but not in vacuoles) described in vegetative mycelia of *H. cylindrosporum* [31] suggest that excess Zn is organelle-sequestered in this species. If the localization of Zn in *Hebeloma* spp. fruit-bodies is the same as in the mycelia and if it is present in vesicles as a free or, e.g., carboxylate bound ion or as a glutathione-Zn complex remains to be elucidated.

In an extensive study scoring the Zn contents in 383 species of basidiomycetous fungi Vetter et al. described the average fruit-body Zn content as 119 mg kg^{-1} of dry tissue weight [40]. Our data obtained for analyzed *Hebeloma* spp. and most *Russula* spp. (Fig. 1) corroborate these results. The authors also reported Zn concentrations in fruit-bodies of *Russula atropurpurea* ranging from 763-1067 mg kg^{-1}. The pronounced capacity of *R. atropurpurea* to accumulate Zn was confirmed also in our survey of fruit-bodies of this species collected from pristine areas [41]. The genus-wide screening was performed to inspect the Zn content in fruit-bodies of species of various phylogenic groups. Most members of the genus fell in the range of 50 to 100 mg of Zn kg^{-1}, but we identified *R. ochraleuca*, *R. pumila* and *R. viscida*, close relatives of *R. atropurpurea*, that showed common fruit-body Zn concentrations of 300 to 1100 mg kg^{-1} (Fig. 1). Although the Zn accumulation potential of these species is exceptional, they could not be regarded as Zn-hyperaccumulators. According to Brooks, species are considered as hyperaccumulating if they deposit in their organs at least 100-fold higher concentrations of a particular element than other species growing over an underlying substrate with the same characteristics [42].

To inspect the Zn-containing complexes in accumulators *R. atropurpurea*, *R. ochraleuca*, *R. pumila* and *R. viscida*, the disintegrated tissues were extracted and the extracts were subjected to SEC. Zn ligands were further characterized by using RP-HPLC and SDS-PAGE as described under Materials and Methods. The SEC chromatograms and isolated Zn-ligands showed the same pattern and characteristics among all four species. Fig. 3 shows data obtained from cell-free extracts of *R. pumila*. The majority of extracted Zn (70 to 85 %) was found associated with a MW fraction of 6 to 9 kDa (Fig. 3A). The size of the complex suggested possible involvement of MTs in sequestration of intracellular Zn. The RP-HPLC showed a multiple-peak cluster pattern (Fig. 3B) similar to that we previously observed with purified 3.4-kDa AsMT1 of *A. strobiliformis* [26]. The higher elution volumes, compared to those of AsMT1, suggested higher MW of the Zn-compex ligands. Considering their proteinaceous nature, the labeled thiol compounds were also resolved using SDS-PAGE. As shown in Fig. 3C, the 9-kDa complex from *R. pumila* contained the labeled MT-like peptide of MW similar to that of the 6.1-kDa rabbit MT1a plus minor larger peptide. Such MT-like peptides were detected also in Zn-complexes from *R. atropurpurea*, *R. ochraleuca* and *R. viscida*. A significant portion of extracted Zn was eluted from the SEC column with the fraction of apparent MW of ≤1 kDa (Fig. 3A), which resembled Zn-complexes of *Hebeloma* spp. extracts (Fig. 1A). Indeed, the RP-HPLC analysis of these fractions revealed the presence of GSH accompanied by uncharacterized minor thiols (Fig. 3D).

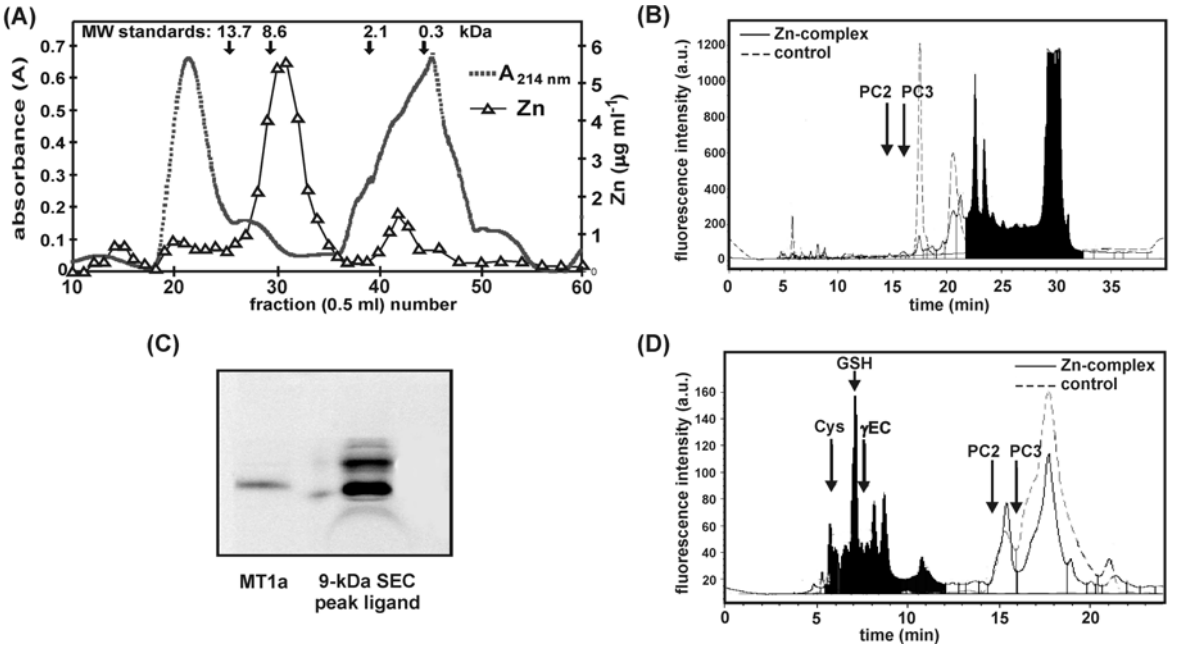

Figure 3: Speciation of Zn in fruit-body of *R. pumila* and characterization of thiol-containing peptides of Zn-complexes. **A)** SEC fractionation of the fruit-body extract. **(B)** RP-HPLC of SBD-labeled peptides from 9 kDa Zn-complex. **(C)** Electrophoretic analysis of SBD-labeled peptides contained in 9 kDa Zn-complex. MT1a denotes the SBD-labeled 6.1-kDa rabbit metallothionen 1a. **(D)** RP-HPLC of SBD-labeled peptides from ≤1 kDa Zn-complex. The elution maxima of the MW standards used in SEC, retention times of GSH, γ-glytamylcysteine (γEC), cysteine (Cys) and phytochelatins PC2 and PC3 on reverse-phase column are indicated by arrows.

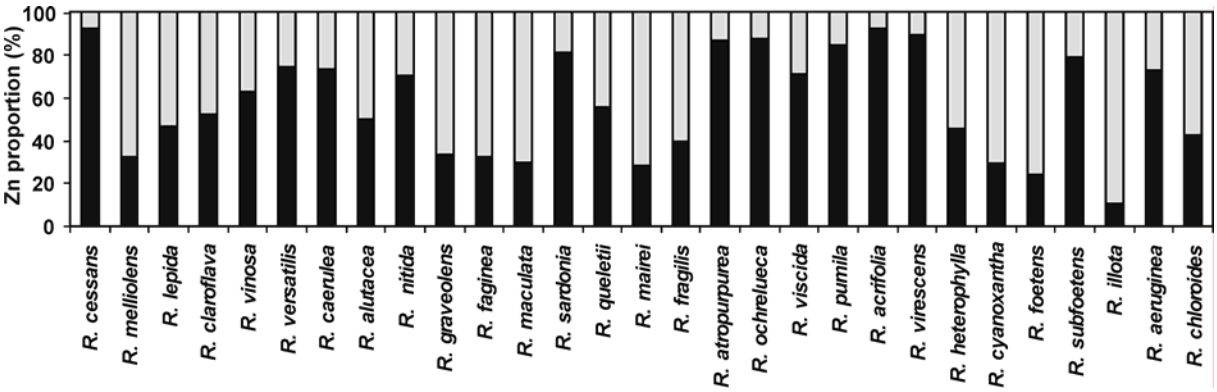

Figure 4: Distribution of Zn between MT-bound (black) and GSH-bound (grey) fractions in fruit-body extracts of *Russula* spp. Zn contents were determined in pooled SEC fractions corresponding to Zn-MT peak and Zn-GSH peak and the results are expressed as % of Zn recovered in Zn-MT plus Zn-GSH fractions (data from the 2010 season collection).

In order to get insights concerning the intracellular Zn in the genus *Russula*, we inspected the speciation of Zn in species of different phylogeny as indicated in Fig. 1. To this end, the individual cell-free extracts were fractionated by SEC and Zn contents were determined in pooled fractions corresponding to Zn-MT peak and Zn-GSH peak. The data summarized in Fig. 4 show that the capacity to deposit Zn in complexes with MT peptides and ≤1 kDa complexes was common and independent of their phylogenetic relation or Zn-accumulation phenotype. These results signify that Zn homeostasis in the genus *Russula* involves both Zn-MTs and formation of

a glutathionatoZn complex (or sequestration of Zn in subcellular compartments?). It should be noted that most eukaryotic cells localize metal-MT complexes in the cytoplasm [18, 19, 36]. It also appears that *Russula* species accumulating high Zn levels preferentially employ MTs to sequestrate the excess metal, a trait that was not detected in *Hebeloma* spp.

CONCLUSIONS

Our data show that *Russula* spp. and *Hebeloma* spp. employ distinct strategies for intracellular sequestration of excess Zn in their sporocarps, indicating that the tolerance against excess Zn would be dominated by different mechanisms. The inspection of the subcellular localization of the metal complexes is under way. Well aware of the fact that the mechanisms underlying extreme Zn accumulation must involve also efficient metal uptake, we are currently challenged with identifying the plasma membrane Zn transporters of *Russula* spp. accumulators by the expression library screening. Better understanding of molecular basis of metal uptake and metallotolerance in EM fungi would lead to improved applications in bioremediation and forestry.

ACKNOWLEDGEMENTS

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ACCUMULATION OF SELENIUM FROM SELENIZED YEAST IN THE CULTIVATED MUSHROOM *AGARICUS BISPORUS*

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ABSTRACT

Agaricus bisporus (button mushrooms) are the most common mushrooms in Europe and on the world market. The nutritive value of that mushroom comes from its chemical composition. The ratio of protein, fats, minerals and amino acids is very suitable for human consumption. The purpose of our research was to increase the accumulation of selenium in mushrooms in order to meet the recommended human daily intake. Based on our previous research, it was determined that mushrooms are good accumulators of selenium from enriched substrate on which they are cultivated. In the study, Sel-Plex (Alltech) was used as a source of selenium. Sel-Plex is an organic source of selenium, selenized yeast which has the approval of the European Union. Sel-Plex application was done in three different ways, by adding compounds in freshly inoculated compost, compost casing layer and to the compost after raking, Sel-Plex was dissolved in water for irrigation, knowing that 1g of Sel-Plex contains 2 mg of selenium. The cultivation was made in the industrial conditions and the total selenium content was determined by optical emission spectrometry with induced coupled plasma, ICP-OES method. Control sample of mushrooms contained approximately 2.118 ppm of total selenium. We achieved the concentration in mushrooms which could meet the daily requirements for adults (50-200 ppm).

Keywords: *Agaricus bisporus*, ICP-OES, Selenium, Sel-Plex

INTRODUCTION

Mushrooms are often called "wild meat" because of the relatively high content of protein and essential amino acids. They have a suitable chemical composition and some medicinal properties, which are of increasing importance in human nutrition.

Agaricus bisporus have a great nutritional and culinary value for human consumption. They contain plenty of protein, less carbohydrates and negligible amounts of fat. Depending on the strain and production techniques these mushrooms contain approximately 5% of proteins which are composed of many essential amino acids. Mushrooms contain 3-4% carbohydrates. During digestion, the human body converts polysaccharides, starch and sugar to glucose known as blood sugar. It provides a much-needed energy for the brain and central nervous system [1].

Mushrooms uptake the necessary organic matter from the substrate on which they are grown through mycelium and brake down them into small components by their enzyme system [2]. Considering the fact that mushrooms contain a high percentage of protein, it is expected that they are able to incorporate high percentage of selenium into amino acids such as selenocistein

and selenomethionine. The content of selenium in mushrooms mostly ranges between 0.57 and 19.46 mg / kg, depending on the type, age and place of finding mushrooms [3, 4].

In nature, selenium exists in two main chemical forms: inorganic and organic. Inorganic forms of selenium are: selenite, selenate and selenide. In many countries, total content of selenium in soil ranges of 0.1 to 2 ppm [5, 6]. Low percent of selenium in foods is related to the endemic occurrence of cardiovascular diseases and disorders of bone and joint system, metabolic disorders and malignancies of the thyroid glands. Glutathione peroxidase containing selenium performs decomposition of hydrogen peroxide and lipid hydro peroxides in tissues and tissue fluids, and together with vitamin E protects cell membranes and other structures of peroxides, thereby inhibiting lipid per oxidation in tissues [7]. There are a few ways for selenium supplementation (selenized salt, pellet, injection), but it is thought that the best way to increase selenium content in the human body is by using plants. Some authors propose that the animals and humans absorb better organic source of selenium. Organic forms of selenium as selenium yeast, proved to be a good source of selenium. The company "Alltech" produced product called Sel-Plex, which is used in this research. The product is produced from yeast *Saccharomyces cerevisiae*, containing organically bound selenium in the form of selenomethionine. The purpose of our research was to try increase the accumulation of selenium in mushrooms in order to meet the human recommended daily intake [8].

MATERIALS AND METHODS

Materials. Sel-Plex (Alltech) was used in the experiment. One gram of Sel-Plex itself contains 1990 mg of selenium. The product is produced from yeast *Saccharomyces cerevisiae* CNCM I-3060, containing organically bound selenium in the form of selenomethionine. Sel-Plex is approved by European Commission, no 1750/2006, for the use in addition to animal feed.
The possibility of assimilation of selenium in the *Agaricus bisporus* fruit bodies was examined. The application was investigated in commercial mushroom growing farm Klraljevo, Serbia. Compost was inoculated with Sylvan spawn A 15. Content of moisture in the compost was aw= 0.68, nitrogen content=1.9% ammonia content=0.08%. The average weight of one bag was 19 kg.

Growing mushroom *Agaricus bisporus* (button mushroom). Seeded substrate was packed in plastic blocks which were sorted on shelves for the production of mushrooms. In the first stage of production (spawn running) the incubation temperature was 25 °C. The incubation period was 18-21 days. Thirteen days after spawing, the compost was covered by casing layer. Raking were done after seven days for the uniform grown of mushrooms. Fructification was at the temperature of 19° C. Mushrooms were harvested in 4 flushes.

Application of Sel-Plex. Sel-Plex was added to the substrate to reach Se concentrations of 35mg/kg of dry substrate, 70mg/kg of dry substrate and 150 mg/kg of dry substrate. Addition of selenium was carried out together with watering of the compost in three stages of mushroom production: after spawning, casing and after raking the casing layer.

Determination of the total selenium content in samples. The total selenium content was determined by using an optical emission spectrometer with induced coupled plasma, ICP-OES Thermo, ICA 6500 Duo. Samples were cut into small pieces and dried (80ºC, 24h). 300 mg of prepared sample was placed in a Teflon dish with 6 ml 65% HNO_3 and 2 ml 30% H_2O_2. The

sample was digested for 40 min (program 10). Subsequently, the sample was diluted with 25 ml of deionized water. Control sample was prepared in the same way. For the determination of total selenium, working solutions were prepared daily by appropriate dilution of 1mg/ml Se (IV) standard solution.

RESULTS AND DISCUSSION

The obtained results indicate that the fruit body of *Agaricus bisporus* mushrooms uptake the most selenium from substrate where it was added immediately after spawning nutrient substrate (see Table 1). The control sample of fruit body contained 3.9 mg/kg d.w. of total selenium. Mushroom samples which developed from substrate with addition of 35 mg/kg d.w. selenium, contained approximately 192.7 mg/kg of selenium, while the samples which were grown on substrate with 140 mg/kg d.w. selenium, contained almost twice. From these results we can conclude that fungus absorbed almost all selenium from the substrate, at the beginning of mycelia growth.

Table 1: Content of selenium in substrate and fruit body of *A.bisposrus* after supplementation of Sel-Plex at the begining of spawn running

Treatment	Se in substrate (mg/kg d.w.)	Se in fruit body (mg/kg d.w.)
Se (0ppm)a	0.4±0.1	3.9±1.1
Se(35ppm)	0.6±0.4	192.7±13.8
Se(70ppm)	6.1±2.3	275.2±3.2
Se(140ppm)	N.A.	353.2±7.5

a control
NA: not analyzed
Each value is shown as the mean ± standard deviation (n = 3).

However, in the experiment where selenium is added to the casing layer, selenium concentration in fruit body was not significantly different comparing to the control samples, except in the case when the higher concentrations were added (see Table 2). Based on these results and the results obtained from the sample in which the selenium were added to the substrate after spawning, it may be concluded that the fungus adopts a greater amount of selenium in early stage of development. So it is supposed that for higher accumulation of Se complete development of mycelium is needed

Table 2: Content of selenium in substrate and fruit body of *A.bisposrus* after supplementation of Sel-Plex in casing layer

Treatment	Se in substrate (mg/kg d.w.)	Se in fruit body (mg/kg d.w.)
Se (0ppm)a	0.1±0.1	4.2±0.8
Se(35ppm)	10.0±0.6	5.4±0.2
Se(70ppm)	13.1±1.2	13.8±1.3
Se(140ppm)	34.9±1.9	28.4±3.6

a control
Each value is shown as the mean ± standard deviation (n = 3).
NA: not analyzed

In Table 3 the results obtained by adding selenium into the casing layer after raking are presented. The control sample contained 2.1 mg/kg of selenium. Samples uptaked more selenium in fruit bodies than samples that were grown on substrate with addition of selenium into casing layer. The reason for that is still unknown and it should be discovered in the future experiments.

Table 3: Content of selenium in substrate and fruit body of *A.bisposrus* after supplementation of Sel-Plex after raking

Treatment	Se in substrate (mg/kg d.w.)	Se in fruit body (mg/kg d.w.)
Se (0ppm)a	1.3±0.1	2.1±1.2
Se(35ppm)	28.4±1.4	19.1±2.1
Se(70ppm)	32.3±0.7	28.1±2.4
Se(140ppm)	40.8±1.5	71.4±0.8

a control
Each value is shown as the mean ± standard deviation (n = 3).
NA: not analyzed

CONCLUSION

Selenium enriched mushrooms are good potential dietary supplement. The results of this study showed that it is possible to grow mushrooms on a substrate enriched with selenium from organic compounds. Negative impact on the growth of mushrooms, yield of mushrooms and time delay were not noticed. The big difference in the content of selenium in fruit bodies depending on the way and time of selenium application and should be explained by the fact that selenium retained in cover. Based on these results, it can be concluded that the product Sel-Plex can be efficiently used as a potential supplement in the production of mushrooms in order to obtain selenium enriched mushrooms. It is necessary to investigate the selenium metabolism in the fruit body. It is necessary to consider the possibilities for using mushrooms with the addition of selenium as a dietary supplement, and as potential functional food in order to correct a deficiency in the human diet. Also, results of our new experiments suggest that Sel-Plex has an inhibiting effect upon the growth of some fungi that occur in the production of mushrooms *Agaricus bisporus* as contaminant, which is another advantage of using this form of selenium (unpublished results).

ACKNOWLEDGEMENT

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POTENTIAL OF *TRAMETES HIRSUTA* MYCELIUM FOR SELENIUM ABSORPTION

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ABSTRACT

Biologically active substances, especially polysaccharides, isolated from fruiting bodies of *Trametes* species have immunomodulating and antitumor activities. Selenium is a trace element which at nutritional levels has antioxidant and numerous anticarcinogenic or preventive effects, while at higher levels it could be toxic. The purpose of this study was to resolve the question of whether various selenium concentrations affect ability of *T. hirsuta* mycelium to absorb and accumulate this trace element. Selenium was used in the form of Na_2SeO_3 and in the following concentrations: 0.3 mg/l, 0.7 mg/l, 1.0 mg/l, and 1.3 mg/l. The absorbed selenium concentration was determined by Atomic Absorption Spectrophotometer. Content of this trace element in the mycelium increased with enlargement of its concentration in the medium. Amounts of absorbed selenium were ranged from 7.54 μg/g (in the enriched medium with 0.3 mg/l) to 55.17 μg/g (in the enriched medium with 1.3 mg/l). Potential of mycelium to absorb this trace element was good. If the selenium concentration in the mycelium is presented as a percentage of its content in the medium it can be seen that absorption level was ranged from 15% to 25%.

Keywords: *Trametes hirsuta*; Mycelium; Selenium absorption

INTRODUCTION

Dynamic industrial and economic development has numerous advantages but also social and environmental consequences. Environment pollution by numerous chemical compounds (metal, metalloids, pesticides, toxic xenobiotics, halogenated and polycyclic aromatic hydrocarbons etc), connecting with higher intensity of UV light and ionizing radiation, climate change, loss of biodiversity, and decrease of natural resources present causal agents of numerous disorders (cancer, heard diseases, hypertension, diabetes, cataract, atherosclerosis, neurodegenerative disorders, etc.), which trigger is oxidative stress [1]. Selenium (Se) is a trace element which participates in biosynthesis of important selenoproteins and selenoenzymes that are parts of the organism defense system [2]. Unfortunately, Se is distributed in different forms and concentrations worldwide. Likewise, Se presence and amount are not good indicators of its bioavailability [3]. This element is easily absorbed in the forms of soluble selenates and selenites and incorporated in the cells in the forms of selenocysteine, selenomethionine, selenoproteins, polysaccharides, and nucleic acids [2]. Numerous studies demonstrated that

mushrooms are good but variable Se sources, depending on the species and substrate [4]. Due to mushroom ability to absorb inorganic Se forms and converse them to bioactive cell compounds they could be used as dietary supplements.

The aim of this study was to research the influence of Se added to the medium on the ability of *Trametes hirsuta* mycelium to absorb and accumulate this trace element.

MATERIALS AND METHODS

Trametes hirsuta BEOFB 30 was collected from *Prunus* sp. in Belgrade. Culture on malt agar medium is maintained in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (BEOFB). Se was used in the form of sodium selenite (Na_2SeO_3) and in the following concentrations: 0.3 mg/l; 0.7 mg/l; 1.0 mg/l; 1.3 mg/l.

The inoculum preparation was contained from several steps: *(i)* inoculation of 100 ml of synthetic medium (glucose, 10.0 g/l; NH_4NO_3, 2.0 g/l; K_2HPO_4, 1.0 g/l; NaH_2PO_4 x H_2O, 0.4 g/l; $MgSO_4$ x $7H_2O$, 0.5 g/l; yeast extract, 2.0 g/l, pH 6.5) with 25 mycelial discs of 7-day-old culture; *(ii)* incubation at room temperature on a rotary shaker during 7 days; *(iii)* washing of obtained biomass 3 times by sterile distilled water (dH_2O); and *(iv)* homogenization of the biomass with 100 ml of sterile dH_2O in a laboratory blender. 5 ml of the prepared inoculum was use for inoculation of 70 ml modified synthetic medium (with glucose in the amount of 65.0 g/l and peptone as nitrogen source in the concentration of 2.0 g/l) enriched with tested Se concentrations. The medium without Se was used as the control. Submerged cultivation was carried out at room temperature on rotary shaker for 21 days. The obtained biomass was filtered, washed 3 times with 50 ml of dH_2O at magnetic stirrer and temperature of 30^0C with the aim of removing the remaining Se on cell wall, and dried at 50^0C to constant weight. Three repetitions for each Se concentration were made.

Dried mycelium (0.09 g), as well as Se-enriched media (2 ml) before inoculation and after mushroom cultivation, were digested with 10 ml of 100% HNO_3 and 3 ml of 100% HCl. The obtained samples were diluted with Milli-Q water, to the final volume of 20 ml, and cooled at 4^0C. Se concentration was measured by hydride generation Atomic Absorption Spectrophotometer (HG AAS) Model SP190 (Pye Unicam, England). A standard curve was obtained from solutions containing Se in the concentrations of 0, 10, 25 and 50 ppb. The obtained values are presented as μg/g of dried biomass or mg/l of the medium.

RESULTS AND DISCUSSION

Se content in the mycelium increased with enlargement of its concentration in the medium. Amounts of absorbed Se ranged from 7.54 μg/g (in the enriched medium with 0.3 mg Se/l) to 55.17 μg/g (in the enriched medium with 1.3 mg Se/l) (Fig. 1). Potential of mycelium to absorb this trace element was good if it is compared with the Se concentrations in the Se-enriched liquid media after sterilization and before inoculation (Table 1). If the Se concentration in the mycelium is presented as a percentage of its content in the medium it can be seen that absorption level was ranged from 15% to 25%.

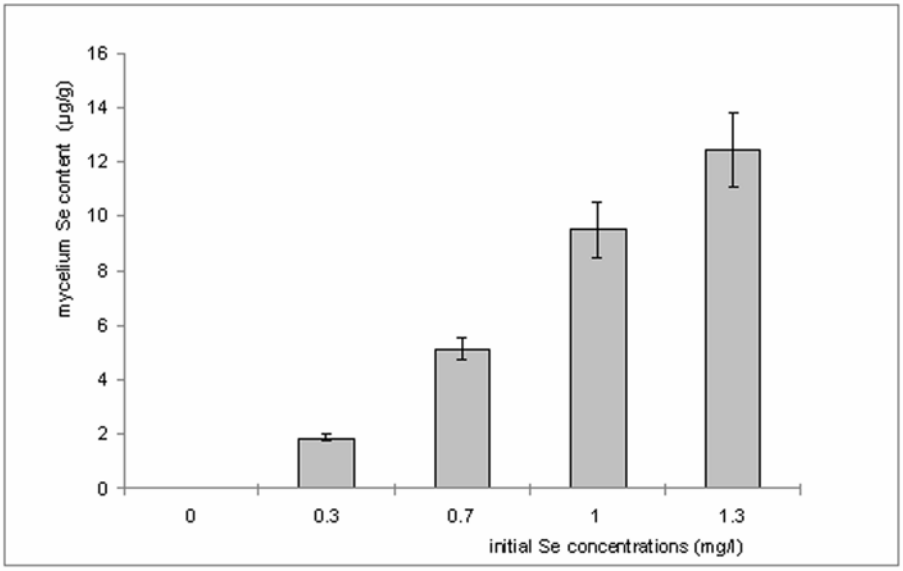

Figure 1. Concentration of absorbed Se in the mycelium of *Trametes hirsute*

The amount of remaining Se in the medium after 21 days of the cultivation was ranged between 6.23 ppb (in the enriched medium with 0.3 mg Se/l) and 23.13 ppb (in the enriched medium with 1.3 mg Se/l).

Table 1: Se concentration in liquid medium

Before sterilization (mg/l)	After sterilization (ppb)	After cultivation period (ppb)
0	0	0
0. 3	096.3	6.23
0. 7	210.0	12.4
1.0	296.3	17.67
1.3	373.0	23.13

Numerous studies have shown that concentration of the absorbed and accumulated Se depends on its form and amount in the medium, as well as mushroom species. Thus, selenized yeast was Se form with more bioavailability than Na_2SeO_3 for absorption and accumulation by *Agaricus bisporus* [5]. Values of incorporated Se in fruiting bodies were 160 µg/g dw, after cultivation in 10 µg selenized yeast-enriched compost, and 110 µg/g dw, in 10 µg Na_2SeO_3-enriched compost. Significant capacity for Se absorption was also noted in *Ganoderma lucidum* (up to 72 µg/g dw) as well as in species of the genus *Boletus* (absorbed Se amount was up to 40 µg/g dw) [2, 6-8]. However, the aim of only few studies was assay of mycelium capacity for Se accumulation [9, 10]. These authors reported that mycelium of *Pleurotus ostreatus* HAI 387 absorbed the highest Se amount after 28 days of submerged cultivation in 1.3 mg/l Na_2SeO_3-enriched synthetic medium. Compared to this species, *T. hirsute* is a poor Se absorber. However, regarding to the fact that it contains up to 22.6% of the dietary reference intake of Se for health adults, man and woman (55 µg/day) recommended by the European Scientific Committee on Food [7, 8], it could be concluded that it should be used as a food supplement.

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PRODUCTION OF *PLEUROTUS´*S LIGNINOLITYC ENZYMES ON COFFEE PULP BY SOLID STATE FERMENTATION

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ABSTRACT

At present, there is a significant interest in solid-state fermentation (SSF) techniques as an efficient biotechnological process for *Pleurotus* cultivation and the production of a wide variety of enzymes. In this research, the production of ligninolityc enzymes by SSF, using six strains of *Pleurotus* spp. (five *Pleurotus ostreatus* and one *Pleurotus pulmonarius*) was studied. The production of laccases enzymes was evaluated during both colonization (Raimbault columns) and fructification (plastic bags) phases of mushroom growth. The influence of mixtures (1:1) of coffee pulp with cedar chip, coconut and cocoa shells in the laccase production was also investigated. *Pleurotus ostreatus* strain CCEBI 3023 produced the highest laccase activity (25 Ug^{-1}) at 96 h of growth on coffee pulp, during the colonization phase in small Raimbault column. Coffee pulp showed a maximum reduction in phenolic and caffeine contents during the first two days of incubation. Laccases appear to be involved in the detoxification of the substrate. When growing on coffee pulp in plastic bags, *P. ostreatus* CCEBI 3023 showed the greatest levels of laccase activity after fructification/harvesting (1,74 U g^{-1}) with respect to the mixtures of coffee pulp and other by-products. As judged by its complex chemical composition, the coffee pulp could act as an inducer of laccase activity. The results were compared with previous studies performed in *Pleurotus* spp. by SSF as well as submerged fermentation.

Keywords: *Pleurotus* spp., ligninolityc enzymes, coffee pulp, solid-state fermentation

INTRODUCTION

Pleurotus ostreatus is the third most important cultivated mushroom for food purpose and also it is a well studied white-rot fungus. The genus *Pleurotus* is related to the bioconversion of agricultural wastes into valuable food products and the use of their ligninolytic enzymes (laccase, manganese peroxidase, etc.) for the biodegradation of organopollutants, xenobiotics and industrial contaminants [1]. Laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) catalyzes the oxidation of ortho- and para-diphenols, aminophenols, polyphenols, polyamines, lignins, and aryl diamines as well as some inorganic ions [1, 2]. Due to the broad variety of reactions catalyzed by laccases, this enzyme holds great promise for many potential industrial applications in biotechnology: bioremediation, green organic synthesis including conductive polymers, and even design of laccase-based fungicidal and bactericidal preparations [3, 4]. There is a significant interest in using solid-state fermentation (SSF) techniques to produce a wide variety of enzymes [5]. However, there is lack of information about the use of basidiomycetes in SSF using glass columns.

Coffee pulp, one of the principal byproducts of wet processed coffee (*Coffea arabica* L.), is rich in carbohydrates, proteins, minerals, and potassium. However, the presence of caffeine, tannins and polyphenols limits its utilization [6]. Nevertheless, SSF of this waste offers an

attractive recycling alternative [7]. Coffee pulp has been also used experimentally in the cultivation of mushrooms, since it represents a direct conversion of an agricultural waste to human food. Among edible mushrooms evaluated for this commercial activity, *Pleurotus* strains appear promising [8-10].

Degradation of lignocellulosic substrates by mushrooms of the genus *Pleurotus* depends on the production and secretion of enzymes such as cellulases, hemicellulases and ligninases. The production of these enzymes is important in substrate colonization and decisive in fruiting bodies production [3]. Although the increase in laccase production by *Pleurotus* mycelia is a well-studied phenomenon, there is not yet enough information on the origin of the overproduction of this enzyme.

This research was aimed to study the capacity of white-rot fungi *Pleurotus* spp. to produce laccase enzyme by SSF during both colonization (Raimbault columns) and fructification (plastic bags) phases of mushroom growth. The lost of toxic components, particularly total phenols and caffeine, was evaluated. The influence of mixtures (1:1) of coffee pulp with cedar chip, coconut and cocoa shells in the laccase production was also investigated.

MATERIALS AND METHODS

Strains. Six strains of *Pleurotus* spp. were studied: *P. ostreatus* CCEBI 3021, CCEBI 3023, CCEBI 3024 y CCEBI 3073 and *P. pulmonarius* CCEBI 3027 were obtained from the Culture Collection of CEBI (Center for Studies on Industrial Biotechnology, University of Oriente, Cuba) and *P. ostreatus* MC 50 from Culture Collection of COLPOS (Campus Puebla, México). All strains were maintained and subcultured in potato-dextrose-agar (PDA, Sigma, U.S.A.) and incubated at 25°C. Two strains (CCEBI 3023 and 3024) were selected for the study of the reduction of toxic components at different column fermenter´s size.

Substrates. Coffee pulp from *Coffea arabica* L. was collected at a coffee processing plant (El Ramón, Santiago de Cuba, Cuba), sun-dried to a moisture content of 10-15%, and then stored at environmental temperature. It was sieved to obtain a particle size of 0.8-2 mm. Coconut shells (*Cocos nucifera,* L.) from "El Cristo", were also sun dried and crushed in irregular fractions bigger than 3 cm. Cocoa shells (*Theobroma cacao* L.) from the farm "La Mandarina" (III Frente), once collected in cacao orchard were sun dried, crushed and screened to select the fraction with a particle size between 1.25 and 4 mm. The cedar chip (*Teona ciliata* Roem) was obtained from the University of Oriente's carpentry.

All the substrates were pasteurized by immersion in hot water at 70-80°C for 60 min. A solution of Benomyl (Bayer, Germany) at 0.02 % was used in order to decrease microbial contamination. In addition to pure coffee pulp, binary formulations (1:1, w/w) were prepared by mixing up the coffee pulp with other by-products (coconut shells, cocoa shells and cedar chip). For fructification studies, the pasteurized substrates were homogeneously mixed with the spawn (5%, w/w) and placed in transparent plastic bags of 2 kg (30x40 cm) [9].

Column fermenters. Colonization studies were carried out in small column fermenters (20 cm large and 25 cm of diameter) with a capacity of 30 g. The reduction of toxic components was also evaluated at medium size column (150 g) and higher size column (750 g). In these experiments the coffee pulp was sterilized 20 min at 121°C and SSF was performed according to Raimbault and Alazard [11].

Enzyme assays. Determinations in colonization studies on Raimbault columns, were made daily during seven days (a duplicate column for each fermentation time). In the case of studies made

with the substrate remnant of plastic bags after fructification/harvesting, the analysis were performed at 60 days of cultivation.

The fermented coffee pulp was extracted with sodium phosphate buffer 0.1 M pH 6.0 (50 mL of buffer per 3 g of remnant substrate) with gentle agitation in an ice bath for 30 min. After that, the suspension was filtered and then centrifuged 10 min at 4 000 rpm. Laccase activity was determined using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate [12]. Enzyme activity was expressed as international units (U), defined as the amount of enzyme required to produced 1 µmol product/min/g (dry weight).

Variation in phenolic content and caffeine in different Raimbault column's size. Phenols present in the substrate were determined by Folin and Ciocalteu reagent [13]. The analysis of caffeine was made by the Kreiser and Martin's method [14]. Samples were extracted in boiling water and filtered prior to injection on to the HPLC column. Results were expressed per gram of dry matter.

Statistical analysis: Data are expressed as means ± S.D. One-way analysis of variance were performed and where significant F values were obtained at the 5% level, differences between individual means were tested using the Duncan test.

RESULTS AND DISCUSSION

Laccase activity was measured daily during seven days of fermentation on coffee pulp of all the *Pleurotus* spp. strains in the small Raimbault columns (Fig. 1). All the *Pleurotus* strains studied grew well in these conditions. At initial growth stages, a lag phase with almost no laccase activity was detected. Subsequently, a rapid increase in the enzyme production was observed until a maximum of activity, which decreased at the end of the fermentation period. A similar pattern of laccase production was found in all the strains on small fermenter [15] in agreement with other reports [16-18]. It is noteworthy that *P. ostreatus* CCEBI 3023 showed the highest laccase activity (25 Ug^{-1}) at 96 h of fermentation, surpassing our reference productive strain CCEBI 3024 (17,9 Ug^{-1}).

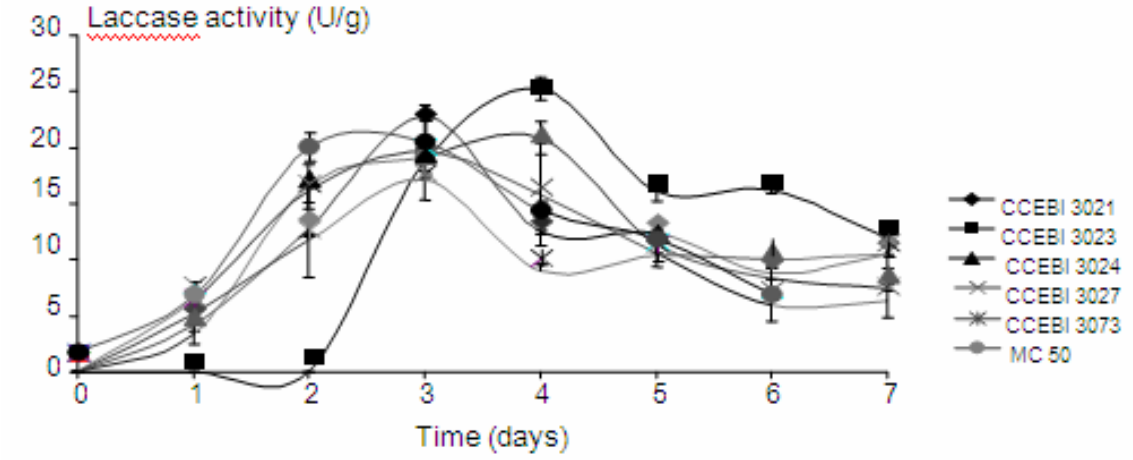

Figure 1: Laccase activity during the cultivation of six *Pleurotus* spp. strains on coffee pulp in small Raimbault columns.

Taking into account the highest laccase activity of *P. ostreatus* CCEBI 3023 found in this communication and the fact that in previous studies *P. ostreatus* CCEBI 3024 was able of decolourizing wastewaters with a high polyphenol content, such as those from pasteurized coffee pulp (laccase activity of 8.53 ± 0.73 UmL^{-1} at day 9 of cultivation) [4], these strains were chosen to assess the reduction of toxic components contained in coffee pulp by SSF at different Raimbault columns size.

Variation in the phenolic content of substrates during the culture cycle is an important aspect in the adaptation of the strain, especially since the capacity of a substrate to resist degradation has been partially attributed to its array of phenolic compounds [4, 16].

In the present study, the coffee pulp showed a reduction in phenolic content during the first two days of incubation. The 53.07% of total phenols were transformed at 24 h by the strain CCEBI 3023, and the 45.51%, at 48 h by the strain 3024. This is particularly important because a rapid reduction in phenolic concentration would accelerate colonization, and consequently, decrease the risks of invasion by mold contaminants.

The changes in enzymatic activity and phenol concentration observed during vegetative development suggest that laccase might be associated with the detoxification of soluble phenolic compounds and lignin degradation.

The caffeine content of coffee pulp also decreased during mycelial growth (Table 1). Caffeine loss was higher at day seven (last day). The results range between 15-24% and were similar to those reported by Salmones et al. [18].

The ability to produce laccase enzymes in relation to the loss of total phenols and caffeine by *Pleurotus* strains was demonstrated. This enzyme could be responsible of the transformation of these toxic compounds [12, 16, 18]. Other enzymes like peroxidases were secreted in minor activities [19]. The complex chemical composition (tannins, lignin, etc.) of coffee pulp could act as an inducer of laccase activity due to the possible enhancement of laccase gene expression.

In view of the significant laccase activity of *P. ostreatus* CCEBI 3023 and its important contribution to the overall ligninolytic enzyme activity, we also examined the extracellular laccase production of this strain cultured on coffee pulp and mixtures (1:1) in plastic bags after fructification and harvesting (60 days).

The results evidenced that coffee pulp favored the production of laccases, being the activity higher on pure coffee pulp than in the mixtures with other by-products (Table 2).

In the case of laccase activity of CCEBI 3024 strain, cultured in transparent plastic bags, a maximum of 2,41 Ug^{-1} was achieved at day 14 of cultivation [20]. The production of laccase enzyme after fructification/harvesting of *Pleurotus* spp. on mixtures of lignocellulosic substrates by SSF is reported for the first time. The highest value found in coffee pulp reflected the influence of the nitrogen content of substrate in laccase production; on the other hand, the mixture with cedar chip had the lowest nitrogen content [21]. Previous studies showed that the production of ligninolityc enzymes is closely related to the nitrogen content of substrates.

Table 1: Caffeine concentration, initial-final values (mg/g) and loss percent (in parentheses) in the coffee pulp during SSF by *P. ostreatus* strains CCEBI 3023 y CCEBI 3024.

	Fermenter type		
Strain	Small	Medium	Large
CCEBI 3023	0.52-0.40 (23.4)	0.58-0.48 (16.6)	0.54-0.41 (22.9)
CCEBI 3024	0.64-0.53 (18.5)	0.56-0.43 (24.0)	0.51-0.44 (15.2)

Table 2: Production of laccase enzyme by *Pleurotus ostreatus* CCEBI 3023 on coffee pulp and mixtures (1:1) at 60 days of cultivation.

Substrates	Laccase Activity (Ug^{-1})
Coffee pulp	$1,745 \pm 0,013^{d}$
Coffee pulp : cocoa shells	$1,234 \pm 0,004^{c}$
Coffee pulp : coconut shells	$0,447 \pm 0,016^{b}$
Coffee pulp : cedar chip	$0,308 \pm 0,036^{a}$

Average of three value ± standard deviation. Distinct letters mean significant differences (ANOVA and Duncan¨s test, $p<0.05$).

The differences between coffee pulp packed in the bags and in Raimbault column lie in the method employed in its treatment. The former is pasteurized (immersion in hot water during one hour) on which losses many soluble components, whereas the later is sterilized (at 121°C during 20 min) and components of the coffee pulp are at greater concentrations.

CONCLUSION

P. ostreatus strain CCEBI 3023 produced the highest laccase activity (25 Ug^{-1}) at 96 h of growth on coffee pulp, during the colonization phase in small Raimbault column. The maximum reduction in phenolic and caffeine contents of coffee pulp was observed in the first two days of cultivation. When growing on coffee pulp in plastic bags, *P. ostreatus* CCEBI 3023 showed the greatest levels of laccase activity after fructification/harvesting (1,74 U g^{-1}) with respect to the mixtures of coffee pulp and other by-products. As judged by its complex chemical composition, the coffee pulp could act as an inducer of laccase activity.

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EFFECT OF SELECTED NITROGEN SOURCES ON Mn-OXIDIZING PEROXIDASES ACTIVITY IN *TRAMETES GIBBOSA*

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ABSTRACT

Trametes gibbosa is an efficient lignin-degrading species due to its ability to produce laccase and Mn-dependent peroxidase that enable degradation of lignin and of a wide range of structurally similar compounds. Wheat straw is a very common worldwide agricultural residue that contains a certain amount of soluble carbohydrates and inducers of lignocellulolytic enzyme synthesis. Whether nitrogen source and concentration could affect activity of Mn-dependent peroxidase and versatile peroxidase in *T. gibbosa*, thereby also affecting wheat straw fermentation, was the question that provided the goal for the present study. *T. gibbosa*, originated from *Fagus moesiaca*, was used for the study. Analyzed inorganic nitrogen sources were NH_4NO_3 and $(NH_4)_2SO_4$ in nitrogen concentrations ranged between 10 and 40 mM, and the organic source was peptone in concentrations from 0.25 to 4.0%. The enzyme activity was determined spectrophotometrically. $(NH_4)_2SO_4$ was the most appropriate nitrogen source for the Mn-dependent peroxidase activity. The maximal activity level was noted at a nitrogen concentration of 15 mM (4479.5 U/l). Slightly lower activity was noted in 1% peptone-enriched medium (4035.5 U/l), and higher peptone amounts caused rapid activity decrease and production was not noted at a concentration of 3%. In the case of NH_4NO_3, Mn-dependent peroxidase activity was increased with enlargement of nitrogen concentration reaching peak at concentration of 40 mM (3669.5 U/l). Production of versatile peroxidase was significantly lower in all studied nitrogen sources and concentrations.

Keywords: *Trametes gibbosa*; Wheat straw; Laccase; Mn-oxidizing peroxidases

INTRODUCTION

Trametes gibbosa degrades lignin and a broad range of structurally similar aromatic pollutants [1-3] due to its ability to produce laccase and Mn-dependent peroxidase [4]. Therefore this species could take participation in various processes, such as biopulping, biobleaching, textile dye discolouration, treatment of agricultural residues, of industrial wastewater, etc. and contribute to the pollution problem solving [5, 6]. Agricultural residues represent prospective substrates for the bioconversion into fungal biomass and production of lignocellulolytic enzymes, but also they could be potential environmental pollutants [7]. Wheat straw is an abundant residue in numerous countries worldwide and a prospective substrate for the bioconversion into fungal biomass and lignocellulolytic enzymes, due to their appropriate chemical composition [8].

The aim of this study was concerned with different production of Mn-oxidizing peroxidases by *T. gibbosa* depending on nitrogen sources and concentrations.

MATERIALS AND METHODS

Trametes gibbosa BEOFB 310 was collected from *Fagus moesiaca* on Suva Mountain (Serbia). The culture on malt agar medium is maintained in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (BEOFB).

The inoculum preparation was contained from several steps: *(i)* inoculation of 100 ml of synthetic medium (glucose, 10.0 g/l; NH_4NO_3, 2.0 g/l; K_2HPO_4, 1.0 g/l; NaH_2PO_4 x H_2O, 0.4 g/l; $MgSO_4$ x $7H_2O$, 0.5 g/l; yeast extract, 2.0 g/l, pH 6.5) with 25 mycelial discs of 7-day-old culture; *(ii)* incubation at room temperature on a rotary shaker during 7 days; *(iii)* washing of obtained biomass 3 times by sterile distilled water (dH_2O); and *(iv)* homogenization of the biomass with 100 ml of sterile dH_2O in a laboratory blender. The ligninolytic enzyme activities were studied after solid-state fermentation of wheat straw. Cultivation was performed at 25ºC in 100 ml flasks containing 2g of wheat straw soaked with 10 ml of the modified synthetic medium (without glucose, with one of inorganic nitrogen sources, NH_4NO_3 or $(NH_4)_2SO_4$, in nitrogen concentrations of 0, 10, 15, 20, 25, 30, and 40 mM, or peptone as an organic source in the concentrations of 0, 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0%). In this way prepared flasks were inoculated with 3 ml of homogenized inoculum.

Samples were harvested after 7 days of cultivation and ligninolytic enzymes were extracted with 50 ml of dH_2O. The obtained extracts were separated by centrifugation and the supernatants were used for determination of the activity of Mn-oxidizing peroxidases [Mn-dependent peroxidase (MnP) and versatile peroxidase (VP)]. Five replicates for each nitrogen source and concentration were prepared to decrease statistical error.

Activities of Mn-oxidizing peroxidases were determined spectrophotometrically (CECIL CE2501 Spectrophotometer) with phenol red (ε_{610} = 22000 M^{-1} cm^{-1}) as a substrate with or without $MnSO_4$ (for MnP and VP, respectively). Enzymatic activity of 1 U is defined as the amount of enzyme that transforms 1 μmol of substrate/min.

RESULTS AND DISCUSSION

Activities of Mn-oxidizing peroxidases were detected after 7 days of solid-state fermentation of wheat straw by *T. gibbosa* BEOBF 310 in the presence of all tested nitrogen sources and concentrations (Fig. 1A-C). The nitrogen sources and concentrations showed significantly different potentials for stimulation of peroxidase production ($P<0.01$).

$(NH_4)_2SO_4$ was the most appropriate nitrogen source for the MnP activity. The maximal activity level was noted at a nitrogen concentration of 15 mM (4479.5 U/l). Further increases of nitrogen concentration up to 40 mM caused a gradual decrease of activity, while concentration of 40 mM showed stimulation effect to the enzyme production (Fig. 1B). Slightly lower activity was noted in 1% peptone-enriched medium (4035.5 U/l), and higher peptone amounts caused rapid activity decrease and production was not noted at a concentration of 3% (Fig. 1C). In the case of NH_4NO_3, MnP activity was increased with enlargement of nitrogen concentration reaching peak at concentration of 40 mM (3669.5 U/l) (Fig. 1A). Production of VP was significantly lower in all studied nitrogen sources and concentrations (Fig. 1A-C). The maximum

activity level was noted in 10 mM $(NH_4)_2SO_4$- and 1.0% peptone-enriched medium (1555 U/l and 1506.5 U/l, respectively).

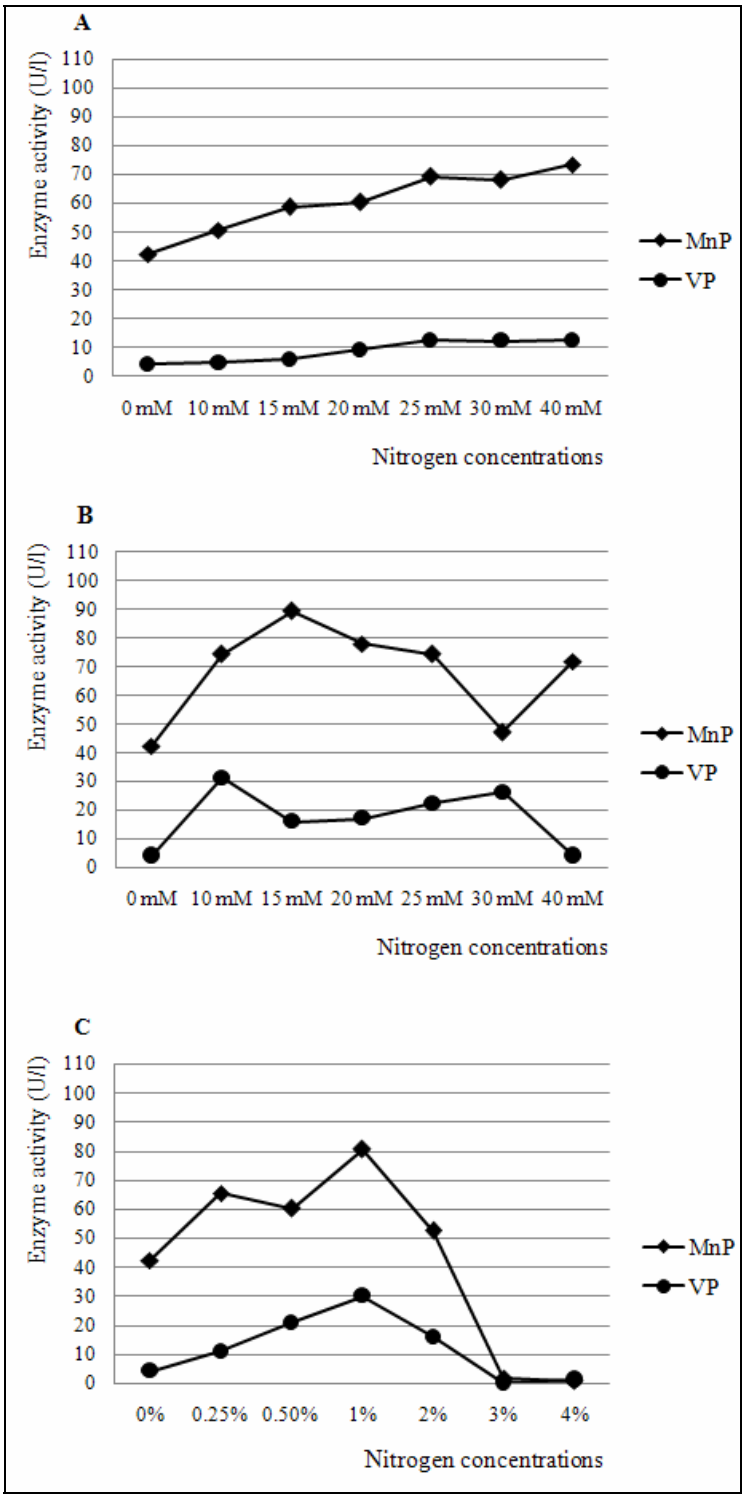

Figure 1: Activity of Mn-oxidizing peroxidases in *Trametes gibbosa* BEOFB 310 depending on nitrogen sources and concentrations: A. NH_4NO_3; B. $(NH_4)_2SO_4$; C. peptone

The obtained results confirmed previous data that enzyme system of *Trametes* species is effective in the process of wheat straw fermentation [9, 10]. According these authors, NH_4NO_3 was an appropriate nitrogen source for the MnP activity. However, regarding to the optimum nitrogen source, *T. gibbosa* showed different responses compared to the other mushroom species. Thus, $(NH_4)_2SO_4$ was an unfavorable nitrogen source and NH_4NO_3 was the appropriate one for *Ganoderma lucidum* [11]. These authors noted a MnP activity twice higher in the NH_4NO_3-enriched medium than in $(NH_4)_2SO_4$-enriched medium. In the case of the observed VP activity level during wheat straw degradation, *T. gibbosa* was a significant producer compared to numerous other white rot species, for example *G. lucidum* [11]. However, peroxidase activity may be enhanced because of higher biomass production and fact that $(NH_4)_2SO_4$ is a better nitrogen source than NH_4NO_3, since ammonium-sulphate can be rapidly used for biomass production *via* amino acids synthesis. Nevertheless, biomass is a parameter that should be monitored in further studies.

According to the obtained results, it could be concluded that *T. gibbosa* could be used in processes of producing feeds and numerous basic commodities for different industrial purposes, as well as in bioremediation processes.

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EXTRACELLULAR CELLULASE PRODUCTION FROM A WHITE-ROT FUNGUS *PORODAEDALEA PINI*

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ABSTRACT

The present study investigated the conditions for extracellular cellulase production of a white-rot fungus *Porodaedalea pini* and compared the enzyme activities between the two culture methods using a fermentor or agitating flasks. The fungus was cultured in the modified Norkrans medium for 24 days, and Avicelase, CMCase, β-glucosidase (BGL), cellobiose dehydrogenase (CDH), and protease activities were assayed every 2 days. BGL activity by flask culture was higher than that by fermentor culture. Avicelase activity by flask culture was slightly higher than that by fermentor culture. The highest activity of CMCase was recognized on the 24th day of the incubation in the flask culture method, whereas the highest activity was found on the 20th day in the fermentor method. CDH activity by flask culture showed much higher value than that by fermentor culture. Protease activity in the both methods did not correspond with change in the protein concentration during culture for 24 days. The flask culture method was proved to produce higher respective cellulases, especially CDH, compared to fermentor method.

Keywords: White-rot fungus; Cellulase; β-Glucosidase; Cellobiose dehydrogenase

INTRODUCTION

After the Kyoto protocol in 1997, many countries in the world have been tackled the reduction of greenhouse gas (GHG) emission. Especially, reduction of CO_2 accounting for about 90% of GHG is immediately required through the globe [1]. In order to grapple with this issue, researches and development for bio-ethanol production are actively undergone around the world. However, most of the raw materials for ethanol production are currently derived from cereals, such as corn and sugar cane, leading to new problems, such as competition to acquire the materials with food supply [2]. Hence, the ethanol production from wood biomass is notified now because the system does not compete with food supply in terms of acquisition of raw materials.

Cellulose in the materials is required to be degraded into fermentable sugars to convert the wood biomass into bio-ethanol. There are two main methods for conversion of the materials into fermentable sugars (saccharification): acid-hydrolysis saccharification and enzymatic saccharification. Acid-hydrolysis saccharification has some merits to hydrolyze cellulose in short duration and to degrade all kinds of polysaccharides, while some demerits to be costly in the facilities durable to strong acids and to have an environmental problem for treating wastes after hydrolysis. In addition, acid-hydrolysis saccharification results in low sugar yield because of further

degradation of produced sugars by strong acids [3]. On the other hand, enzymatic saccharification has some merits that saccharification conditions are rather moderate, the saccharification is less problematic in waste treatment, and that the saccharification results in higher sugar yield because of no further degradation of released sugars. However, enzymatic saccharification has some demerits that it requires rather longer reaction time and huge amount of enzymes [4]. The background for enzymatic saccharification of wood biomass, therefore, requires the better enzymes with higher saccharification efficiency.

Cellulose-degrading microorganisms are widely distributed in the nature, whereas limited microorganisms can degrade crystalline cellulose completely. Among such microorganisms, moulds, such as *Trichoderma ressei*, and white-rot fungi, such as *Phanerochaete chrysosporium* have powerful cellulases. These microorganisms have common features to secrete some extracellular cellulases with different properties to degrade crystalline cellulose, resulting in complete degradation of crystalline cellulose by additive effects of those cellulases [5].

A white-rot fungus *Porodaedalea pini* belongs to Hymenochaetaceae in Basidiomycetes. *P. pini* is known to have powerful enzymes because it thins the wide range of cell walls after degrading most of lignin in secondary walls and compound middle lamellae [6]. In addition, this fungus has been investigated for its culture conditions to produce extracellular cellulases, and it has been suggested that the fungus produces highly active β–glucosidase compared to other cellulases assayed, such as Avicelases and CMCase [7].

The present study assayed four extracellular cellulases, i.e. Avicelase, CMCase, β–glucosidase (BGL), and cellobiose dehydrogenase (CDH), and protease from *P. pini* in time-course experiments, and investigated the activity patterns of these enzymes in order to establish efficient production system for highly active cellulases of this fungus. Moreover, the two culture methods, flask culture and fermentor culture were compared to select better one for respective cellulases.

MATERIALS AND METHODS

Fungal material. A white-rot fungus *Porodaedalea pini* (Brot.) Murrill. WD1174 was used for the experiments. This fungus was obtained from the Institute of Forestry and Forest Products, Tsukuba, Japan. The fungus was precultured in Petri dishes (9 cm i.d.) containing potato-dextrose-agar medium (BD Difco, Becton, Dickison and Company, Sparks, MD, USA) at 25ºC in the dark.

Flask-agitating culture. The fungus was cultured in 2 L Erlenmeyer flasks containing modified Norkrans medium [8] as a basal medium. A flask contained the following culture components: modified Norkrans medium components (30.00 mM glucose, 16.65 mM asparagines, 18.37 mM KH_2PO_4, 5.28 mM $MgSO_4 \cdot 7H_2O$, 4.28 mM NaCl, 2.25 mM $CaCl_2$, 0.05 mM $FeCl_3 \cdot 6H_2O$, 770 nM $ZnSO_4$, 40 nM thiamine hydrochloride), 27.8 mg Avicel (Funacel II, Funakoshi, Tokyo, Japan), additional solution (139.8 mg glucose, 693.3 mg $MgSO_4 \cdot 7H_2O$, and 1,042.8 mg urea in 9.7 mL H_2O), 56.25 mL bovine calf serum (BCS) (HyClone, South Logan, Utah, USA), and 1,250 mL H_2O. The medium solution in flasks except for additional solution and BCS was autoclaved for 30 min at 121ºC. Additional solution was filter-sterilized with Millex-GV filter (0.22 μm, Merck Millipore, Darmstadt, Germany) and added to the flasks. The pre-sterilized BCS was added to the autoclaved culture solution in a laminar flow cabinet.

Mycelial disks (6 mm in diameter) were prepared by punching out fungal pre-cultures in Petri dishes, and then the 30 disks per a flask were inoculated to the culture medium. Total 3 flasks

containing culture medium were agitated by a shaker (NR-150, TAITEC Corp., Koshigaya, Japan) at 100 rpm in the dark for 24 days. The culture experiment was repeated 3 times.

Fermentor culture. The components of the culture medium were same as those of flask-agitating culture, and total 2.5 L culture medium was prepared. The 60 mycelial disks were inoculated to the culture medium. The culture of the fungus was carried out with a fermentor (MBF, EYELA, Tokyo, Japan) under aeration and agitation at 100 rpm in the dark at 25ºC for 24 days. The fermentor culture was repeated 2 times.

Preparation of crude enzyme solution. Each 30 mL of culture solution was collected from each flasks every second day for flask-agitating culture. Each 30 mL of culture solution was sampled 3 times every other day for fermentor culture. The collected culture solution was filtered through Miracloth (Calbiochem, Darmstadt, Germany), and then the filtrate was centrifuged at 1,500 x *g* for 15 min at 4ºC. After the supernatant was moved into dialysis tubes, the tubes were placed in measuring cylinders, and then the space between cylinder and tube was packed with polyethylene glycol (mean molecular weight 20,000 ± 5,000, Wako Pure Chemical Industries, Osaka, Japan), which resulted in dehydration and concentration of culture solution. The obtained solution was moved to another dialysis tube and dialyzed against 20 mM succinate buffer (pH 5.5) overnight. The obtained solution was used as crude enzyme solution for enzyme assays.

Enzyme assay. Protein concentration of the crude enzyme solution was determined by Bradford method [9] with using chicken ovalbumin as a standard.

Avicelase and CMCase activities were determined as the amount of total sugars released from the substrates by the crude enzyme preparation. A reaction mixture (1 mL) contained the crude enzyme solution (0.5 mL) and 0.5% (w/v) Avicel (Funacel II, Funakoshi) or carboxymethyl cellulose (CMC) sodium salt (Wako Pure Chemical Industries) aqueous solution (0.5 mL). The reaction mixture was incubated for 6 h at 37ºC under reciprocal agitation at 45 rpm. After incubation, reaction mixture was boiled for 5 min. The reaction mixture was then centrifuged at 1,500 x *g* for 15 min at 4ºC. The amount of total sugars released in the reaction mixture was determined by phenol-sulfuric acid method [10]. One katal (kat) was defined as the amount of enzyme preparation to release 1 mol of total sugars from Avicel or CMC per second.

β-Glucosidase (BGL) activity was determined as the amount of *p*-nitrophenol (*p*NP) released from *p*-nitrophenyl β-D-glucopyranoside (*p*NPG) (Wako Pure Chemical Industries) by the enzyme preparations. A reaction mixture (3 mL) contained the crude enzyme solution (1 mL), 4 mM *p*NPG, and 50 mM acetate buffer (pH 5.0). The reaction mixture was incubated for 30 min at 50ºC under reciprocal agitation at 45 rpm. After incubation, ice-cold 0.5 M Na_2CO_3 solution (1 mL) was added to the reaction mixture. The amount of *p*NP released in the reaction mixture was determined by the absorbance at 405 nm [11]. One kat was defined as the amount of enzyme preparation to release 1 mol of *p*NP from *p*NPG per second.

Cellobiose dehydrogenase (CDH) activity was determined by monitoring the reducing activity of cytochrome c (bovine heart sample, C-2037, Sigma-Aldrich, St. Louis, MO, USA) by the enzyme preparation. CDH activity was assayed at 30ºC in a 100 mM sodium acetate buffer (pH 4.2) containing 1.5 mM cellobiose and 0.15 mM cytochrome c. The activity was determined as the time-course change in absorbance at 550 nm [12]. One kat was defined as the amount of enzyme preparation to reduce 1 mol of cytochrome c per second.

Protease activity was measured by hydrolyzing Azocoll (Wako Pure Chemical Industries). Azocoll (10 mg) was incubated with 1 mL of the enzyme preparation at 30ºC under reciprocal agitation at 60 rpm for 1 h. After incubation, 700 μL of the colored supernatant was removed, and then the pH was adjusted to 5.0 by adding 300 μL of 500 mM sodium acetate buffer (pH 5.0). The absorbance of the obtained solution was measured at 550 nm [13].

RESULTS AND DISCUSSION

Fig.1 shows the time-course changes in Avicelase specific activity for flask-agitating and fermentor cultures. In flask-agitating culture, each replicate did not show any common and constant trends in the activity (Fig.1A). In fermentor culture, the activity of 2nd replicate increased dramatically after 18th day of culture and showed the maximum (16.8 nkat/mg) on 20th day (Fig.1B). The maximal activity of 2nd replicate was about 4 times higher than that of 1st replicate.

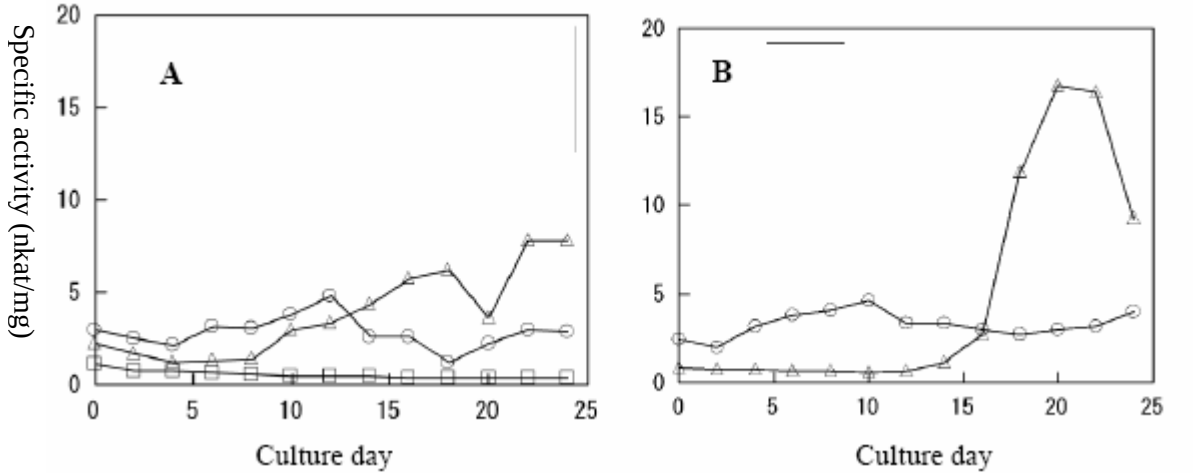

Figure 1: Time-course changes in average Avicelase specific activity
A: Flask-agitation culture; B: Fermentor culture
○: 1st replicate; △: 2nd replicate; □: 3rd replicate

Time-course changes in CMCase specific activity are shown in Fig. 2. In flask-agitating culture, the activity mostly ranged from 0 to 6 nkat/mg, while in the 2nd replicate the activity showed more than 10 nkat/mg and the maximum on the 24th day of culture (Fig. 2A). In the case of fermentor culture, the specific activity in both replicates exhibited lower values (0~4 nkat/mg). However, the pattern of activity change was different between 1st and 2nd replicate, the former showed the maximum on the 4th day, whereas the latter on the 20th day (Fig. 2B).

Fig. 3 shows time-course changes in BGL specific activity. Although the flask-agitating culture did not show any common trends among 3 replicates, some distinct higher peaks of the activity were observed on 8th and 20th day of culture for the 1st replicate, and on 4th, 10th, and 20th day for the 2nd replicate (Fig. 3A). In contrast, the 3rd replicate did not show any distinct peaks but very low activity through the time-course experiment. On the other hand, the fermentor culture gave the highest activities (31.5 and 147.2 nkat/mg) on 12th and 18th day in the 1st and 2nd replicate, respectively (Fig. 3B).

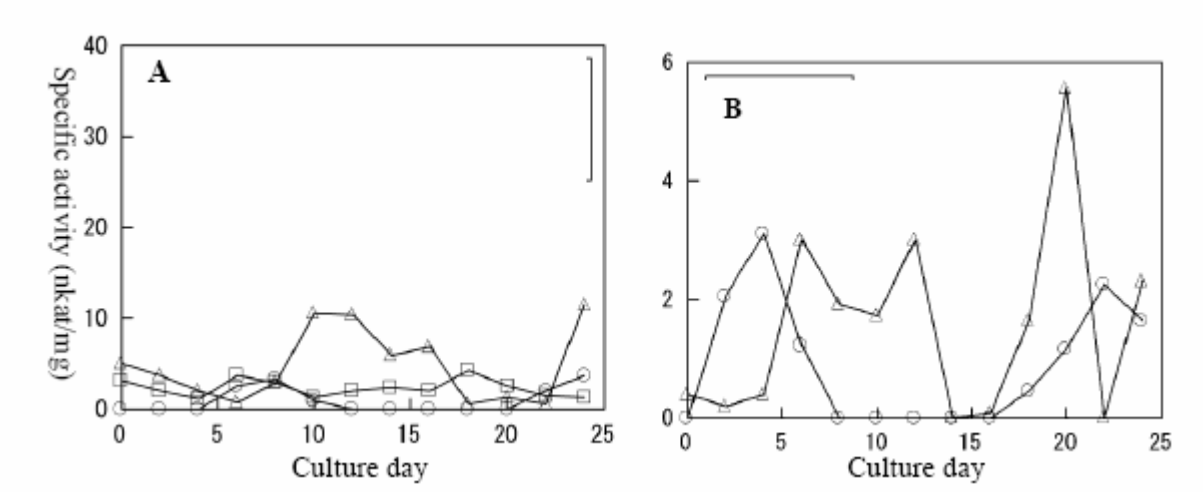

Figure 2: Time-course changes in average CMCase specific activity
A: Flask-agitation culture; B: Fermentor culture
◯: 1st replicate; △: 2nd replicate; □: 3rd replicate

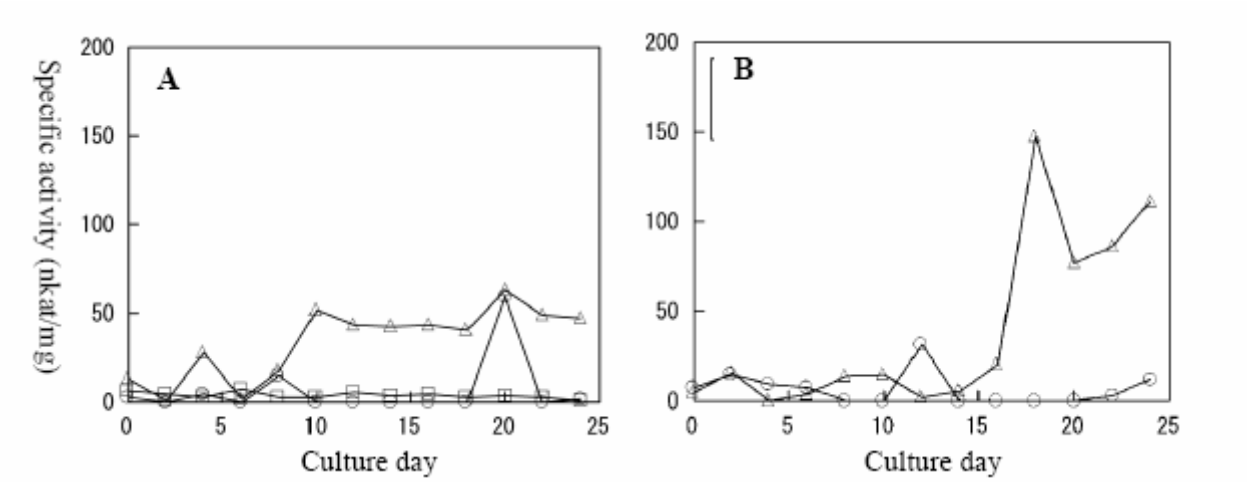

Figure 3: Time-course changes in average BGL specific activity
A: Flask-agitation culture; B: Fermentor culture
◯: 1st replicate; △: 2nd replicate; □: 3rd replicate

Time-course changes in CDH specific activity are shown in Fig. 4. In flask-agitation culture, the activity in the 1st and 2nd replicates increased after 12th day of culture, reached the maximum on 20th day, and decreased thereafter, though all replicates did not any common trends through the time course (Fig. 4A). In the case of fermentor culture, the maximal activity (80.1 nkat/mg) in the 1st replicate was about 20 times higher than that (4.3 nkat/mg) in the 2nd replicate. In addition, the activity in the 1st replicate was much higher than that in the 2nd replicate through the time-course experiment (Fig.4B).

In the present study, BGL and CDH activities exhibited rather higher values compared to Avicelase and CMCase in both flask-agitation and fermentor culture. Habu et al. [11] reported that addition of BCS to the culture medium significantly enhanced CDH activity as well as BGL for a white-rot fungus *Phanerochaete chrysosporium*. Hence, addition of BCS to the culture medium is considered to have also enhanced both activities for *P. pini* in this study.

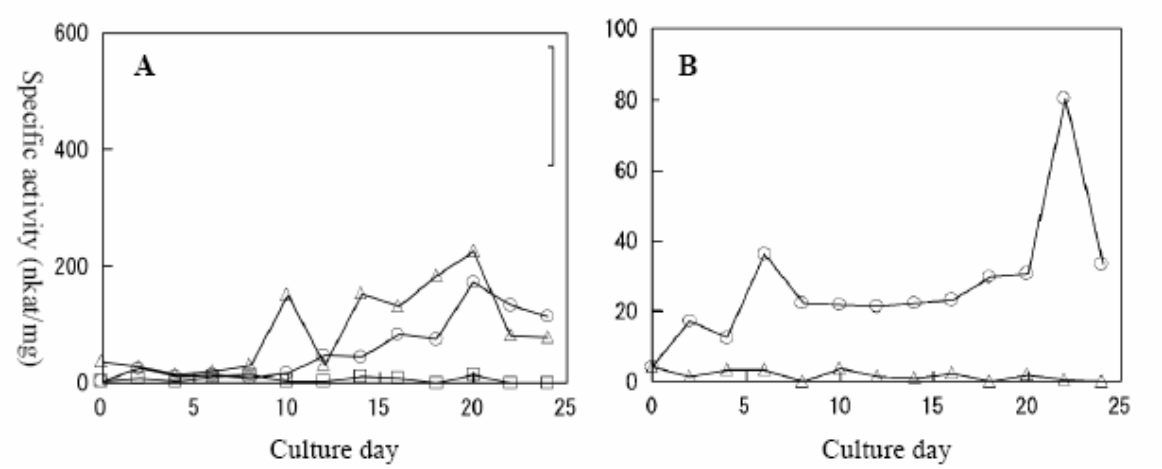

Figure 4: Time-course changes in average CDH specific activity
A: Flask-agitation culture; B: Fermentor culture
○: 1st replicate; △: 2nd replicate; □: 3rd replicate

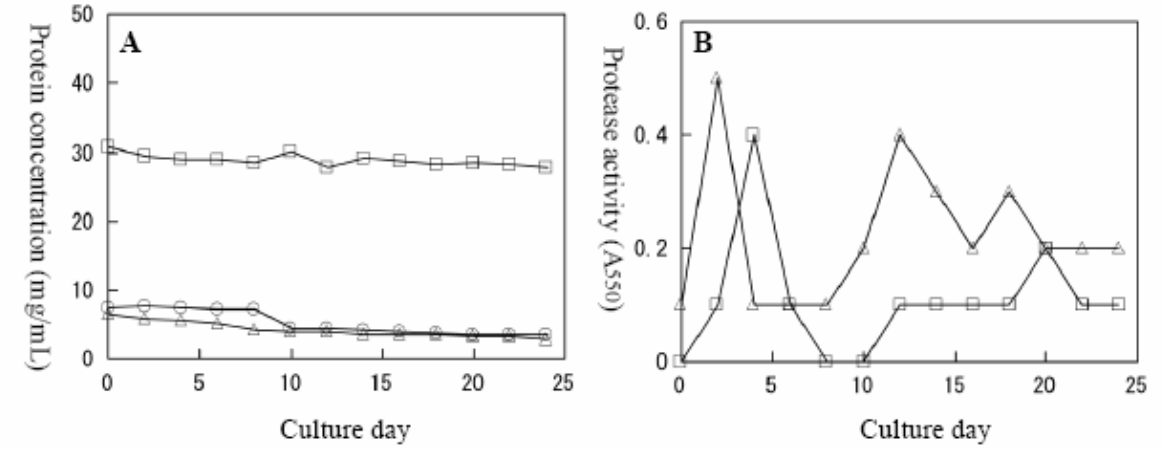

Figure 5: Time-course changes in protein concentration (A) and protease acitivity (B) for falsk-agitation culture
○: 1st replicate; △: 2nd replicate; □: 3rd replicate

Fig. 5A shows time-course changes in protein concentration for flask-agitation culture. In the 1st replicate, protein concentration decreased to almost 3 mg/mL from 8th to 10th day of culture and then decreased gradually. Protein concentration in the 2nd and 3rd replicates decreased gradually. It was suggested that marked decrease in protein concentration for the 1st replicate might be caused by protease from the fungus. Hence, time-course change in protease activity was measured in the 2nd and 3rd replicates (Fig.5B). Dosoretz et al. [14] have measured time-course changes in protease activity and protein concentration in the culture medium of a white-rot fungus *P. chrysosporium* in order to examine the effects of protease on the ligninolytic enzymes from the fungus. They confirmed that protein concentration in the culture medium had decreased dramatically concomitant with the increase in protease activity. On the other hand, significant decrease in protein concentration was not observed for flask-agitation culture in this study, although maximal activities of protease were recognized on 2nd day of 2nd replicate and on 4th day of 3rd

replicate, respectively (Fig.5). Hence, protease seems to less affect decrease in protein concentration for flask-agitation culture.

Protein concentration of 1st replicate also gradually decreased in fermentor culture. Thus, time-course change in protease activity was measured for 2nd replicate in fermentor culture (Fig.6). Protease activity slightly increased after 12th day of culture, while protein concentration decreased drastically after 12th day and showed almost constant lower values after 18th day. Protein concentration was dropped down to 1/24 from 12th to 18th day of culture, whereas protease activity increased about 1.2 times during the same period. These results suggest that protease caused decrease in protein concentration to some extent in fermentor culture of *P. pini*.

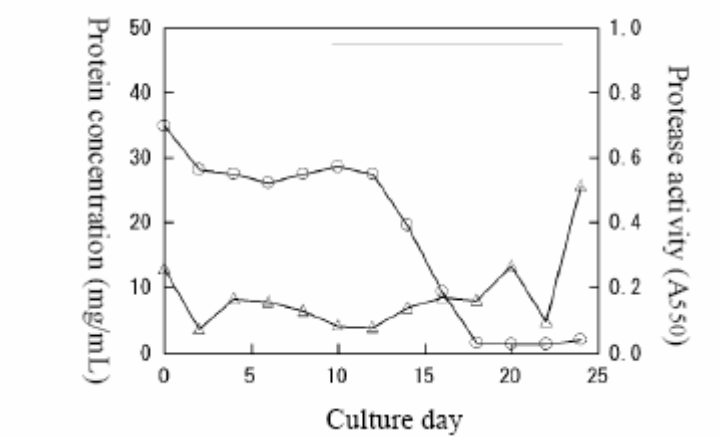

Figure 6: Time-course changes in protein concentration and protease acitivity for fermentor culture.
○ Protein concentration; △ : Protease activity

The maximal specific activities of each cellulase in respective cultures are summarized in Table 1 for both flask-agitation (9 cultures in total) and fermentor culture (6 cultures in total). The maximal specific activities of Avicelase, CMCase, BGL, and CDH for flask-agitation culture were 18.8, 31.8, 154.3, and 571.7 nkat/mg, respectively, indicating that all cellulase activities in flask-agitation culture were higher than those in fermentor culture. Especially, CMCase and CDH in flask-agitation culture exhibited about 6 and 7 times higher values compared to those in fermentor culture.

Table 1: Maximal specific activities (nkat/mg) of each cellulase in respective cultures for flask-agitation and fermentor culture

Cellulase	Flask-agitation	Fermentor
Avicelase	18.8	16.8
CMCase	31.8	5.5
BGL	154.3	147.2
CDH	571.7	80.1

CONCLUSIONS

The conditions for extracellular cellulase production from a white-rot fungus *P. pini* were investigated, and each cellulase (Avicelase, CMCase, BGL, and CDH) activity was compared between flask-agitation and fermentor culture in the present study. As the results, it was indicated that flask-agitation culture has a potential to produce cellulases with higher activities, especially CDH, compared to fermentor culture.

ACKNOWLEDGEMENT

We are grateful to Professor Naoto Habu, Faculty of Agriculture, Utsunomiya University, Japan, for helping us to prepare crude enzyme solution.

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SCREENING OF FUNGAL STRAINS PRODUCING CELLULASES BY SOLID FERMENTATION

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ABSTRACT

Cellulosic substrates are represented mainly by wood but also by recyclable waste products such as bran and wheat straw, peanut shell, sawdust and olive cake. Adding value to agricultural and industrial by-products by fermentation, such as cellulase production, is an attractive biotechnological option. Fungi are the primary source of the enzymes required to convert plant biomass into sugars, bio ethanol and other fermentation products and for improving the digestibility of feed. Isolates of cellulolytic strains from various samples (soil, plant debris, decaying wood) of the Yakouren forest litter (wilaya of Tizi-Ouzou-Algeria) were grown on selective agar media containing cellulose as sole carbon source. Screening of selected strains was carried out on Mandel's agar medium containing either amorphous or crystalline cellulose. Strain BY had a zone of hydrolysis of 25 mm diameter after 7 days at 30 °C compared to 30 mm for *Trichoderma reesei* reference strain used. In addition, strain BY was the only strain that hydrolyzed crystalline cellulose. Solid substrate fermentation studies were conducted using strain BY. Cultures were incubated at 30 ° C for 8 days. Filter paper activity of about 46 U/g dry matter was observed after 5 days of fermentation. Dry matter increased during the fermentation from 47.7 mg to 59.0 mg. In contrast, the pH decreased gradually from 6.4 to 6.14. Further research is required to identify other enzymes such as CMCase, the ß-glucosidases and xylanases, and to study the degradation of other substrates such as wheat straw, sawdust, wood and cornbran.

Keywords: Screening; Fungal cellulases; Wheat bran; Solid-state fermentation; Filter paper activity.

INTRODUCTION

Lignocellulosic biomass consists mainly of 40-60% cellulose, 15-30% hemicellulose and 10-30% lignin. There is growing global interest in the environmental impact of solid lignocellulosic waste generated by different agricultural activities. Industrial techniques are being used to find ways to reduce and, if possible, add value to these cellulose-rich wastes. Cellulose is the basic structural material of plant cell walls and the macromolecule is the most abundant and widely synthesized in the plant world. It is a renewable energy source virtually inexhaustible and untapped to date.

The solid state fermentation process is one of the most applied in industry with low investment and operation costs. This has made it an attractive alternative for the production of cellulases, the enzymes that break down cellulose, which are widely used in industrial processes in the food and pharmaceutical industry. However, the most promising use in the current economic climate seems to be the production of alternative fuels. Cellulosic residues hydrolyzed

by cellulases, provide a mixture of sugars. Fermentation of these residues can lead to products such as acetone, butanol, ethanol or methane.

Filamentous fungi that produce cellulases are usually grown on solid substrates in an environment that is similar to their natural environment. Their vegetative growth consists of aerial branched hyphae that colonize the porous solid matrix. In addition, filamentous fungi can grow at humidity levels as low as 12% and they tolerate high osmotic pressure, characteristics that are not usually found in yeasts and bacteria.

The exploitation of agricultural by-products such as wheat straw, peanut shell, and sawdust, by fermentation is a very interesting biotechnological approach for the production of cellulases due to their high cellulose content. This paper describes screening fungal strains for their cellulolytic activity and then testing one strain for its ability to produce cellulases in a solid substrate fermentation process.

MATERIALS AND METHODS

Fungal strains. Isolates of cellulolytic fungal strains were obtained from various samples (soil, plant debris, decaying wood) of the Yakouren forest litter (wilaya of Tizi-Ouzou, Algeria) using a selective medium containing cellulose as the sole carbon source.

Preparation of amorphous cellulose[1] Micro crystalline cellulose (Avicel) (10 g) was suspended in concentrated phosphoric acid (85%; 200 ml) with occasional stirring and constant cooling. The swollen microcrystalline cellulose was washed several times with cold deionized water. After incubating overnight with 3% (w/v) Na_2CO_3, the thick suspension of swollen cellulose was washed several times with cold deionized water. The amorphous cellulose was drained and stored at 4°C.

Screening of fungal strains for cellulase activity.
The isolated strains were screened for cellulase activity on an agar medium containing either amorphous or crystalline cellulose. The screening medium used is that of Mandel [2] containing in g/L: urea: 0.3; KH_2PO_4: 2; $(NH_4)_2SO_4$: 1.4; $MgSO_4$, 7 H_2O: 0.3; soy peptone: 0.75; yeast extract: 0.5; $CaCl_2$: 0.4; amorphous cellulose or crystalline cellulose: 10; agar: 20 and in mg/L: $FeSO_4$, 7 H_2O: 5; $MnSO_4$, 7 H_2O: 1.08; $ZnSO_4$, 7 H_2O: 1.4; $CoCl_2$: 2. The Mandel's medium (containing either amorphous or crystalline cellulose) was poured into sterile dishes (22 x 22 cm) and cooled. An agar plug of each strain was inserted into a corresponding hole in the medium. The plates were incubated at 30 °C until the appearance of transparent zones around the holes corresponding to the zones of hydrolysis of cellulose. *Trichoderma reesei* strain was used as a reference organism.

Production of cellulases from strain BY by solid fermentation
Subculture of the strain. Strain BY was sub cultured on Petri dishes containing malt medium and incubated at 28 °C for 10 days.
Preparation of substrate. Wheat bran (20 g) was moistened to 50% and placed into 500 ml Erlenmeyer and then autoclaved at 121 °C for 20 min.
Inoculum obtention**.** Ten mls of a sterile solution of 0.2%, Tween 80 was added to a Petri dish containing the culture. The mycelium was scraped from each Petri dish and the mycelial suspension was recovered into a sterile vial using a sterile pipette.
Substrate inoculation. Three mls of mycelial inoculum was used to inoculate the substrate (8.25 mg / g substrate). The substrate was inoculated and incubated at 30 °C for 7 days.

Enzyme extraction. Two hundred mls of distilled water were mixed with 10 g of the fermented substrate, the pH of the mixture was measured and the mixture was centrifuged at 10,000 rpm at 4 °C for 15 min. The supernatant contained a crude enzyme extract.

Monitoring the growth of strain BY.

Measurement of dry matter. A test sample of from 0.5g to 1 g of fermented substrate was used to determine the % dry matter during the period of fermentation.

Monitoring changes in pH. The pH was measured during the period of fermentation

Determination of enzyme activity. The cellulase activity was measured using the filter paper activity (FPA) assay, expressed in filter paper units (FPU) according to the method of Ghose [3]**.** This method measures the release of reducing sugars produced in 60 min from a mixture of enzyme solution (0.25 ml) and of citrate buffer (0.05 M; pH 4.8; 0.5 ml) in the presence of 25 mg of Whatman N° 1 (1 x 3 cm strip) and incubated at 50°C. The released sugars were analyzed by a nitrosalicylic acid method [4]. All simples were analyzed in triplicate and mean values were calculated. All activities were described in International Units (IU) where one unit of activity is defined as the amount of enzyme required to liberate 1 µmol of reducing sugars in 1 minute. Filter paper activity was expressed by U/ g of dry matter.

RESULTS AND DISCUSSION

Twelve fungal strains were isolated on cellulose agar medium and then sub cultured onto malt medium for further study (Table 1). Of the 12 strains screened, two *Trichoderma sp.* gave a clear zone of cellulose hydrolysis of 17 mm on the amorphous cellulose medium. Strain 4 (BY) gave a zone of 25 mm on amorphous cellulose as well as 15 mm on the crystalline cellulose medium. A 30 mm zone of hydrolysis was observed around the *Trichoderma reesei* on amorphous cellulose medium only (Table 1).

Table 1: Diameter of hydrolysis zones of the strains isolated

Strains	Hydrolysis zone (mm)	
	Amorphous Cellulose	Crystalline Cellulose
1	-	-
2	-	-
3	-	-
4 (BY)	25	15
5 (*Trichoderma sp*)	17	-
6 (*Trichoderma sp*)	17	-
7	-	-
8	-	-
9	-	-
10	-	-
11	-	-
12	-	-
Trichoderma reesi	30	-

In the solid substrate fermentation study, Figure 1 shows that the dry matter increased during fermentation and reached 59% in the seventh day of fermentation. The pH dropped during the fermentation from 6.4 to 6.05 rising again slightly to 6.14 (Figure 2). The cellulase activity was

measured using the filter paper activity (FPA) assay and expressed in filter paper units (FPU). The maximum activity was found after 5 days of fermentation with 46 U / g dry matter (Figure 3).

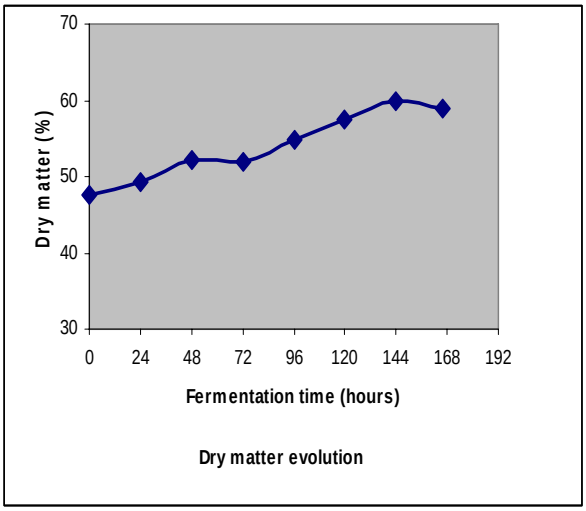

Figure 1: Dry matter evolution

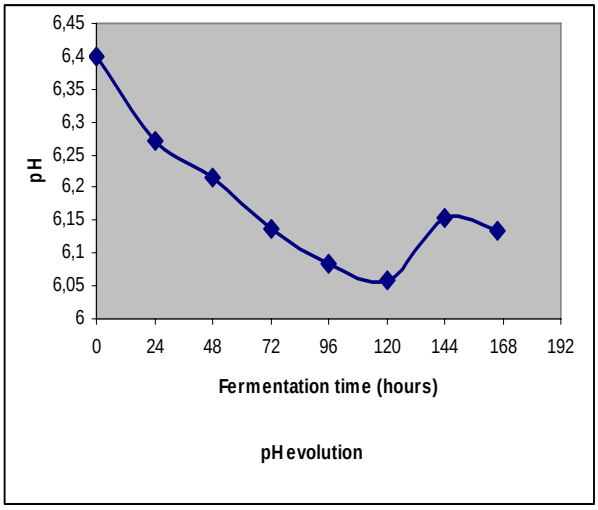

Figure 2: pH change during fermentation

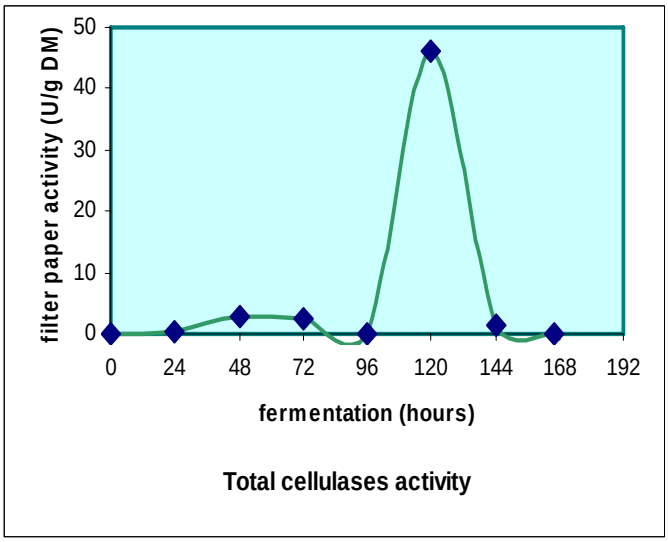

Figure 3: total cellulases activity

CONCLUSION

The present study identified an interesting cellulolytic strain – Strain BY. It has a zone of hydrolysis of 25 mm on amorphous cellulose and 15 mm of crystalline cellulose. However, a clear zone of 30 mm in diameter was observed for *Trichoderma reesei* on amorphous cellulose. These results are preliminary and further investigations will include assays for CMCase, B-glucosidases and xylanases. Other substrates such as wheat straw, sawdust, wood and corn bran will also be investigated.

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We would like to thank the world Society for Mushroom Biology and Mushroom Product and the sponsors of the conference.

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MYCELIUM GROWTH AND YIELDING OF *AGROCYBE AEGERITA* (BRIG.) SING. ON DIFFERENT SUBSTRATES.

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ABSTRACT

High yield and good quality of the carpophores is the most important issue that modern mushroom growers focus on during the cultivation work. Crop commodity is usually done on the sawdust of deciduous trees. *Agrocybe ægerita* is an edible mushroom characterized by high content in protein, easily digested by human gastrointestinal. This experiment was set to investigate mycelial growth and yield of two strains of *A. ægerita* on different substrates. In the laboratory experiment, mycelial growth on 8 agar media (PDA, Standard, wheat, MA, CYM, potato-carrot based, 2 sawdust extract: alder, beech and birch (1:1)) and 5 sawdust substrates (birch, beech, oak, maple, alder) was investigated. Petri dishes (Ø 9 cm) for agar media and biological tubes (18 cm long and Ø 2.5 cm) for sawdust substrates were used. In the cultivation studies, two kinds of sawdust substrates were used: birch, beech and mixture of beech and alder (1:1). Each of the sawdust was moisturized up to 70%. After sterilization the substrate was inoculated with mycelium (on grain) of the investigated strains and incubated at 25°C. Later, when mycelium has completely overgrown the substrate the temperature was decreased to 15-17°C to initiate primordia formation. The cultivation was enlightened 10 h/d with Day-Light lamps (500 lx). One crop was harvested after 5 weeks. The carpophores of black poplar mushrooms were picked up in clusters.

The laboratory experiment showed no statistically important difference between the mycelial growths of the investigated strains. The best growing agar media were PDA, MEA and wheat, both strains showed slowest mycelium growth on CYM. The linear mycelial growth was the best on the beech and birch sawdust. The two investigated strains differed significantly within the yield on the beech and birch sawdust. The best substrate for cultivation of *A. ægerita* was birch sawdust. The weight of single carpophores as well as the weight of single cap, as the edible part, was measured. Both investigated strains were characterized by big and heavy carpophores.

INTRODUCTION

The interest of researches all over the world in *Agrocybe aegerita* (Brig) Sing. – black poplar mushroom is continuously growing together with new knowledge obtained on its fabulous properties. *Agrocybe aegerita* is an edible mushroom characterized by high content of protein, easily digested by human gastrointestinal [1, 21]. Its flavor qualities were valued already by the ancient Greeks and Romans and the cultivation of this species reaches almost 2000 years [18]. The taste of the fresh carpophores is mild and makes a very good composition for the poultry and fish dishes, giving them a gentle pork flavor [14]. In nature black poplar mushroom grows in clusters on living and decaying stumps of mostly deciduous trees such as: poplar, willow, black poplar, ash, elderberry, black locust and Brazilian araucaria [20]. The cap of *A. aegerita* is convex, expanding to plane at maturity. Cap diameter is up to 20 cm, yellowish gray to grayish brown, darker towards the center. Gills are at first gray, with spore maturity becoming chocolate brown. Stem is white, adorned with a well developed membranous ring, usually colored brown

from spore fall [16]. *Agrocybe aegerita* gained special attention for its healing properties [3]. It was found to be medically active in several therapeutic effects such as antitumor, antifungal, antioxidant, nerve tonic, hypercholesterolemia and hyperlipidemiê [4, 8, 15, 19]. Extracts from fruiting bodies of *A. aegerita* acquired antimutagenic activities and might play enormous role in cancer prevention [11]. Currently numerous studies are undertaken in order to extend knowledge of those properties as well as wider exploitation of *A. aegerita* in medicine.

First stage of growing mushrooms is mycelium production on agar media. The growth rate of mycelium depends on the type of media and substrate as well as of species and strain of mushroom [2]. The best agar media for maternal mycelium production is the one on which the mycelium growing rate is the quickest and the quality of mycelium hyphae is the best. Choosing the best agar media determinate the shortest time for mycelium production, hence the mushrooms yield production.

High yield and good quality of the carpophores is the most important issue that modern mushroom growers focus on during the cultivation work. Crop commodity, which very often depends on the speed of mycelium growth, is usually done on the sawdust of deciduous trees [12, 17]. The growth speed of mycelium on the growing substrate not always means high and good quality yielding. Therefore after choosing the best substrate for the mycelium growth it has to be verified in cultivation conditions. The aim of this study was to determinate the best substrates for mycelium growth as well as the best substrates for cultivation and fruiting bodies development of *A. aegerita*.

MATERIALS AND METHODS

Materials. Strains of *A. aegerita,* indicated as AE02 and AE05, used in our experiment came from collection of cultivated and medicinal mushrooms of the Department of Vegetable Crops, Poznań University of Life Sciences. The material used for inoculation was granular mycelium prepared according to the recipe recommended by Lemke [7]. Maternal and granular mycelium of examined strains was prepared in the biological laboratory of Department of Vegetable Crops of Poznań University of Life Sciences. Granular mycelium was prepared on wheat grains.

Laboratory experiment. Two separate experiments were established, one for agar media and the other for sawdust substrate. In first experiment mycelium growth on 8 agar media (PDA, Standard, wheat, MA, CYM, potato-carrot based, 2 sawdust extract: alder, beech and birch (1:1)) was investigated (Table 1). Petri dishes (Ø 9 cm) filled with analyzed agar media were inoculated with slices of maternal mycelium (Ø 0.5 cm) placed centered. Mycelium was incubated in 25°C and 80-90% of relative air humidity.

Second experiment was set to asses the mycelium growth on 5 sawdust substrates: birch, beech, oak, maple, and alder. Sawdust was moisturized up to 70%. After sterilization and cooling to the temperature of 21°C the substrate was placed in the biological tubes (18 cm long and Ø 2.5 cm), inoculated with granular mycelium of investigated strains and incubated in 25°C and relative air humidity 85-90%.

Experiments were established in fully randomized design, in 6 replications on Petri dishes; in 4 replications and 2 series in biological tubes. On Petri dishes diameter of media occupied by mycelium was measured after 9 days from inoculation. On biological tubes thickness of substrate mass occupied by mycelium was measured after 18 days from incubation.

When comparing the experimental results, the analysis of variance for factorial experiments was applied using Newman-Keuls test on the level of significance α=0.05. The results of experiments were discussed on the basis of mean values from 1 cultivation cycle.

Table 1: Composition oft he agar media used to study the mycelial growth of *A. aegerita*

Agar Media	Composition (+ fill to 1 liter of distilled H_2O)
Malt Extract Agar (**MEA**) Bioskop/ Lab Empire	- 20.0 g maltose extract - 22.0 agar
Potato Dextrose Agar, Bioskop pH 5.6 +/- 0,2(**PDA**)	- 20.0 g PDA - 22.0 g agar
Potato-carrot agar (**PCA**)	- 100 g carrot - 100 g potato - 1 g glukose - 22 g agar
Wheat	- 200 g wheat grain extract - 3 g glukose - 22 g agar
Sawdust - alder	- 50 g alder sawdust - 3 g glukose - 22 g agar
Sawdust – beech+birch	- 100 g sawdust (1:1) - 3 g glukose - 22 g agar
Complete Yeast Medium **CYM**	- 0.5 $MgSO_4$ $7H_2O$ - 0.46 g KH_2PO_4 - 1.0 g K_2HPO_4 - 2.0 g peptone - 2.0 g yeast extract - 20 g glukose - 22 g agar
Standard	- 1.0 g KH2PO4 - 1.0 g NH4NO3 - 0.5 g MgSO4 - 3.0 g sacharose - 2.0 g glukose - 1.0 g maltose - 20 g agar

Cultivation experiment. The discussed experiment was established in an air conditioned chamber and the cultivation was conducted in plastic bottles of capacity of 600 ml. The substrates used in the experiment were two kinds of sawdust substrates: birch, beech and a mixture of beech and alder (1:1). Each sawdust was moisturized up to 70%. After sterilization and cooling to the temperature of 21°C the substrate was inoculated with granular mycelium of investigated strains and incubated at 25°C and relative air humidity 85-90%. Once the substrate was overgrown by mycelium the temperature was decreased to 15-17°C to initiate primordia formulation. The cultivation was lighted 10 h/d with Day-Light lamps 500 lx.

Experiments were established in fully randomized design, in 4 replications and 2 cultivation cycles. Harvest of *A. aegerita* carpophores was carried out for a period of 6 weeks. The carpophores of black poplar mushrooms were picked up in clusters, no single carpophores were cut out from the sawdust. Yields and dry matter content of carpophores were determined on

the basis of harvested fresh fruiting bodies calculated per 100g of substrates dry matter. For morphological features of carpophores, 10 fruiting bodies were sampled from each repetition and measurements of cap diameter, diameter and length of the stipe together with single cap and carpophore weight were performed.

When comparing the experimental results, the analysis of variance for factorial experiments was applied using Duncan's test on the level of significance α=0.05. The results of experiments were discussed on the basis of mean values from 1 cultivation cycle.

RESULTS AND DISCUSSION

In the first experiments, mycelial growth measurements were performed to investigate the influence of the type of media and substrates on colonization rates. Mycelium growth rate is different between the species and sometimes even within the strains of mushroom and depends on the type of media and substrate [9]. Bilay *et al*. [2] investigated growth rate of 30 species and strains of mushrooms on different agar media. They concluded that different species vary concerning the nutritional requirements. Mycelium of both examined strains presented similar growing rate on all evaluated agar media except from Complete Yeas Media (CYM), where mycelial growth was the slowest (5.2 cm / 9 d), which corresponds with investigation of Kim *et al*. [6] who also obtained the best results on CYM agar media. Other 7 media presented statistically insignificant growth differences, however there was a tendency where mycelium of both strains showed quickest growth on PDA (7.3 cm/ 9 d), followed by MA (7.2 cm/ 9 d) and wheat and birch/beech (7.1 cm/ 9 d) agar media (Fig.1). Comparing both examined strains, regardless agar media the quickest growth was represented by the strain AE05 with 7.0 cm/ 9 d where AE02 was 6.5 cm/ 9 d, the difference being statistically significant. The results of our experiment show the mycelium growth for 9 d depends on the used medium; however the differences between media are not statistically significant. This makes *A. aegerita* mycelium easy to reproduce, which confirm investigation of other authors who used for maternal mycelium productions different agar media obtaining quick growth on each media [2, 6].

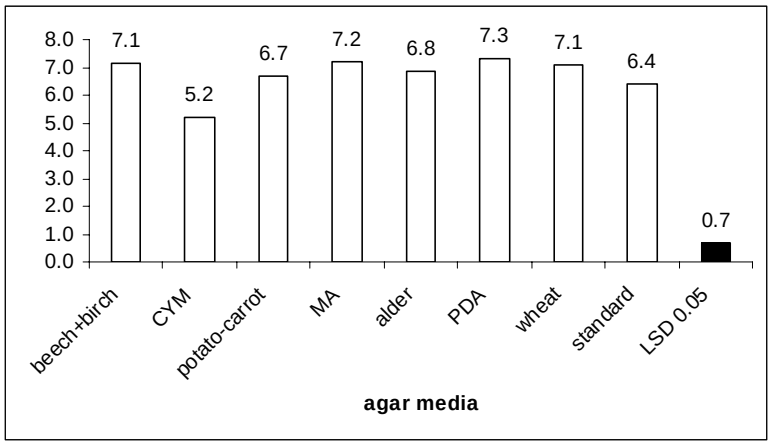

Figure 1: Growth rate of *A. aegerita* strains on different agar media [cm / 9 days].

In the nature carpophores of *A. aegerita* appear commonly on many species of trees, the cultivation substrate is mostly chosen by its availability [14]. In our experiment, both of examined strains showed similar growing rate regardless used sawdust. Mycelium of both strains (AE02 and AE02) showed the quickest growth on beech sawdust (7.1 cm /18 d), little slower growth but statistically significant was on birch sawdust (6.8 cm /18 d), and alder sawdust (6.7 cm /18 d). Definitely the slowest mycelial growth of examined strains was on oak sawdust (4.0

cm /18 d). Growth of mycelium on the maple sawdust was moderately fast (5.6 cm /18 d) (Fig. 2).

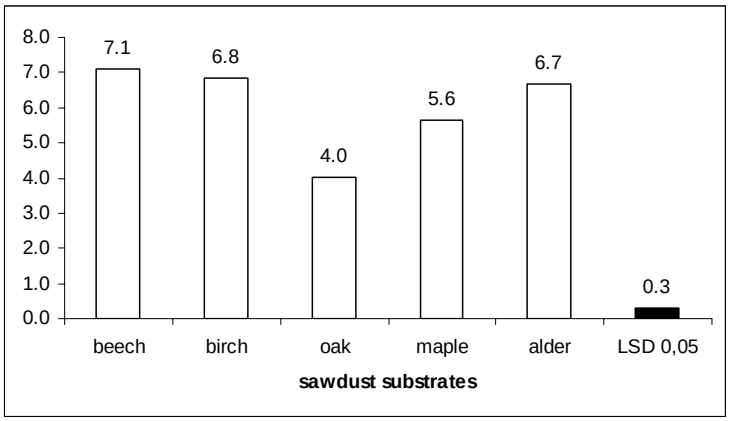

Figure 2: Growth rate of *A. aegerita* strains on different growing substrates [cm / 18 days]

In published works, average yield of *A. aegerita* is 0.5 kg of mushroom on 2.5-3.0 kg of substrate. Yield of carpophores of investigated strains was different and depended on the used cultivation substrate [10, 13]. The highest yield was obtained on substrate composed from beech and alder sawdust (39.5 g/100g of substrate) and birch (36.8 g/100g of substrate; Fig. 3). However mycelium growth rate was statistically quickest on the beech sawdust, yielding on substrate of only beech sawdust was much lower than above mentioned substrates (8.7 g/100g of substrate). Obtained results do not correspond with previous experiments conduced by Sobieralski *et al*. [13], where the highest yield was, on the contrary, obtained from beech sawdust much higher yielding – 49.7 g/100 g of substrate, however yield from birch sawdust was similar (46.1 g/100g of substrate). Zadrazil [22] states the better mycelium growth, as well as higher yield, of *A. aegerita* can be obtained by increasing inorganic nitrogen content or by addition of protein-rich additives in the substrate.

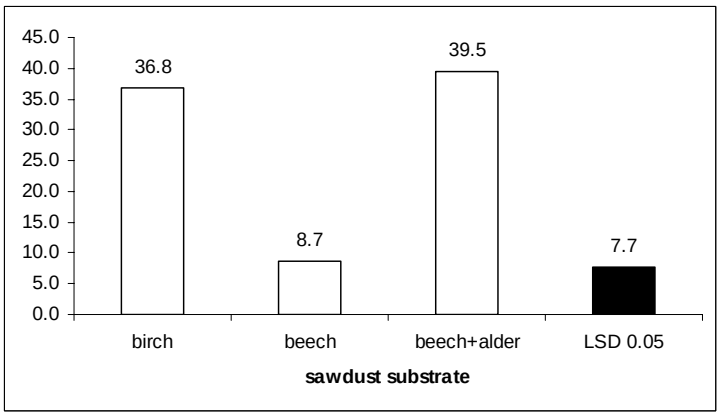

Figure 3: Yield of *A. aegerita* on different substrates [g/100 g substrate DM]

Dry matter (DM) content in the carpophores of cultivated mushrooms is one of the important factors describing the quality of the fruiting bodies. Generally DM content in fruiting bodies of cultivated mushrooms ranges from 7.9 to 11.4% in *Agaricus bisporus* [5], 8.0% in *Pleurotus ostreatus*, 14.3% in *Boletus edulis* [1] and depends on the cultivation substrate used [5]. Content of dry matter in our experiment was estimated after drying the collected carpophores at 105°C for 24 hours. The highest yield based on dry matter content of harvested mushroom was

obtained again on the composition of beech and alder sawdust (3.2 g/100g of substrate DM) and birch (2.9 g/100g of substrate DM), the beech sawdust was only 1.2 g/100g of substrate DM (Fig. 4). However percentage of dry matter within the obtained carpophores compared to the yield harvested was higher in the beech substrate (15.7 %), when on beech/alder and birch it was much more lower (only 8.1 and 8.0 % respectively) (Fig. 5), this corresponds with the results obtained by Bauer Petrovska & Kulevanova [1] where DM content of *A. aegerita c*arpophores was 10.2%.

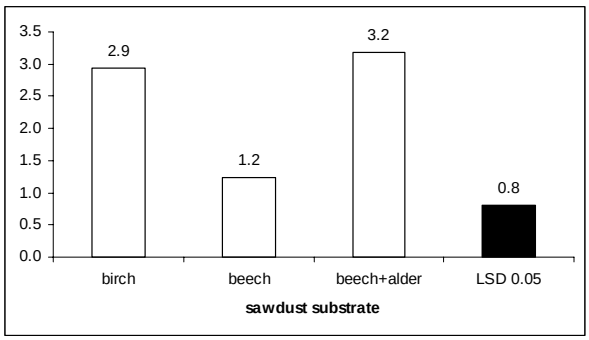

Figure 4 Yield of dried carpophores of *A. aegerita* cultivated on different substrates [g/100g of substrate DM]

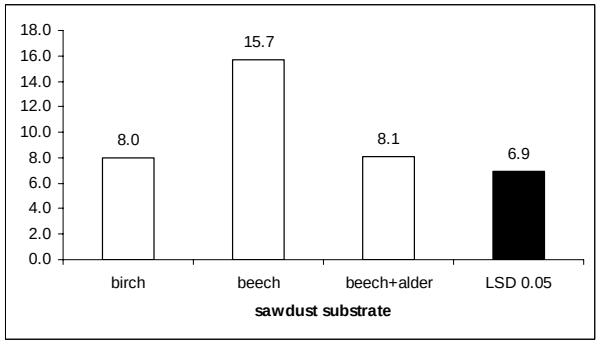

Figure 5: Dry matter content in carpophores of *A. aegerita* cultivated on different substrates [% DM]

Carpophores of *A. aegerita* show differences in their morphology depending mostly on the type of substrate used for cultivation [16]. The morphological features of carpophores in our experiment differed among used cultivation substrate. Carpophores harvested from birch substrate characterized with the heaviest caps (Fig. 6) and carpophores (Fig. 7) (2.2 and 3.7 g respectively), those collected from beech/alder substrate were little bit lighter (1.9 and 3.4 g). However fruiting bodies collected from beech/alder sawdust have bigger caps (3.3 cm) (Fig. 8) and longer stipes (4.8 cm) (Fig. 9) than on birch substrate (3.1 and 4.2 cm respectively). Carpophores harvested from beech sawdust were much lighter (2.0 g), had smaller caps (2.7 cm) and shorter stipes (3.0 cm).

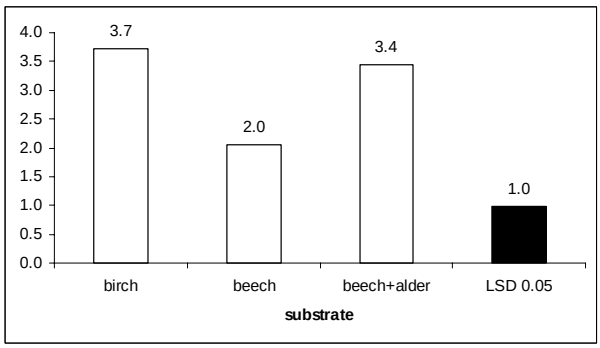

Figure 6: Average weight of carpophores of *A. aegerita* cultivated on different substrates [g/ carpophore]

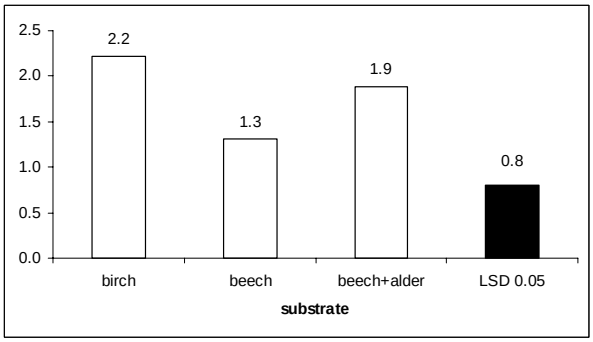

Figure 7: Average weight of caps of *A. aegerita* cultivated on different substrates [g / cap]

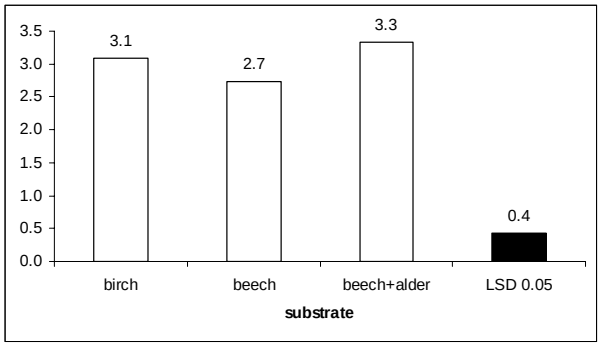

Figure 8: Average cap diameter of *A. aegerita* cultivated on different substrates.
[cm / cap]

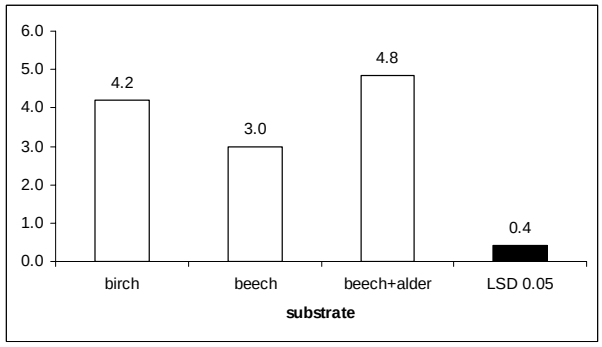

Figure 9: Average stipe length of *A. aegerita* cultivated on different substrates.
[cm / stipe]

CONCLUSION

1- The experiment showed no statistically important difference between the mycelium growth of investigated strains.
2- Suggested agar media for maternal mycelium growth were PDA, MEA and wheat and birch/beech agar media.
3- Both strains showed slowest mycelium growth on CYM comparing to other investigated agar media.
4- The mycelium growth was the best on the beech and birch sawdust, therefore both were used in cultivation experiment.
5- Yield of investigated strains depended on the substrate used for cultivation.
6- The best substrate for cultivation of *Agrocybe aegerita* was birch sawdust and composition of beech and alder sawdust.
7- The carpophores of both investigated strains characterized with the big and heavy carpophores.

ACKNOWLEDGEMENTS

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BIOMETHANE DIGESTATE FROM HORSE MANURE, A NEW WASTE USABLE IN COMPOST FOR GROWING THE BUTTON MUSHROOM, *AGARICUS BISPORUS* ?

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ABSTRACT

Mushroom cultivation is a direct utilization of their ecological role of organic matter degradation in the bioconversion of solid wastes generated from industry and agriculture into edible biomass, which could also be regarded as a functional food or as a source of drugs and pharmaceuticals. Significant changes are expected in the integrated management of wastes streams in the future due to the use of plant biomass for biofuel and energy production and other non-food crops. On the one hand these activities may use the same wastes than those allowing mushroom cultivation today and problem of competition for supplying may occur. On the other hand these new activities will generate new wastes to be treated and valorise by the mushroom industry.

Horse manure is a component of the compost used to grow the button mushroom, *Agaricus bisporus* and other *Agaricus* species. We studied the methanisation of horse manure and utilisation of the digestate produced by the biogas reactor as waste for growing *A. bisporus*. Methanisation was efficient and the compost obtained from the digestate in mixture with wheat straw and low quantities of mushroom compost as source of aerobic and thermophilic microflora allowed to grow *A. bisporus*, but with lower yields than in conventional mushroom composts. Horse manure methanisation digestate was also used without composting by incorporation into the compost at spawning compared to a proteinic supplementation and in the preparation of casing soil in substitution of a part of peat moss. No beneficial effect was observed and with increasing rate of digestate incorporated, negative effect appeared (unproductive area).

Keywords: composting, button mushroom, horse manure, methanisation.

INTRODUCTION

Because many natural resources are limited, sustainability becomes an important concept in maintaining the human population, health, and environment. Mushroom cultivation is a direct utilization of their ecological role in the bioconversion of solid wastes generated from industry and agriculture into edible biomass, which could also be regarded as a functional food or as a source of drugs and pharmaceuticals. In Europe, mushroom industry mainly focuses in the cultivation of only few species under standardised cultivation conditions, the button mushroom, *Agaricus bisporus*, being largely dominant. Today, wheat straw and horse or poultry manures are the main wastes used for the button mushroom cultivation, but changes in climatic conditions and agricultural practices may results locally in difficulties for supplying, with the limitation of transportation as an objective. Concerning the integrated management of wastes streams in the

future, significant changes are expected due to the use of plant biomass for biofuel and energy production and other non-food crops. On the one hand they may use the same wastes than those allowing mushroom cultivation and problems of competition for supplying may occur. On the other hand these new activities will generate new wastes to be treated and valorise by mushroom industry.

In the present investigation, the possibility to develop an integrated process of biogas production and mushroom cultivation from horse manure was studied. The objective is to state how Anaerobic Digestion of horse manures can be used to produce both renewable energy as methane and an edible biomass as button mushroom.

MATERIALS AND METHODS

Anaerobic Digestion of horse manure. Horse manure on wheat straw was purchased by a racing stable of horses. The optimal conditions for methane productions were studied in a pilot reactor of 30 L filled with 90 % of Horse manure and 10 % of garden wastes and load at a rate of 5.5 g organic matter per L per day during the process. The inoculum was from two reactors working with horse manures on wheat straw and corn. A process of thermophilic (55°C) dry (moisture content of less than 75%) anaerobic fermentation was used to produce biogas. Digestate was collected every week from the reactors and stored at 4°C before being used for growing *A. bisporus*.

Composting. Digestate collected every week in 5L or 30L reactors were stored at 4°C before being used for composting experiments. Composting was performed in reactors for aerobic solid state fermentation containing approximately 3 kg of raw materials in which temperature is managed [1]. The studied parameters of composting were the composition of the mixture raw ingredients: digestates, wheat, straw, horse manure, mushroom compost ready to be spawned, wheat bran, gypsum. The microbial quality of the obtained composts was estimated by measuring the metabolic activity through the hydrolysing activity of fluorescein diacetate and the number of fungal and bacterial colony forming units at both 28°C and 45 °C as in Libmond & Savoie [2]. Analyses of the chemical composition of composts were performed by a private laboratory with normalised methods. The efficiency of the produced compost as cultivation substrate for *A. bisporus* was evaluated by measurement of mycelial growth rates in Petri dishes and small boxes inoculated with commercial spawn.
After the optimisation of the formulation in the reactors, an experiment was performed at a larger scale with a conventional process of composting in two phases for estimation of mushroom yield in 0.09 m² trays filled with 8 Kg of compost spawned at 0.8 % as in previous works [3].

A commercial substrate (Renaud Champignon, Pons, France) obtained with wheat straw and horse manure as main ingredients was used as control.

Digestates as supplement in button mushroom cultivation. Phase II mushroom compost was obtained from France Champignon Substrate Company for all experiments. It was compost made from wheat straw and horse manure. The plots consisted of plastic trays (0.09 m²) filled with 9 kg of pasteurized compost (100 kg/m^2) set in a randomized block experimental design with six replicates. Hybrid white strain of *Agaricus bisporus* (Sylvan A15) was inoculated at the rate of 0.8 %. The compost temperature during spawn-run (13 days) was maintained around 25°C. The casing soil was made of 50 % limestone and 50 % peat. Digestates collected from INRA-LBE Narbonne reactors were stored at 4°C before being used for compost and casing experiments. They were mixed manually with compost or casing at the different rates used in experiments. Mushrooms were grown under French standards of culture with controlled environmental

conditions in CTC facilities. Mushrooms were harvested (without trimming the stipe) daily, at market maturity i.e. veil unbroken and gills not exposed, for the crop duration of three weeks, and this for each plot. In this way, the weight of mushrooms was registered daily. The yield was expressed as kg/m^2.

RESULTS AND DISCUSSION

Anaerobic Digestion of horse manure. The average production of biogas containing 50 % of CH_4 was 80 $^+$/- 20 L.j^{-1}. The transformation yield of the dry matter to biogas was of 40 %.

Composting. Digestates may not be used directly as cultivation substrate for *A. bisporus*. There microbial and chemical compositions resulting from anaerobic digestion are not favourable to the mushroom. Mycelium inoculated directly in digestates does not grow from the spawn grain. During various experiments of composting in laboratory scale reactors we observed that one of the difficulties with this material used as main component was the production of ammonia that necessitated long time of composting. Consequently we tested mixtures of digestate and wheat straw in which digestate did not represent more than 60 % (dry weight) of the raw ingredients and where commercial compost was used as an inoculum. Based on the mycelial growth rate tests, analyses of the microbial quality, pH and organic matter measurements and ammonia odour, the selected mixture was (dry weight basis): 45 % wheat straw, 30 % digestate, 11 % gypsum, 9 % wheat bran, 5 % mushroom compost. Composting of this formula at laboratory scale for 20 days followed the temperature patterns presented in Fig 1, and allowed to obtain compost on which the mycelial growth rate was at mean 2/3 that measured in commercial compost used as control.

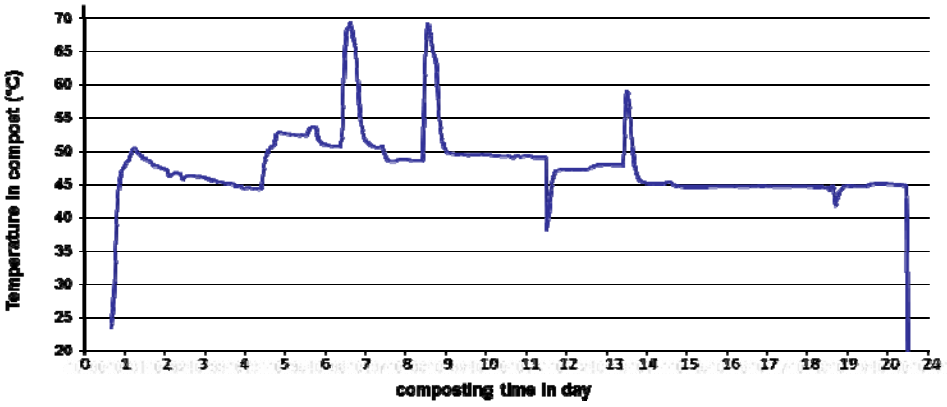

Figure 1: Temperature recorded in the substrate during composting at laboratory scale in fermenters.

Table 1 shows their chemical compositions and that of the initial mixture before composting. The differences in mineral composition may be noticed and is due to the high quantity of gypsum we used. The values of the other parameters of the chemical compositions are close.

The above mixture of raw ingredients was used for the production of 50 Kg of compost with a conventional composting process performed in trays. The recorded mean temperature during composting is reported in Fig 2. The yields of mushroom for 5 weeks of harvest were 25.9 ± 5.68 and 15.2 ± 1.64 Kg/m² (means of 6 replicates) for the control (commercial

mushroom compost) and the compost from digestate respectively. The mushroom mean weights were not different in the treatments.

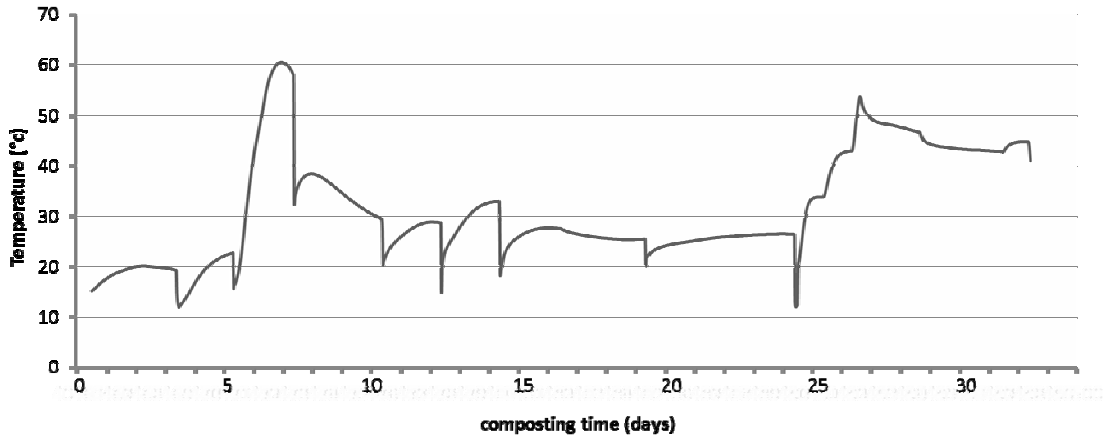

Figure 2: Temperature recorded in the substrate during conventional composting at pilot scale

Table 1: Chemical composition of the mixture of raw ingredients including digestate, of obtained compost, and commercial compost as control

	mixture of raw ingredients	compost from digestate	commercial compost
Minerals (g Kg-1)	288.6	345.5	303.3
K	25.9	35.5	32.1
Mg	5.5	7.11	6.4
Ca	66.4	80.1	55.8
Na	1.8	2.3	2.8
S	65.5	84.4	35.8
Organic C	356	323	348
N (Kjeldahl)	17.8	21.5	23.1
C/N	20.0	15.0	15.1
water soluble OM (% of total OM)	29.5	29.8	32.5
% of OM non soluble in acid detergent	45.8	52.1	48.8
Hemicelluloses (% of total OM)	24.7	18.1	18.7
Cellulose	5.1	5.1	2.9
lignin and cutin	40.7	47.1	45.9

The present work shows that digestates from dry anaerobic digestion of horse manure for production of biogas might be used as raw ingredients of composting in place of horse manure for the cultivation of *A. bisporus*. However it is necessary to add an inoculum of aerobic thermophilic microbial communities. This inoculum can be obtained by introducing mushroom compost obtained with conventional ingredients or by inoculation of pure cultures of useful microorganisms either bacteria [4] or fungi [5]. We only obtained half of the yield of a commercial cultivation substrate, but the simple composting process used here could be optimised.

Digestates as supplement in button mushroom cultivation. Incorporation of digestates into the compost at spawning is compared to the effect of a proteinic supplementation. In that case,

compost was supplemented with Promycel 600 at spawning at the rate of 0.8 %. One trial was conducted to investigate the effect of two rates of digestates (2% and 5%). Non-supplemented compost produced 23.48 kg/m² of mushrooms (Fig. 3). With commercial supplementation, yield increased very significantly to 33.38 kg/m² (+ 42 %). In the same time lower increases in yield were observed with addition of digestates to the compost (+ 12 % to + 21 %). Digestates can't be a substitute to commercial supplementation to improve mushroom yield, but might be used with some beneficial effects.

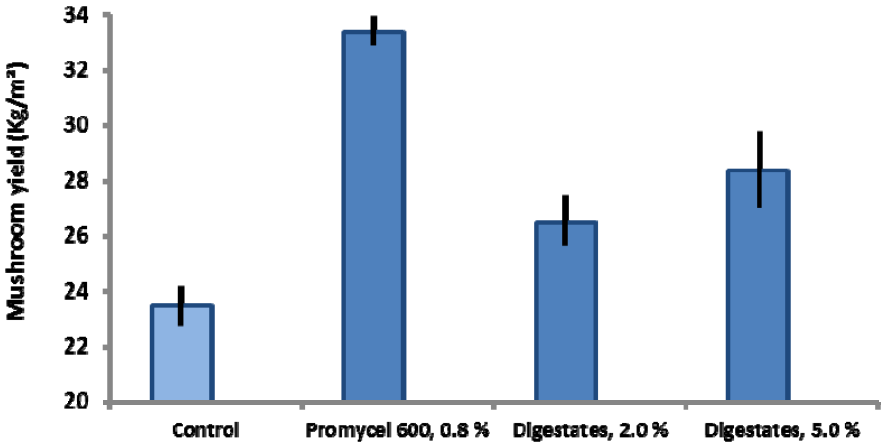

Figure 3 : Effect of 2 rates of digestates, used as supplementation at spawning, on total yield

Incorporation of digestates into the standard casing soil was made at different rates (5-10-15 and 20%). Compost cased with commercial casing (control) produced 32.79 kg/m² of mushrooms (Fig. 4). On casing with digestates, we noted the same level of mushroom production compared to the control, despite the presence of "black" area in the casing soil. Only the highest rate of digestates (20%) gave a significant lower yield (28.24 kg/m²). In our conditions, we can conclude that digestates may be added in casing soil, but without beneficial effect on *A. bisporus* mushroom production.

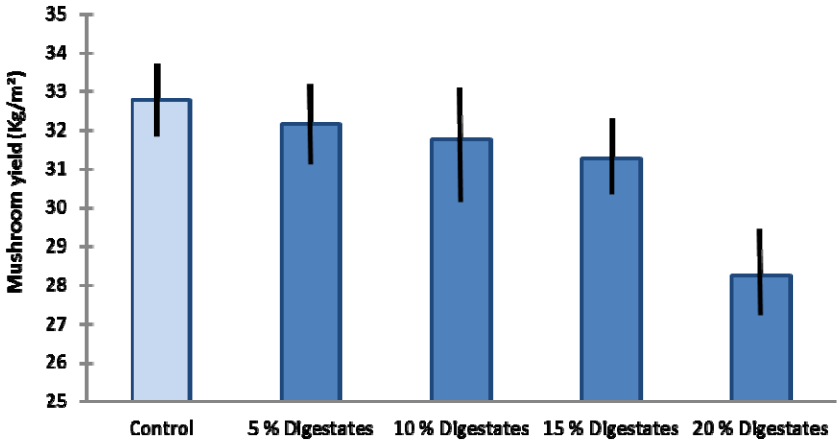

Figure 4: Effect of 4 rates of digestates incorporated into casing on total yield.
Error bars are standard deviations

CONCLUSION

Dry and thermophilic anaerobic digestion of horse manure on wheat straw is efficient for the production of Methane and digestate produced as waste of this process proved to be a raw material that can be used in mixture with others for the composting process aiming at producing a cultivation substrate for the button mushroom *A. bisporus*. However mushroom yields obtained in this study were lower than those obtained with compost used in Europe and further improvements are needed. The use of digestate as supplement, without composting was less efficient for the mushroom production.

Integrated management of horse manure for biomethane and mushroom production might have an economic interest if it is used in parallel with the current way of compost production from horse manure.

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MAGUEY MUSHROOM: AN EDIBLE SPECIES CULTIVATED FOR THE FIRST TIME

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ABSTRACT

Saprophytically in the pulque maguey, a mushroom called "menanacatl" (metl = maguey, nanacatl = mushroom) grows and produces fruiting bodies in summer with increasing moisture and heat. The aim of this study is to obtain fruiting bodies of the IE-771 strain isolated from pulque maguey in Alchichica Puebla, Mexico, cultivated in a mixture of agave bagasse and barley straw 1:1 (AB-BS 1:1), barley straw (BS) as the control, as well as to identify them morphologically and determine their proximate composition. To achieve this goal, the optimal fermentation time for mycelium growth on pulque maguey bagasse was previously established and the result was 7 d. Based on this data pulque maguey bagasse fermented for 7 d and barley straw for 3 d were mixed 1:1 on a wet basis. After 30 d of incubation, they were moved to the production room for their fructification. The biological efficiency (BE) and production rate (PR) were evaluated using a wild strain (IE-771) on the two substrates. The BE and PR on barley straw reached 44.95 % and 0.270 respectively whereas the BE and PR on agave bagasse-barley straw was 62.45 % and 0.378 respectively. The mushroom obtained was identified as *Pleurotus dryinus* and its approximate analysis is within the parameters recorded for this species except for fat and crude fiber which indicated higher values.

Key words: Agave wastes, *Pleurotus dryinus*, Mexico, edible mushroom cultivation

INTRODUCTION

One of the most notable plants found within the Mexican landscape is the maguey or agave (*Agave* spp.) appearing in arid and semiarid regions of the country. *Agave* spp. are xerophytic plants adapted to live in bad weather conditions with long periods of drought and strong temperature fluctuations between day and night [1]. From around 200 species of the genus *Agave* that exist in America, 150 of them are to be found in Mexico distributed mainly in the central part of the country [2]. Agaves in Mexico have had and still have great economic and cultural importance for many people. They have been used for at least 5000 years, making Mexico the center of domestication and diversification through human selection [3]. Agaves are used as a source of food, drink (tequila, mezcal, pulque), medicine, fuel, shelter, ornament and are a source of tough fibers, extracted from the leaves (ixtle), as fertilizer, building material and in the development of agricultural implements among other uses. Of the drinks made with this plant, pulque is considered the oldest and most traditional and was used by pre-Hispanic priests in ritual ceremonies as witnessed by numerous manuscripts and is still currently produced [2]. "Agua miel" is the liquid sap obtained from the maguey *Agave salminana* which is the ingredient for making pulque that contains a moderate amount of alcohol. Pulque had its heyday in the nineteenth century with the use of rail transport, as due to its perishable nature, it could not be drunk in places remote from centers of production [4]. Its preparation continued and it was considered a profitable industry in Mexico during the first 70 years of the XX s [5]. Later, as a result of a complex interaction of economic, technological and social variables, its

demand decreased by 60% and was replaced by beer that could be stored longer and was economically viable, healthy and hygienic [5,6,7]. This has resulted in a drastic reduction in the cultivation of pulque maguey which, associated with the faults of genetic improvement and overexploitation could in the future lead to its extinction [8]. This would also affect the survival of pulque maguey mushrooms that grow saprophytely between the leaves "pencas" of the plant, feeding on the lignocellulosic residues on dead leaves. On the other hand, the mushroom is also edible and has been collected from the wild for consumption since ancient times. Because of this, the aim of this study is to identify taxonomically the maguey mushroom and obtain fruiting bodies, through their culture using an agave bagasse- barley straw mixture 1:1 ratio with barley straw as the control, and obtain the approximate composition of the cultivated mushroom.

MATERIALS AND METHODS

Strain and spawn preparation. The strain used in this study was obtained from a mushroom collected from pulque maguey in Alchichica Puebla, Mexico (19° 24' N and 97° 23' W) (G. Mata collection No. 728) and maintained in liquid nitrogen in the Strain Collection of the Institute of Ecology AC Xalapa, Mexico, registered as IE-771. Agar inoculum was made on potato dextrose agar medium (PDA), supplemented with wheat extract according to Guzman *et al*. (2002) [9], sterilized for 20 min at 121 °C and 15 psi, placing 25 ml in Petri dishes of 90 mm ø. The medium was inoculated with the strain and incubated at 28 °C for one month and then kept refrigerated at 4 °C. To prepare the primary spawn or master, grain sorghum was used (*Sorghum vulgare* Pers), placing 50 g of sorghum and 40 g of water in glass jars, sterilized for 1 h at 121 °C and 15 psi, reaching a relative humidity of 50%. To make grain spawn, 1 cm^2 of mycelium of IE-771 was transferred in a laminar flow hood and incubated for one month at 28 °C in darkness. The secondary spawn was prepared using sorghum seeds previously soaked in water for 24 h. 175 g of sorghum was placed into plastic bags and sterilized for 1 h at 121 °C and 15 psi. After cooling, 25 g of primary spawn were transferred into new bags with sterilized sorghum seeds. The bags were incubated in a dark room at 28 °C until the mycelium fully covered the grain and then kept stored under refrigeration (5 °C) in dark conditions. To reactivate the spawn before use in the inoculation of the substrate, the bags were incubated at 28 °C for 12 to 24 h [9].

Cultivation. We used the mixture agave bagasse-barley straw 1:1 and barley straw as the control. The barley straw was cut into 5 cm, and then it was soaked for 24 h and fermented for 3 d at room temperature. For use as agave bagasse, maguey leaves were sun-dried, soaked for 24 h and fermented at room temperature for 7 d [10] and cut into pieces of approximately 5 cm. Agave bagasse and barley straw were mixed in proportion 1:1 (w / w). The substrates were pasteurized for 18 h using steam at 65 °C [11] and cooled at room temperature before inoculation. Then the substrates were placed in polyethylene bags (60 x 40 cm) by mixing the spawn 5% (w / w), up to 4 kg. The inoculated bags were incubated at 28 ° C in total darkness. On the second day of incubation the bags were cut with a knife to allow the mycelium to breathe (12 in total). The bags were kept in the incubation area until completely covered with mycelium and primordia appeared. At the end of incubation period, the bags were transferred to the production room with light and dark periods of 12/12 h at 23 °C and a relative humidity of 80-85%, to encourage the development of fruiting bodies. Once collected, the fruiting bodies were cut, weighed and measured manually, 4 crops were evaluated in each substrate. The productivity of the strain was analyzed using as parameters the Biological Efficiency (BE) = g

fresh fruiting bodies /100 g dry weight based substrate [12] and precocious using Rate Production (RP) = BE / total number of days evaluated from the day of incubation.

Statistical analysis. 24 replicates were used with the mixture of agave bagasse, barley straw 1:1 and 13 replicas with barley straw. The data obtained was analyzed using an analysis of variance (ANOVA) to determine significant differences in both substrates and when a significant difference was found, an average comparison Tukey's multiple range test at 0.05% significance was applied, using the Statistica software version 7.0.

Morphological identification. For microscopic observation, radial tangential and transverse hand cuts were made to the basidiomas with a razor. Mounted in 70% alcohol and then in a solution of KOH 5% and 1% phloxine. The size and shape of spores were observed and sections were observed under a microscope using Melzer's reagent [13].

Proximate analysis. The proximate composition of cultured mushroom was determined according to the Association of Official Analytical Chemists methods (AOAC) [14]. The moisture content was determined through oven drying at 105 °C for 24 h. The ash content was determined by incinerating at 600 °C in a muffle furnace for 6 h or until pale gray or white ash was obtained. The total protein content was determined by the Kjeldahl method using a conversion factor of 4.38 [15]. The fat content was determined by the Soxhlet method and dietary fiber by the enzymatic gravimetric method. The carbohydrate content was calculated by difference [16].

RESULTS AND DISCUSSION

Environmental conditions of wild maguey mushroom. The Climate of the town of Alchichica, where the wild mushroom grows (Fig. 1) is semi-dry with summer rains. The annual average temperature reported is 12.9 °C, June being the hottest month with 15.4 °C average temperature and the coldest being January with 9.2 C. Total annual precipitation is 372.0 mm and the rainfall is concentrated in June to 76.5 mm on average, and January is the driest month with 5.0 mm. [17].

Substrate conditioning. For substrate preparation we based ourselves on previous research employing grown *Pleurotus ostreatus* using tequila agave bagasse obtained from the central part of the agave called the "piña" [18]. Continuing with the same study using the same mushroom, it is recommended that the agave bagasse used as substrate is fermented in order to obtain the softening of the fiber and when mixed with wheat straw, it increases the absorption of water, retains moisture better, reduces the presence of contaminants and forms a better consistency [19]. This is confirmed by the results obtained by Bernabe-Gonzalez, *et al*. [20], where they grow *P. pulmonarius* in mezcal maguey bagasse (*Agave cupreata* L.) fermented for 7 days and mixed with rice straw in ratios of 2:1 and 3:1, where they obtain a BE of 111% and 120% respectively. Given these considerations, we measured the mycelial growth of the strain IE-771 in agave bagasse obtained from the leaves, fermented for different times (0, 3, 5 and 7 d), reporting the best growth at 7 d of fermentation (data not shown). Then we proceeded to find the right mix of agave bagasse- barley straw in order to find the best BE with mixtures of agave bagasse-barley straw 1:0, 1:1, 1:3 and 0:1 in a previous study (data not shown). In each of the mixtures we used maguey bagasse fermented for 7 d and barley straw for 3 d at room temperature finding that the BE was the best for mixing agave bagasse-barley straw 1:1 ratio.

Figure 1: Maguey mushroom from Alchichica Puebla, Mexico. It can be observed that the mushroom grows on the dead leaves of the maguey ("pencas").

The production of fruiting bodies. The time between substrate inoculation to the primordia appearance in both AB-BS 1:1 and BS was 30 d, and later in the production room, the appearance of fruiting bodies took between 10 and 12 d. The highest BE recorded was obtained in the mixture AB-BS 1:1 (Table 1). This is consistent with the results obtained by mixing cereal straws with other agro-industrial wastes [19.20]. The PR is also higher in the AB-BS 1:1 mixture, although the values obtained in this research are much lower than those reported using mixtures of straw with agro-industrial waste, where the PR is 0.8 to 3.41 range [20]. With regard to crops, stocking grams obtained in each of the 4 flushes show no significant differences between the two substrates used AB-BS 1:1 and BS (Table 1).

Table 1: Productivity (g) of the strain IE-771 in two substrates

			Crops			
Substrate	BE [1]	PR [2]	1st	2nd	3rd	4th
AB-BS 1:1	64.2[a]	0.378[a]	226.1[a]	176.1[ab]	169.6[ab]	163.3[ab]
BS	45.0[b]	0.270[b]	186.8[ab]	108.3[b]	75.1[b]	130.3[b]

[1] Biological efficiency (%), fresh mushrooms (g) / dry weight of substrate (g). [2] Production Rate (%) (BE / total number of production days, from inoculation). Means in a column with different letters are significantly different ($p < 0.05$, Tukey).

For the analysis of group size, the averages were obtained by dividing the total weight of the mushrooms in a group by the total number of fungi of the same group. The results (Table 2) show no significant differences among the same group size in both substrates. We could not compare the weight of the pileus obtained because research consulted on the size measure only diameter but not the average weight per group. [21]. However, the size-weight data may prove valuable for producers looking to improve the quality of their crops or for their taxonomic identification. In addition, this mushroom has organoleptic qualities very acceptable to the consumer as the color and turgidity can be maintained under refrigeration for at least 2 weeks, its smell is pleasant, unlike those reported for European species [22].

Table 2: Average production (g) of the mushrooms by group size diameter of pileus

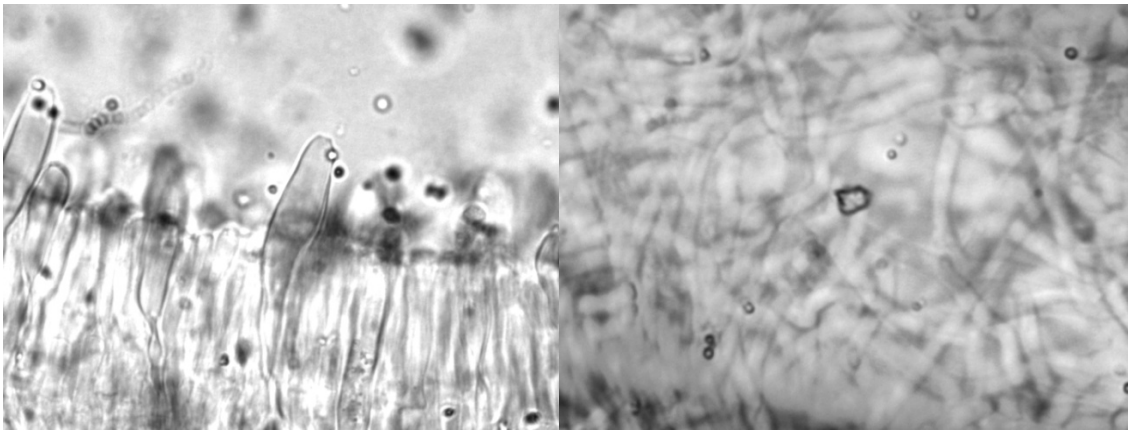
	Production by size group				
Substrate	G1(0-4.9cm) [1]	G2(5-9.9 cm)	G3(10-14.9 cm)	G4(15-19.9 cm)	G5(>20 cm)
AB-BS 1:1 [2]	9.2[d]	25.6[c]	58.5[b]	105.5[a]	181.1
BS [3]	7.5[d]	20.2[cd]	54.4[b]	97.3[a]	-------

[1] G1-G5 correspond at mean diameter of pileus. [2] Agave bagasse-barley straw 1:1. [3] Barley straw. Means in a column with different letters are significantly different ($p < 0.05$, Tukey.

Morphological identification. In the macro-morphological description, fruiting bodies grown from mycelium of the strain IE-771 obtained from a maguey mushroom were observed for the first time. Morphologically, there is a viscid pileus, flat in the center to fibrillar on the periphery, creamy yellow, clear in the center to dark on the periphery with white flakes of 10 cm in diameter. De-current white lamellae. White stipe solid to semi hollow, fleshy to leathery down, covered with fine hairs or hirsute at the base, which is a little wider, measuring 6 to 8 cm long and 1 to 1.5 cm wide (Fig. 2). Veil well developed in the primordial and there remains a delicate ring but it is thick and fibrillar in the apical part. All white (Fig. 3). Micromorphological descrpción spores were found of (12.5-) 13-14 (-15) x 3.5-4 (-5) µm, cylindrical to ellipsoid, smooth thin wall. Basidia of (40-) 42-44 (-46) x 6-7 (-8) µm, cylindrical to slightly clavate, quadrispore of thin wall (Fig.4). Cystidium elements of 42-52 x 7-7.5 µm, clavate, hyaline, thin wall, basidioles protruding slightly. Absent cheilocystidium or similar to basidioles. Hyphal system dimitic with skeletal hyphae (Fig. 5). Generative hyphae of 3.5- µm, thin wall, and occasionally branched. Scarce eskeletic hyphae, of 5 µm, thick wall. The veil has generative hyphae of 3.5-4 (-4.5) µm, hyaline, wall thickened slightly thicker. The above descriptions are consistent with *Pleurotus dryinus* (Pers.:Fr.) P. Kummer [23, 24, 25] with which different authors [23, 25] mention that the stipe is velvety with pubescent villose towards the base. *Pleurotus dryinus* has been identified in Asia (Siberia, Russia), Europe (Norway, Netherlands, Germany, Italy, Spain, Austria, Croatia, Slovakia), Africa (Egypt) and North America [22]. Recently in Georgia, Russia, *P. dryinus* has been distinguished among other white rot basidiomycetes due to the high activity of lygnocellulolytic enzymes identified in its mycelium [26].

Proximate analysis. Edible mushrooms are considered healthy foods because they are rich in protein and vitamins and low in calories and fat [27, 16]. The protein content is higher than for many vegetables, but less than in meat and milk [28]. For the present study, despite the fact that maguey mushrooms have been consumed since ancient times in Mexico collected from the wild, as yet their nutrient composition has not been studied (Table 3). In general, in the case of *Pleurotus* the protein content is reported in a range of 17.8 to 53.3%, the fat content varies from 1.1 to 11.7%, and its ash content is reported in a range from 6.7 to 15.4% [29]. It can be seen that the percentage of protein, fat and crude fiber, is within the ranges reported for this genus, but the ashes reported in this study are below the lower limit. Other authors place the range of proteins in *Pleurotus* between 18 and 35% and fat content from1 to 2.4% [30]. Based on these criteria, the fat content of the mushroom is above the upper limit. In the case of the crude fiber reported for *P. columbinus* and *P. pulmonarius* 7.57% and 11.7% are reported respectively in the fruiting bodies [15], and in our analysis there is a higher fiber content of 23.93% perhaps because we used the full mushroom and stipe was very fibrous.

Figures 2 - 3. Fruiting bodies of the strain IE-771 obtained in barley straw. **2:** Remnants of the veil on the edge of the pileus and the delicate ring on the stipe. **3:** Primordium showing the veil and the ring.



Figures 4 - 5. Microscopic characteristics of *Pleurotus dryinus* obtained from the strain IE-771. **4:** basidia and basidioles. **5:** hymenophoral trama (100 x).

Table 3: Proximal analysis in dry basis of *P. dryinus* grown in AB-BS 1:1

Proximal Analysis	
Crude Protein	27.00 %
Fat	2.45 %
Crude Fiber	23.93 %
Ash	6.13 %
Carbohidrate	40.49 %

CONCLUSIONS

Maguey mushrooms were cultivated for first time using mixtures of maguey leaves bagasse and barley straw and achieved a better BE than using barley straw only. However, PR values show very low precocity. According to the taxonomic study of strain IE-771 it was identified as *Pleurotus dryinus* (Pers.: Fr) P. Kummer, pending confirmation by molecular analysis. An proximate analysis shows that the mushroom has a high content of fiber and fat in

relation to other species of the genus. In agave bagasse-barley-straw 1:1mixture some mushrooms showed pileus of more than 20 cm in diameter.

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EFFECTIVENESS OF COMPOST TEA FROM SPENT MUSHROOM SUBSTRATE ON DRY BUBBLE (*LECANICILLIUM FUNGICOLA*)

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ABSTRACT

Dry bubble, caused by the fungus *Lecanicillium fungicola* (Preuss) Zare & W. Gams, is a serious and common disease of white button mushroom. The most widely used control method against this disease is strict hygiene in the growing installation accompanied by the application of prochloraz. However, the gradual decrease in the sensitivity of *Lecanicillium fungicola* to prochloraz [1] and the relatively short persistence of the fungicide [2] mean that alternative methods of control need to be found. Among biocontrol methods considered as an alternative to chemical products is the use of aqueous extracts (compost tea) made from agricultural wastes. Indeed, the results obtained with this method to control dry bubble have proved encouraging in *in vitro* trials [3].

Here, we study the possibility of using compost tea made with spent mushroom substrate as a biocontrol method against the disease. Its effectiveness was evaluated in a mushroom growth cycle artificially infected with *L. fungicola*. Two compost teas were tested, one obtained from spent mushroom substrate with mineral soil as casing layer and the other with peat. One, two or three drench applications with these compost teas were carried out. Three controls were used: one pure control, one inoculated with *L. fungicola*, and the other inoculated and treated with prochloraz. In all treatments the yield of mushrooms, with and without *L. fungicola*, was calculated. The application of the compost tea made from substrates using peat was the most effective. In this treatment, infected mushrooms represented 14.6 to 26.2% of the total crop, while in the control to which prochloraz was applied 35.6% of mushrooms showed signs of the disease. The best results were obtained when the compost teas were applied close to the beginning of harvesting.

Keywords: Biological control; Compost tea; Spent mushroom substrate; *Lecanicillium fungicola*; *Agaricus bisporus.*

INTRODUCTION

Verticillium fungicola (Preuss) Hassebrauk, recently classified as *Lecanicillium fungicola* (Preuss) Zare & W. Gams [4], is the causal agent of dry bubble disease and the principal micoparasitic fungus of white button mushroom [*Agaricus bisporus* (Lange) Imbach] in Spain [5]. Methods of control include the application of fungicides (prochloraz) and the observation of strict measures of hygiene. However, some data confirm that the sensitivity of *L. fungicola* to prochloraz is gradually diminishing [1], and that its persistence in the casing layer falls considerably at the end of the second flush [2]. Taken together, these observations suggest that protection against dry bubble is less than desirable. It must also be borne in mind that the voluntary withdrawal of prochloraz has been suggested during the evaluation of active substances set in motion by the EU (Directive 91/414/ECC). This implies that in the near future mushroom crops will be left without protection against dry bubble, so that present day management strategies should include reinforcing hygiene practices accompanied by a search for new control methods.

The use of water-based composts of agricultural wastes has been proposed as an alternative biocontrol to the use of chemical products in the control of foliar pathogens [6, 7]. The bibliography consulted in this respect includes mention of the use of compost teas in the fight against *L. fungicola*. For example, Dianez *et al*. (2006), obtained good results in controlling nine pathogens, among them the mycopathogen *L. fungicola* using several grape marc aerated compost teas. Gea *et al*. (2009*)* analysed the *in vitro* efficacy of several non-aerated compost teas (NACT) obtained from agricultural wasted for controlling *L. fungicola*. The results obtained provide hope that dry bubble disease can be controlled by the use of compost teas made from agricultural wasted with no pre-sterilisation step.

In this work, we present the results obtained concerning the *in vitro* efficacy of compost tea made from spent edible mushroom substrate against *L. fungicola* and concerning its use for controlling dry bubble disease in mushroom crops artificially infected with *L. fungicola*.

MATERIALS AND METHODS

***In vitro* efficacy of compost tea against *L. fungicola*.** Spent mushroom substrate (SMS) mixed with peat moss in a 1:1 (v/v) proportion was used to prepare the compost tea. The ratios of compost to water were 1:4 and 1:8 (w/v), while fermentation times were 1, 7 and 14 days. The mixtures were incubated at 20ºC without stirring and were finally filtered through two layers of muslin. Three different culture media were prepared with each of the six compost teas obtained, using 1.5% agar-water as basic medium (1:1, v/v). The first of these media was elaborated by mixing the basic medium with compost tea with no treatment (CT). The second was prepared by mixing the basic medium with the compost tea, previously autoclaved for 20 minutes at 121ºC (AT). The third medium was elaborated from the basic medium mixed with microfiltered compost tea (MT), first at 25 μm and then through Millex® 0.22 μm filters. A control with 1.5% agar-water and sterile water (1:1, v/v) and a positive control (FC) with the same agar and the fungicide prochloraz-manganese 46% WP (1:1, v/v) (Sporgon®, AgrEvo, Valencia, Spain), giving a final concentration of 50 ppm of active ingredient (a.i.), were also prepared. For each compost tea treatment and control, five Petri dishes were inoculated centrally with a 5 mm diameter mycelial disc of *L. fungicola* [7, 8]. Three different isolates of *L. fungicola* per treatment and control were used. Two perpendicular colony diameters were measured on each dish after incubation in the dark at 20ºC for 12 days. The experiments were carried out in duplicate.

The results are expressed as percentage inhibition of mycelium growth of the *L. fungicola* isolates for each of the treatments assayed with respect to mycelium growth obtained with the sterile water control. The mean values were examined using analysis of variance (ANOVA) after transformation. Significance of treatments was determined using the Tukey-HSD test ($P = 0.05$). Data were analyzed using Statgraphics® Plus v. 5.1 (Statistical Graphics Corp., Princeton, NJ).

Effectiveness of compost tea in a mushroom crop artificially infected with *Lecanicillium fungicola*. The compost tea used was made from SMS (60% *Agaricus* SMS and 40% *Pleurotus* SMS) treated with steam at 70ºC for 12 hours to eliminate pathogenic organisms. The material was then re-composted for 57 days. The ratio of compost to water was 1:4 (w/v), while fermentation time was one day. The mixture was incubated at 20ºC without stirring and was finally filtered through two layers of muslin.

A cropping trial was set up in an experimental mushroom growing room, according to standard practices used in mushroom farms in Spain. *A. bisporus* was cultivated in experimental trays (16 l in volume, 870 cm^2 in area) filled with 6 kg of commercial mushroom compost spawned at 1% (Gurelan 45 strain, Gurelan S. Coop., Huarte, Pamplona, Spain). Spawn-run took place for 15 days in a cropping room set at 25ºC and 95% relative humidity (RH). On day 0 of

the cropping cycle, trays of spawn-run compost were cased with a 30 mm layer of a casing soil made with mineral soil + *Sphagnum* peat 4:1 (v/v). One day after casing (day 1), a spore suspension of *L. fungicola* (10^6 spores ml^{-1}) was sprayed onto the surface of the casing layer (120 ml per m^2).

Two different compost teas were used: one obtained from an SMS in which mineral soil + sphagnum peat 4:1 (v/v), denominated "*mineral soil*" was used as casing layer , while the other was obtained from a substrate with a casing based on Topterra®, type peat, which we shall refer to as "*peat*". The compost teas were applied at a rate of 1.2 litres per m^2 at the following times: the first (R1) was applied with the irrigation water on the same day as casing (day 0), the second (R2) three days after casing (day 3), and the third (R3) seven days after casing (day 7). Three controls were used: one inoculated with spores of *L. fungicola*, in which irrigation was carried out with water alone (CI); another, also inoculated, but including the fungicide prochloraz at the third irrigation (P); and a pure control (C), consisting of water and no spores. A randomised complete block design with six replicates was used.

The effectiveness of the compost against *L. fungicola* was tested during the first three flushes (F1, F2 y F3), by comparing the productions of healthy mushrooms and those infected with *L. fungicola* obtained in each treatment, with the production obtained for the controls.

RESULTS AND DISCUSSION

In vitro* efficacy of compost tea against *L. fungicola. Table 1 depicts the mean percentage of mycelial growth inhibition for the three isolates of *L. fungicola* treated with compost teas and the fungicide prochloraz. The grouped data show the behaviour of the *L. fungicola* isolates in each of the three culture media prepared with compost tea (CT, AT and MT) and with the fungicide prochloraz (FC). Table 1 also shows the effect of the extraction times used (1, 7 and 14 days) to obtain the compost teas and the effect of the dilutions of compost and water on *L. fungicola* mycelium growth.

Table 1: Effects of treatments with compost teas and the fungicide prochloraz, extraction times and dilution on growth of mycelium of three isolates of *L. fungicola*.

		n	Mean ± Standard deviation[2]
Effect of treatment with compost tea and fungicide on growth of *L. fungicola*	CT[1]	360	95.74 ± 9.79 c
	AT[1]	360	11.51 ± 8.50 b
	MT[1]	358	6.99 ± 8.33 a
	FC[1]	360	95.34 ± 6.78 c
Effect of extraction times (days) on *L. fungicola* mycelium growth	1	120	100.00 ± 0.0 b
	7	120	98.94 ± 16.19 b
	14	120	88.39 ± 13.81 a
Effect of dilution on *L. fungicola* mycelium growth	1:4	180	96.12 ± 10.25
	1:8	180	95.37 ± 9.57

[1]CT: culture medium prepared with 1.5% agar-water and filtered compost tea (1:1, v/v); AT: culture medium prepared with 1.5% agar-water and compost tea autoclaved for 20' at 121ºC (1:1, v/v); MT: culture medium prepared with 1.5% agar-water and microfiltered compost tea (1:1, v/v); FC: culture medium prepared with 1.5% agar-water and prochloraz 46% in manganese complex (1:1, v/v).
[2]Meand followed by the same letter do not differ significantly ($P \leq 0.05$) according to Tukey-HSD test.

The percentage of inhibition was lowest in the microfiltered (MT) and autoclaved (AT) compost teas (7 and 15%, respectively), while the inhibition attained with filtered tea (CT) was 96%, which exceeds that obtained with the fungicide prochloraz (FC). This shows that the compost

teas obtained by autoclaving and microfiltration lose a substantial part of their activity, hence their little effect on mycelium growth, suggesting that the inhibition is produced by the presence of microorganisms in the aqueous extracts which would compete for the nutrients and space [7]. Therefore, processes like microfiltration and heat sterilisation eliminate these microorganisms from the teas, diminishing their ability to suppress the disease [6]. In contrast, Yohalem *et al*. (1994) found that aqueous extracts of spent mushroom composts fermented anaerobically maintained their inhibitory properties after autoclaving and microfiltration, even maintaining their effect on the germination of *Venturia inaequalis* conidia for at least four months when stored at a -20 ºC, at 4 ºC and at room temperature. Diánez *et al*. (2006) obtained up to 60% inhibition of *L. fungicola* using a compost tea of microfiltered grape marc incubated for one day.

As can be seen in Table 1, the best results were obtained with extraction times of 1 and 7 days, whereas Diánez *et al*. (2006) obtained 100% growth inhibition of *L. fungicola* mycelium after two week extraction. Several studies have indicated that suppression of disease varies widely with the fermentation time, increasing with fermentation times up to a maximum level, after which it decreases [6]. In this sense, Weltzien (1991) suggested that a 5-16 day extraction period is necessary to reach a given level of control over the disease, although the ideal fermentation time must be established for each host-pathogen-type compost used [6].

As regards the dilutions studied, no statistically differences were found, and in both cases high inhibition percentages were obtained. Scheuerell & Mahaffee (2002) maintained that it was unclear how the compost/water ratio influences disease suppression, and suggested a maximum dilution level of 1:10.

Effectiveness of compost tea in a mushroom crop artificially infected with *Lecanicillium fungicola*. The healthy mushroom yields obtained with the different treatments are shown in Fig. 1. As expected, the lowest yields (9.36 kg/m^2) were obtained with the control inoculated with *L. fungicola* (CI), and the highest (16.86 kg/m^2) with the pure control (C). In general it can be seen that the treatments involving compost teas based on peat provided better results (12.71-14.35 kg/m^2) than those based on mineral soil (9.77-12.32 kg/m^2). It should also be noted that the yields obtained with all the compost tea treatments based on peat and treatment 23 using mineral soil-based compost tea were higher than those obtained with the prochloraz control (11.19 kg/m^2).

Fig. 2 shows the yields of mushrooms with *L. fungicola* obtained with the different treatments. This time, the control inoculated with *L. fungicola* (CI) provided the best results (8.26 kg/m^2), while dry bubble hardly appeared in the pure control (0.1 kg/m^2). The yields of mushrooms with *L. fungicola* harvested in the treatments with peat-based compost tea (2.45-4.51 kg/m^2) were lower than those obtained with the mineral soil-based compost teas (3.80-6.18 kg/m^2). In both cases the figures are lower than those for the inoculated control. A comparison of the results obtained with compost teas and those obtained with the prochloraz treatment (P) (6.09 kg/m^2) shows that this last treatment led to a higher production of mushrooms with *L. fungicola*, with the exception of the mineral-soil based treatment R1. That is, the application of irrigation containing compost teas, especially those based on peat, controls dry bubble better than the application of the fungicide prochloraz. It can also be seen that irrigation with compost tea applied on days close to harvest (irrigations 2 and 3) favour disease control even further.

Taking into account that the phytotoxic effect of the compost teas was not very pronounced, and that the control of *L. fungicola* obtained with them was even better than that obtained using the fungicide prochloraz, such compost teas made with the substrates of edible mushrooms can be recommended as an alternative means of dry bubble disease control.

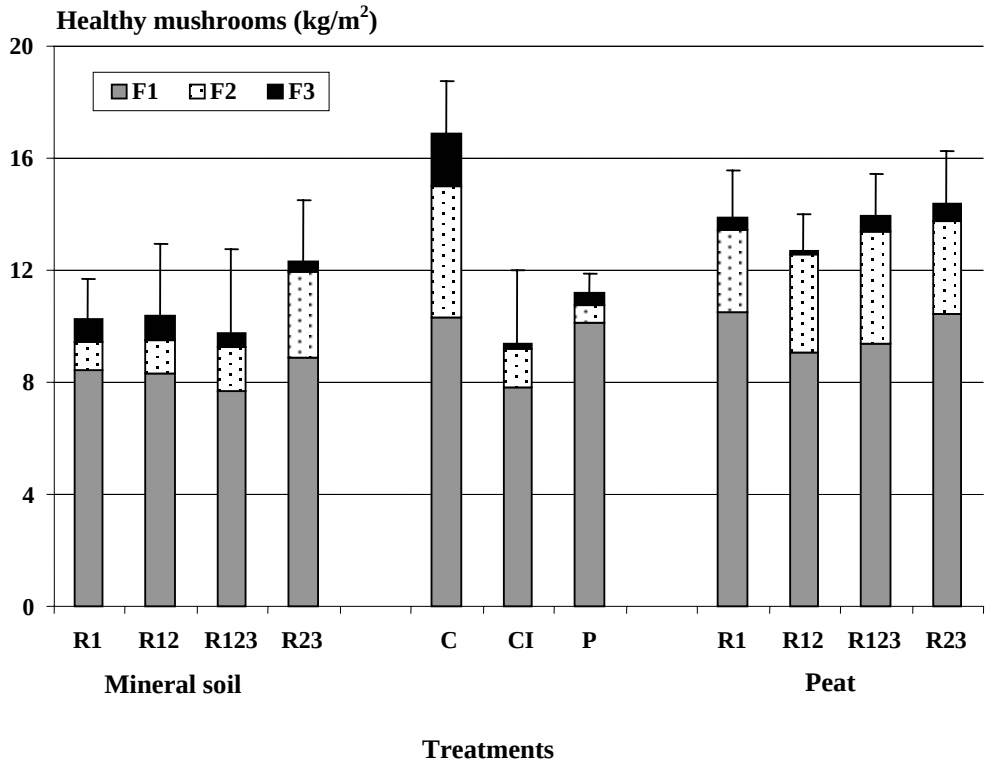

Figure 1: Healthy mushroom production for each of the treatments

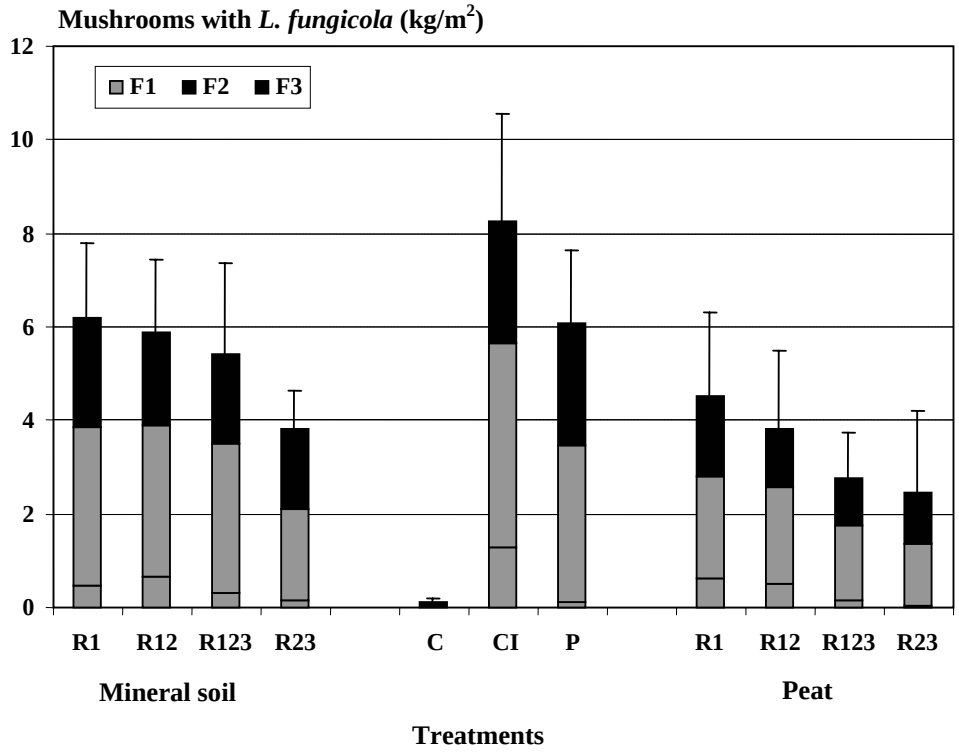

Figure 2: Production of mushrooms with *Lecanicillium fungicola* for each treatment

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TOXICITY OF COMPOST TEA FROM SPENT MUSHROOM SUBSTRATE AND SEVERAL FUNGICIDES TOWARDS *AGARICUS BISPORUS*

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ABSTRACT

The control methods used against fungal diseases in mushroom crops usually involve the application of fungicides and strict hygiene practices. The availability of fungicides within the mushroom industry is limited not only by strict regulations (Directive 91/414/ECC) but also by the fact that both pathogen and crop are fungi. The use of aqueous extracts (compost tea) made from agricultural wastes has been proposed as a potential biological control method to reduce the use of chemical products [1, 2].

In the present investigation, we evaluate the toxicity of several fungicides (carbendazim, iprodione, thiophanate-methyl, prochloraz and thiabendazole) used during mushroom production, and the resulting residue levels. The toxicity of spent mushroom compost tea, applied to the casing using different irrigation programmes, was also examined. A mushroom crop cycle was run and the yield and earliness of the crop were evaluated. All the fungicides, except thiabendazole, led to reductions in yields that varied from 4% (carbendazim) to 15% (iprodione). Higher MRL values than are permitted were detected with carbendazim, iprodione and thiophanate-methyl, while no such problem was observed with prochloraz and thiabendazole. Mushroom production fell by between 4% (one application) and 10% (three applications) when compost tea was used. Compost tea also slightly delayed the production - by one day when three applications were made. Regardless of this, the less pronounced decrease in yield and the absence of any residue-related problems suggest that compost tea is suitable for mushroom cultivation. Its effectiveness observed *in vitro* for controlling *L. fungicola* would also suggest that spent mushroom compost tea can be considered a suitable biocontrol substance for use against dry bubble disease.

Keywords: Toxicity; Fungicides; Compost tea; Biological control; *Agaricus bisporus*

INTRODUCTION

There are several reasons for using compost tea made from spent mushroom substrate (SMS) for the biological control of dry bubble disease, among them the promising results mentioned in the literature with water-based composts made from agricultural wastes, which have been proposed as an alternative to chemical products for the control of foliar pathogens [1, 3]. Favourable results have also been obtained *in vitro* with several compost teas made from agricultural wastes (SMS mixed with amended light peat, olive oil husk + cotton gin trash composted and mixed with rice husk, grape marc compost and cork compost) against the fungus *Lecanicillium fungicola* [2]. The ease with which such products can be obtained should also be taken into account as well as the revision process of active substances set in motion by the European Union,

which will inevitably lead to the withdrawal of several fungicides at present authorised for use with mushrooms.

Before using the compost tea made from SMS as biocontrol substance we evaluated its toxicity towards mushroom mycelium. A comparative analysis was also made with five fungicides, two authorised in Spain (iprodione and procloraz) for use in mushroom and three that are included in Annex I of Directive 91/414/EEC (carbendazim, thiophanate-methyl and thiabendazole). Lastly, an analysis was made of the residues found in harvested mushrooms to ascertain whether any of the fungicide treatments exceeded the permitted maximum residue limits (MRL).

MATERIALS AND METHODS

Phytotoxic effect of compost teas on mushroom mycelium. The compost tea used was made from SMS (60% *Agaricus* SMS and 40% *Pleurotus* SMS) treated with steam at 70 ºC for 12 hours to eliminate pathogenic organisms. The material was then re-composted for 57 days. Table 1 shows the physical, chemical and biological characteristics of the SMS after re-composting.

Table 1: Physical, chemical and biological characteristics of the SMS after re-composting

Parameter	Value
Bulk density $_{fresh}$ (g/ml)	0.609
Moisture (%)	50.4
pH 1:5 (w/v)	7.8
Electrical conductivity$_{25}$ 1:10 (w_{dry}/v) (µS/cm)	5,265
Nitrogen (%)	1.34
Ash (%)	64.62
Organic matter (%)	35.38
C/N ratio	15.3
Water-holding capacity (kg/kg)	1.95
Bulk density $_{dry}$ (g/ml)	0.302
Particle real density (g/ml)	2.118
Total porosity (%)	85.7
Acari	Predators
Nematodes	Saprophagous
Trichoderma	Absence

Non-aerated compost teas (NACT) and aerated compost teas (ACT) were used [1]. The ratio of SMS to water was 1:4 (w/v), while fermentation time was 1 day. The mixtures were incubated at 25 ºC with stirring (ACT) and without (NACT), and were finally filtered through two layers of muslin. The pH and electrical conductivity (EC_{25}) were measured in both compost teas with the following results: in the case of ACT, pH was 7.6, and EC_{25} was 5,190 µS/cm; for NACT, pH was 7.6, and EC_{25} was 5,245 µS/cm.

A cropping trial was set up in an experimental mushroom growing room, according to standard practices used in mushroom farms in Spain. *A. bisporus* was cultivated in experimental trays (16 l in volume, 870 cm^2 in area) filled with 6 kg of commercial mushroom compost spawned at 1% (Gurelan 45 strain, Gurelan S. Coop., Huarte, Pamplona, Spain). Spawn-run took

place for 15 days in a cropping room set at 25 ºC and 95% relative humidity (RH). On day 0 of the cropping cycle, trays of spawn-run compost were cased with a 30 mm layer of a casing soil made with mineral soil + sphagnum peat 4:1 (v/v). The compost tea was applied to the casing mixture at 100 ml per tray. Three different treatments were made with each compost tea (ACT and NACT) – 1R (one drench application on the same day as the casing material was applied, day 0); 2R (two drench applications, days 0 and 2) and 3R (three drench applications, days 0, 2 and 6). A control treatment was irrigated with water alone. A randomised complete block design with five replicates was used.

The phytotoxicity of the three tea treatments was evaluated during the first three flushes (F1, F2 y F3) by comparing the yield with that obtained in the control. In addition, the earliness of each treatment was assessed, and expressed as the number of days between casing and harvesting of the first flush.

Phytotoxic effect of different fungicides on mushroom mycelium. The phytotoxicity of the five fungicides was assessed in a crop cycle during which the chemicals were applied as shown in Table 2: (I) with the first irrigation water (day 0) or (II) with the second irrigation water (day 5), with 100 ml per tray, using the same volume of water in the control. The cultivation conditions were the same as those described above. A randomised complete block design with five replicates was used.

Table 2: Fungicides and doses used

Commercial name	Active substance	Dose
Bavisfor 50 (IQV)	Carbendazim 50% WP	0.1%
Rovral wp (AGRODAN)	Iprodione 50% WP	0.1%
Topsin 70 wg (BAYER)	Thiophanate-methyl 70% WG	0.1%
Sporgon (BASF)	Prochloraz 46% WP	0.05%
Textar 60t (TECNIDEX)	Thiabendazole 60% SC	0.1%

The phytotoxicity was evaluated as in the previous experiment, based on yield and earliness. Fungicide residues were also analysed in the mushrooms from the first two flushes: iprodione residues were determined by gas chromatography, and the other fungicides by liquid chromatography.

Statistical analysis. The data obtained were evaluated by analysis of variance using the statistical package Statgraphics Plus v. 4.1. A Tukey test was used to establish significant differences between means, Tukey-HSD (p=0.05).

RESULTS AND DISCUSSION

Phytotoxic effect of the compost teas on mushroom mycelium. Yield and earliness data for the different treatments are shown in Table 3. As can be seen, there was a small reduction in the yield (3-4%) in the trays which were irrigated once with compost teas. This decrease increased with the number of times the teas were applied, reaching 10% of the yield with three applications of aerated compost teas. This may be related with the high EC_{25} of the teas, which may increase

the conductivity of the casing layer, hindering mushroom fructification [4]. However, the statistical analysis of the data pointed to no significant differences between the treatments.

Table 3: Total mushroom yield, in kg/m^2 (mean value ± SD) and percentage compared with the control, and earliness (mean value ± SD) for each of the compost tea treatments

Treatment		Yield (kg/m^2)	%	Earliness (days)	
Control		20.00 ± 1.80	100	21.07 ± 0.34	a*
	1R	19.20 ± 2.55	96.00	21.86 ± 0.48	b
ACT	2R	18.60 ± 1.59	93.00	21.87 ± 0.58	b
	3R	17.97 ± 1.30	89.85	22.08 ± 0.64	bc
	1R	19.42 ± 2.26	97.10	22.41 ± 0.44	bc
NACT	2R	18.23 ± 1.87	91.15	22.46 ± 0.28	bc
	3R	18.87 ± 1.83	94.35	22.50 ± 0.22	c
		p = 0.4963		p = 0.0007	

*Different letters indicate significant differences ($p < 0.05$) between means.

As regards earliness, there was a slight delay in the harvest of the first flush compared with the control in all treatments using compost teas, regardless of the number of applications. This delay was slightly longer with the NACT extract (1.2-1.4 days) than with ACT (0.8-1 day).

Phytotoxic effect of the fungicides on mushroom mycelium; analysis of residues. Table 4 shows the yield and earliness data for the different fungicidal treatments carried out. Statistical analysis of the production data points to statistically significant differences between treatments.

Table 4: Total mushroom yield, in kg/m^2 (mean value ± SD) and percentage compared with the control, and earliness (mean value ± SD) for each of the fungicide treatments

Treatment	Yield (kg/m^2)		%	Earliness (days)	
Control	22.79 ± 1.41	bc*	100	20.81 ± 0.45	a*
Carbendazim – I	21.99 ± 0.96	abc	96.49	21.85 ± 0.76	bcd
Iprodione – I	19.25 ± 1.70	a	84.47	23.12 ± 0.83	f
Thiophanate-methyl – I	20.48 ± 1.68	ab	89.86	23.21 ± 0.48	f
Prochloraz – I	20.58 ± 2.13	ab	90.30	21.87 ± 0.71	bcd
Thiabendazole – I	23.91 ± 0.90	c	> 100	21.95 ± 0.74	bcd
Carbendazim – II	20.83 ± 7.91	abc	91.40	22.18 ± 0.66	cde
Iprodione – II	19.29 ± 1.63	a	84.64	22.61 ± 0.71	def
Thiophanate-methyl – II	20.48 ± 2.15	ab	89.86	22.85 ± 0.96	ef
Prochloraz – II	20.00 ± 1.44	ab	87.76	22.70 ± 0.87	def
Thiabendazole – II	22.94 ± 2.18	bc	> 100	21.68 ± 0.94	abc
	p = 0.0005			p = 0.0005	

* Different letters indicate significant differences ($p < 0.05$) between means.

Of particular note is the decreased yield obtained with iprodione for either time of application, with decreases around 15% in both cases. Decreases of around 10% were obtained with thiophanate-methyl and prochloraz, also regardless of the moment of application. Similar values were obtained with the trays treated with carbendazime at the second irrigation time, while the same substance led to a 5% decrease when applied with the first irrigation water. Lastly, the application of thiabendazole did not lead to production losses regardless of application time; in fact, the yields obtained were slightly higher than with the control for both application times.

Earliness showed a similar trend, with iprodione and thiophanate-methyl delaying the beginning of harvesting by up to 2.5 days, while thiabendazole led to a shorter delay (of hardly one day). Carbendazim and prochloraz led to an intermediate behaviour, the former leading to a delay of one day and the latter to a delay of 2 days.

The results obtained for the analysis of the different active substances in the mushrooms harvested from the first two flushes are shown in Table 5. It should be noted that thiophanate-methyl decomposes into carbendazim, so that an analysis of both active compounds was made in this case. The results show that the MRL was exceeded in all the treatments involving iprodione, carbendazim and thiophanate-methyl, especially when applied at the second irrigation time. However, the application of prochloraz and thiabendazole supposed no problem residue when applied at the times and doses specified herein.

Table 5: Fungicide residues (ppm) detected in mushrooms at the first two flushes

Treatment	MRL	First flush	Second flush
Carbendazim – I	0.10	0.42	0.14
Iprodione – I	0.02	0.03	0.06
Thiophanate-methyl – I*	0.10	< 0.10 (0.34)	< 0.10 (0.36)
Prochloraz – I	2.00	< 0.05	< 0.05
Thiabendazole – I	10.00	1.01	1.44
Carbendazim – II	0.10	0.88	0.84
Iprodione – II	0.02	0.18	0.28
Thiophanate-methyl – II*	0.10	< 0.10 (0.37)	< 0.10 (0.86)
Prochloraz – II	2.00	< 0.05	< 0.05
Thiabendazole – II	10.00	0.87	1.08

* Carbendazim residues were also analysed (in parentheses).

CONCLUSIONS

The results obtained in the analysis of residues rule out the application of the fungicides iprodione, thiophanate-methyl and carbendazim at the doses and application times specified in the text. In the case of iprodione and, to a lesser extent, thiophanate-methyl, this conclusion was lent weight by the fall in production recorded. The other two fungicides used, prochloraz and thiabendazole, showed no residue problems and, in the latter's case, no effect on production was noted, although it fell by 10% when prochloraz was used. In contrast, the application of compost teas led to falls in production of less than 10% regardless of the number of applications. However, there were no associated residue problems.

Therefore, the less pronounced decrease in yields recorded with compost teas, the absence of residue problems and the *in vitro* efficacy observed for the control of *L. fungicola* [2] suggest that they may be considered as a biological alternative for the integrated control of this disease.

ACKNOWLEDGEMENT
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COOKING GOD'S OWN FOOD IN GOD'S OWN COUNTRY – THE EASY WAY

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ABSTRACT

Mushrooms are the most exquisite of the gastronomic treasures of the world and were in use for edible purpose from time immemorial. With more than 38,000 varieties mushrooms are popular not only in restaurants but in homes too. Each locality is blessed with a natural mushroom flora which varies according to climate vegetation etc. However in the world market the most important mushroom is the button mushroom. But owing to the sophisticated technology and cost of cultivation the more popular ones cultivated are the oyster mushrooms and the tropical milky mushrooms. Mushrooms are best when consumed fresh. Most of the recipes can be prepared with any of the edible varieties but the recipes presented here are prepared using oyster mushrooms (*Pleurotus*), milky mushrooms (*Calocybe*), Jew's ear mushrooms (*Auricularia*) and button mushrooms (*Agaricus* sp.).

A variety of soups can be prepared with oyster mushrooms. Soups can be plain mushroom soup or sweet corn mushroom soup, tomato mushroom soup, cauliflower mushroom soup etc. It can also be blended with the mixed vegetable stock or chicken stock to suit the occasion.

A variety of snacks like burger, cutlets, fritters etc. can be prepared with milky and oyster mushrooms. Mushroom preparations also serve as excellent main dishes and side dishes. Mushrooms can also be cooked with fish and meat products. The oyster mushrooms and milky mushrooms are best for these preparations.

Jew's ear mushrooms are excellent for wine preparations.

The main ingredients and easy method of preparation of more than a dozen recipes with superb illustrations are dealt with in this paper.

Key words : Oyster mushroom; soups; Jew's ear mushroom; milky mushroom; button mushroom.

INTRODUCTION

Kerala State is blessed with a diversity of climate and is rich in its natural flora and diversity of crops cultivated. Mushrooms are known for their high quality protein and the same in fresh mushrooms is twice as high as that of vegetables [3]. Modern mushroom cultivation produces more protein per unit area and gives higher productivity than any other form of agriculture.

The State of Kerala exudes a rustic charm and is characterized by elegant coconut palms and lusting paddy fields. The state has a captivating variety of back waters and lakes. Kerala - Gods Own Country enjoys unique geographical features, an equable climate, long shoreline with serene beaches, tranquil stretches of emerald backwaters, lush hill stations and exotic wildlife,

waterfalls, sprawling plantations and paddy fields, enchanting art forms, magical festivals, historic and cultural monuments.

Though Kerala is one of the small states in the country there prevail all types of climate at any time in the year. The cultivation of the oyster mushrooms has gained much popularity because it can be grown round the year at any place in Kerala. Milky mushrooms are popularly known as the summer edible mushroom. Recently the medicinal mushroom *Auricularia* is gaining popularity.

Use of mushrooms as food and medicine have been found in the inscriptions, sculptures and scriptures of many ancient civilization. Mushrooms were believed by the Greeks to give strength to their warriors while Romans regarded them as the "Food for the Gods" [1]. Undoubtedly mushrooms are basically consumed for their texture flavour and aroma.

It was the French during the reign of Louis XIV who first started cultivating mushrooms systematically. Even before that people who lived in caves used to collect mushrooms from the wild and consume them. But collection from the wild often lead to poisoning owing to their inability to distinguish the poisonous ones from the edible varieties [2]. But now with the availability of choice species vulnerable for cultivation mushrooms which were once considered as a delicacy or as the food of gods has now become the food of common man.

The protein in mushroom is considered to be of high quality containing all the essential amino acids required for an adult individual. Some of the amino acids like tryptophan and lysine which are absent in vegetable protein are present in mushroom protein. They are also known to be excellent sources of riboflavin nicotinic acid and pantothenic acid. Minerals such as calcium phosphorus and potassium are present along with a fair quantity of copper and iron all of which are essential for the formation of bones, teeth and also good for eye sight [7].

Mushrooms blend well with most of the vegetables and spices to form delicious items of food [4], [5]. Salads, soups, snacks, main dishes, side dishes, pickles and even sweet preparations are possible with mushroom. It can also be sun dried and stored or marketed as mushroom powder. In India mushroom dishes can be divided into North Indian dishes and South Indian dishes [8]. North Indian dishes are mostly prepared with button mushrooms while in South India oyster mushrooms are preferred.

MATERIALS AND METHODS

The oyster mushrooms used for the study were procured from the All India Co-ordinated Research Project on Mushroom laboratory of the Institute at the Vellayani Campus. Milky mushrooms and *Auricularia* were collected fresh from the fields as well as from experimental beds. The button mushrooms were bought from the local traders. The mushrooms were thoroughly washed in running water before preparation of recipes. For certain recipes mushrooms were used as such but for others it was sliced before use.

The following dishes were prepared using Oyster mushrooms (*Pleurotus* sp.), Button mushrooms (*Agaricus* sp.), Jew's ear mushrooms (*Auricularia* sp.) and Milky mushroom (*Calocybe* sp.) [6], [10].

The ingredients and method of preparation are dealt with in detail.

Recipe 1. SWEET CORN MUSHROOM SOUP

Ingredients

Mushrooms	-	200 g
Shallots	-	3 g
Butter	-	30 g

Corn flour	-	1 tsp.
Ground Cardamom	-	¼ tsp.
Ground pepper	-	¼ tsp.
Milk	-	2 cups
Egg	-	1
Salt	-	to taste

Method

Melt butter fry onions and chopped mushrooms mix the corn flour in two cups of mil and boil for ten minutes in shallow pan. Before removing from fire add beaten eggs white sprinkle pepper powder, cardamom, salt and serve hot.

Recipe 2. MUSHROOM VADA

Ingredients

Oyster mushrooms	-	200 g
Channa dal	-	100 g
Shallots (shredded)	-	50 g
Chilli powder	-	2 tsp.
Ginger shredded	-	1 tsp.
Asafoetida powder	-	¼ tsp.
Salt to taste		
Oil for deep frying		

Method

Soak the channa dal for 6 hours. Wash the mushrooms, drain and remove the stipe from the pileus. Grind the dal very coarsely. Mix in the chilli powder, shallots, ginger, asafoetida powder along with a pinch of salt. Uniformly coat the surface of the mushrooms with this mixture and deep fry in oil.

Recipe 3. MUSHROOM BIRIYANI

Ingredients

Long grained fine rice	-	1 cup
Mushrooms sliced	-	1 cup
Ghee	-	50 g
Onion	-	2
Capsicum	-	2
Chilli powder	-	1 tsp.
Coriander powder	-	1 tsp.
Turmeric powder	-	a pinch
Ground masala paste (cinnamon: clove : cardamom 1:2:2)	-	1 tsp.
Pepper	-	½ tsp.
Salt to taste		

Method

Wash the rice in water, add 3 pieces each of cinnamon, cardamom and clove with 2 cups of water and salt.

Pressure cook for 15 minutes and keep aside. Fry the onions, capsicum, add the mushrooms marinated with turmeric powder, chilly powder, coriander powder and ground masala paste. Sprinkle water, salt to taste and cook for 5 minutes. Mix with the cooked rice, garnish with fried onions and nuts bake and serve hot.

Recipe 4. SIMPLE MUSHROOM STEW

Ingredients

Button mushrooms	-	200 g
Carrot (cooked)	-	100 g
Peas (boiled)	-	100 g
Potato (cooked)	-	100 g
Ginger grated	-	1 tsp.
Green chillies	-	2
Onion sliced	-	2
Turmeric powder	-	a pinch
Masala powder (cinnamon:clove:cardamom 1:2:2)	-	1 tsp.
Oil	-	2 tsp.
Coconut milk (first extract and second extracts)	-	2 cups each
Salt	-	to taste
Curry leaves	-	a few

Method

Wash and chop the mushrooms. Heat oil in a pan, add the mustard seeds, and curry leaves. After the mustard seeds splitter, saute the onions, green chillies and grated ginger.

Add the chopped mushrooms, turmeric powder salt and bring to boil in 2 cups of second extract of coconut milk. When it boils add masala, potatoes, carrot and peas. Add the remaining two cups of first extract of coconut milk and simmer in moderate heat for 5 minutes.

Recipe 5. MUSHROOM MUSSEL FRY

Ingredients

Mushrooms (thickly sliced)	-	200 g
Mussels (Medium sized)	-	25 nos.
Ginger	-	2 pieces of 1" size
Garlic	-	6 flakes
Pepper	-	½ tsp.
Chilli powder	-	2 tsp.

Onion	-	2
Coriander powder	-	1 tbsp.
Salt to taste		

Method

Wash and scrub the mussels. Boil in water for 10 minutes. Discard the shells removing the beards attached to the flesh and keep aside. Put ginger and garlic and blend fairly in 200 ml of water. Heat oil, saute onions till translucent add the paste from the blender along with pepper powder, chilli and coriander powder. Stir fry for 3 minutes. Add the mushroom and mussels. Mix well and bring to boil. Lower heat and let mussels and mushrooms steam for 15 minutes. Serve immediately while hot.

Recipe 6. HONEY COATED MUSHROOM DOUGH NUTS

Ingredients

Mushroom powder	-	100 g
Rice flour	-	50 g
Honey	-	100 ml.
Grated coconut	-	100 g
Molasses	-	100 g

Method

Fry the mushroom powder and rice flour along with grated coconut in low heat. Mix well with molasses, make into balls and smear with honey to make delicious dough nuts.

Recipe 7. AURICULARIA WINE

Mushroom (Auricularia)	-	500 g
Sugar	-	500 g
Wheat kernels	-	100 g

Method

Wash clean the mushrooms in running water, allow to dry and slice. Arrange one third of mushrooms in the bottom layer of a clean glass jar. Sprinkle the sugar over the top along with wheat kernels. Then arrange the next lot over the first one sprinkle sugar, wheat kernels. For first 15 days allow for aerobic fermentation then tightly close the lid keep undisturbed for a month. Then strain the contents into clean bottle and serve as such.

Recipe 8. AURICULARIA FRITTERS

Ingredients

Fresh Mushrooms (Auricularia)	-	500 g
Besan flour	-	300 g
Chilly powder	-	3 tsp.
Asafoetida powder	-	1 tsp.

Oil - 350 ml.
Salt to taste

Method

Wash the mushrooms as such and leave to drain off water. Make a thick paste with besan, water, chilly powder, asafoetida powder and a pinch of salt. Dip the mushrooms in the batter one by one to get a smooth cooking. Deep fry till golden brown.

RESULTS AND DISCUSSION

About 70 different recipes have been standardized at the College of Agriculture, Vellayani, Kerala, India. Most of the recipes have been prepared with oyster mushrooms and a few recipes with button mushrooms, milky mushrooms and Jew's ear mushrooms.

The feasibility of Auricularia in recipes was explored during a post graduate study and it gave tremendous result. "Auricularia wine" and "Auricularia fritters" were excellent preparations. It has good keeping quality too. Sale of value added products like soup powder and pickles are also gaining popularity.

Lip smacking soups can be prepared using fresh and dried mushrooms [12]. Soups can be either plain mushroom soup or mixed with other vegetables like tomato, cauliflower etc. Ready to use soup mix is being standardized which enable us to prepare the soup in a couple of minutes. The recipe of the soup given here is very simple and at the same time very tasty.

Sweet corn Mushroom soup

Mushroom Vada

Mushroom stew

Mushroom macaroni curry

Mushroom mussel fry

Mushroom mutton mince

Mushroom kofta curry

Mushroom peas masala

Table 1: Composition of cultivated mushrooms and some common vegetables

Name	Calories	Moisture	Protein	Fat	Carbohydrate	Protein % (dry wt.)
Greenpeas	98	74.3	6.7	0.4	17.7	26.1
Beans	35	88.9	2.4	0.2	7.7	21.6
Cauliflower	25	91.7	2.4	0.2	4.9	28.8
Potato	83	73.8	2.0	0.1	19.1	7.6
Beet root	42	87.6	1.6	0.1	9.6	12.9
Celery	18	93.7	1.3	0.2	3.7	20.6
Mushroom	16	91.1	2.4	0.3	4.0	26.9

A large variety of snacks can be prepared using Oyster mushrooms. These are very easy to prepare and also affordable for the common people.

Mushrooms go well with fish too. A variety of main dishes and side dishes can be prepared using them. “Mushroom shark relish”, “Mushroom roe delight”, Mushroom mussel fry” etc. are very tempting dishes. Mushroom curry along with boiled tapioca is an excellent dish to be served for lunch.

Egg savouries using mushrooms are quite common in India. Eggs have been found to enhance the taste of mushrooms. “Mushroom mint omlette”, “Mushroom egg curry” etc. are very delicious milky mushroom preparations [11].

As part of the technology transfer, mushroom trainings were imparted to beneficiaries identified by the Centre. During the trainings various preparations of mushrooms were consumed by trainees and based on the characteristics such as appearance, colour, flavour, taste, texture and overall acceptability they are graded. The best recipes selected by them are

promoted. Some of the recipes narrated here are the ones selected exclusively for their taste and overall acceptability. Besides some of the recipes displayed in the poster have won prizes at competitions sponsored by private food marketing and non governmental organizations (NGO's).

CONCLUSION

The changing food habit, increasing health consciousness have led to a gradual increase in demand of fresh and processed mushrooms among urban and rural consumers. However such products must suit the taste and choice of common buyers. The mushroom recipes have always been a gourmet's delight. It not only provides delicious dishes but also a very balanced cholesterol free and almost fat free items. Thus there remain ample scope for popularizing mushroom cultivation and development of mushroom based recipes in God's own country.

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Use of electron microscopy to study the basidiospore production in *Agaricus brasiliensis*

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INTRODUCTION

Agaricus brasiliensis ss. Heinemann is a medicinal mushroom native to Brazil. It was identified at first as *A. blazei* Murrill but, after that, it was recognized as *A. brasiliensis* or *A. subrufescens* (Wasser et al., 2002; Kerrigan, 2005). A former study has shown this mushroom species doesn't show a classical dikaryotic stage during its life cycle (Dias et al., 2008) and its life cycle remains unclear.

OBJECTIVES

The objective of this work was to study the basidiosporogenesis and the intraspecific variation in the number of basidiospores produced per basidium in *A. brasiliensis* using transmission (TEM) and scanning electron microscopy (SEM).

MATERIALS AND METHODS

The biological material corresponded to fresh basidiocarps resulting from the fructification of *Agaricus brasiliensis* strains CS1, CS2, CS7, CS9 and CS10. A standard protocol for electron transmission microscopy and for scanning electron microscopy was used (Bossola and Russell, 1999).

RESULTS

Basidium and basidiospore development occurs asynchronously during basidiocarp production.

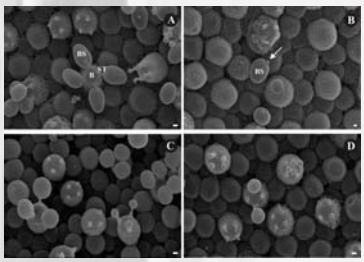

Figure 1. A. Basidiospores (BS) linked to basidium (B) through sterigmata (ST). B. Free basidiospore showing the hilar appendix (arrow). C. Asynchronous development of basidia and basidiospores in the same gill region. D. Different basidiospores at distinct developmental stages in the same basidium. Bars: A = 1 µm; B = 2 µm; C-D = 1 µm.

The basidiospores are generally produced free, but may also be enveloped by an extracellular matrix.

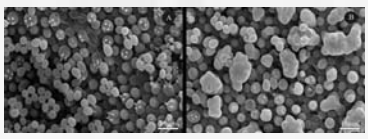

Figure 2. A. Free basidiospores. B. Basidiospores of strain CS2 involved by an extracellular matrix with unknown chemical composition. Bars: A-B = 10 µm.

The basidiospore cell wall is composed of three distinct layers.

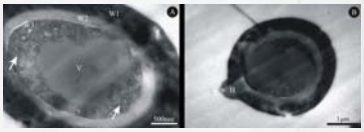

Figure 3. A. Basidiospores hydrolyzed and fixed in Karnovsky's showing three-layered cell wall (W1, W2 e W3) and vacuole (V). B. Basidiospore hydrolyzed and fixed in Karnovsky's showing the hilar appendix (H). Bars: A = 500 nm; B = 1 µm.

The conspicuous presence of lipid bodies was also observed in the fungal basidiospores, while nuclei, mitochondria, vacuoles, and dolipore septa could only be visualized in the basidia.

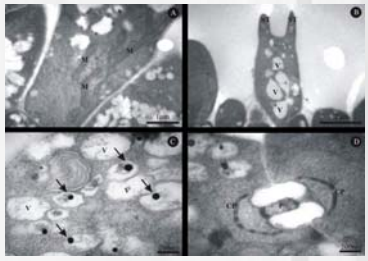

Figure 4. A. Mitochondria (M). B. Sterigmata (ST) and vacuoles (V). C. Spheric bodies indicated by arrows. D. Dolipore septum, formed by pore (P) and pore cover (PC). Bars: A = 1 µm; B = 2 µm; C-D = 200 nm.

The presence of connection hyphae linking basidia was observed for the first time in *A. brasiliensis*.

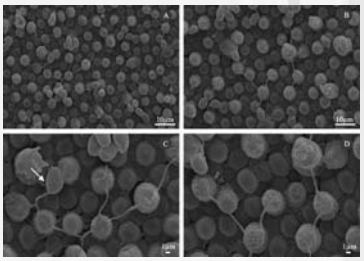

Figure 5. A-B-C-D. Basidia linked by connection of hyphae. The arrow indicates a basidiospore linked to a connection hypha, Bars: A-B = 10 µm; C-D = 1 µm.

A. brasiliensis produces predominantly tetrasporic basidia, but this trait may vary depending on the strain in question. For certain strain, such as CS2 and CS7, the frequency of bisporic and trisporic basidia was similar or higher than that of tetrasporic ones.

CONCLUSIONS

The results indicating that *A. brasiliensis* is amphithallic, however, this behavior is variable and depends on the strain in question.

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Detection and comparative expression of *028-1* (thermotolerance) and *HspA* (heat shock protein) genes in *Agaricus bisporus* strains adapted to high temperatures

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Current cultivars of *Agaricus bisporus* require for a temperature of 23 - 25°C for stubstratum colonization by the mycelium and fruit optimally at 16 to 18°C. Some wild strains were shown to be able to produce fruiting bodies at temperatures up to 25°C (FHT+ strains). Generally, a temperature over 32°C stops mycelial growth and fruiting [1]. To study the genetic basis of thermotolerance and its role in thermal adaptation of *A. bisporus* var. *bisporus* (a few percentage of strains are able to fruit at 25°C), var. *burnettii* (100 % fruit at 25°C) and var. *eurotetrasporus* (no strain fruit at 25°C), the expression levels of two genes (*hspA*, *028-1*) were measured using quantitative PCR. The gene *028-1* was shown to be involved in thermotolerance of vegetative mycelium [2] and *hspA* is a gene of heat shock protein which level of expression in primordia has been correlated with the resistance to the pathogen *Lecanicillium fungicola* [3].

Materials and Methods

***Agaricus bisporus* primordia and fruiting bodies.** Ten strains from CGAB (INRA, France) (Table 1) representative of variety *bisporus* and variety *burnettii* and collected under tropical, temperate and cold climates in America and Europe were selected for previous work [1]. Spawn was prepared on cooked rye grains purchased by Euromycel (France). Cultivation was performed in 0.09 m² crates filled with 8 Kg of compost spawned at 0.8 % [3], 4 replications. Incubation was for 13 days at 25°C. After casing and post incubation for 7 days, half of the trays were moved in a climatic room regulated at 17°C. Primordia and fruiting bodies produced at 17°C and 25 °C were harvested (Fig.1) . Gills were removed and the samples stored at -80°C until RNA extraction.

Fig.1: Primordia (1) and fruiting bodies (2)

Heat shock on vegetative mycelium. Five wild strains from CGAB (INRA, France) were selected: 3 FHT+ (table 1) and 2 FHT- (Bs0261, Bs0679). They were grown in liquid sterile Cristomalt agar medium (barley malt powder, Dif.A1) for 14 days at 25 °C in darkness. Then replicates were divided into two groups. One group was continuously cultivated at 25 °C (control treatment), and the other one at 32 °C for 12 h to potentially induce expression of the thermotolerance and heat shock genes. The mycelium was harvested directly with a spatula, deposited in tubes of 2 ml and frozen at -80°C until RNA extraction and purification.

RNA extraction and Q-PCR. The frozen samples were ground in liquid nitrogen and total RNA extracted with Qiashredder™ and RNeasy® Minikits. M-MLV Reverse Transcriptase Kit (Invitrogene) was used to obtain cDNA from mRNA in 800 ng of total RNA. qPCR were performed using a Thermocycler 1.5 Q-PCR (Roche Diagnostic) and the LightCycler® FastStart DNA MasterPLUS SYBR Green I kit (Roche). *EF1-α* was used as housekeeping gene. Standard curves were obtained for each of the primer pairs (Table 2), Pair wise fixed reallocation randomization test on *Ct* values was used to compare the level of gene expression in the two treatments with $p \leq 0{,}001$ using REST-384® software.

Table 1: *Agaricus bisporus* strains FHT+

Strains	Variety	Yield (kg/ 100 kg) at 17°C	Yield (kg/ 100 kg) at 25°C
Bs0094	*burnettii*	28.0	31.4
Bs0190	*bisporus*	21.0	16.3
Bs0419B	*bisporus*	18.0	15.7
Bs0470	*bisporus*	19.2	4.3
Bs0483	*bisporus*	16.4	16.0
Bs0571	*bisporus*	14.6	18.7
Bs0661	*bisporus*	19.5	10.7
Bs0705*	*bisporus*	12.7	5.9
Bs0738*	*burnettii*	26.4	30.4
Bs0739*	*bisporus*	20.3	1.9

* Used for high temperature treatment on mycelium

Table 2: Primers for Q-PCR

Genes	Primers	
	Code	Sequence
EF1-α	EF3L	TGGTCGTGTTGAGACTGGTA
	EF2R	GGGTCGTTCTTGGAATCAGA
HspA	HS3L	CTCCAGTGTCACACAGAC
	HS4R	ATCCTTCAAGACCTTCTC
028-1	TH5L	AAGTGCGATGGTACCAAGGT
	TH5R	GGATATGGAACTCCACAGCG

Results and Discussion

Relative gene expression in primordia and sporophores at both temperatures. At 17°C the level of *hspA* transcript was significantly higher in primordia than in sporophores for 8 strains (ratios from 2.9 to 8.5) whilst there was no significant difference between the two development stages for Bs0094 (var. *burnettii*) and Bs0483 (var. *bisporus)*. At 25°C, primordia and sporophores did not differ in *hspA* expression for the two var. *burnettii* strains and Bs0419B whilst the other strains showed higher transcript levels of *hspA* in primordia (ratios 1.9 – 14.6). Differences in *028-1* expression were low (ratios 1.1 - 1.9 at 17°C, and 1.2 -2.4 at 25°C).

Relative gene expression in primordia obtained at 25°C *vs* 17°C. Differential expression of the *028-1* gene was significant in 4 strains, but with a notable ratio (6.5 fold) in only Bs0094 (Fig. 2A). The expression level of *hspA* was significantly lower at 25°C than at 17°C in 4 strains. Neither the mushroom yield at 25°C nor the variety correlated (p=0.05) with the variation in *028-1* or *hspA* expression with the fruiting temperature.

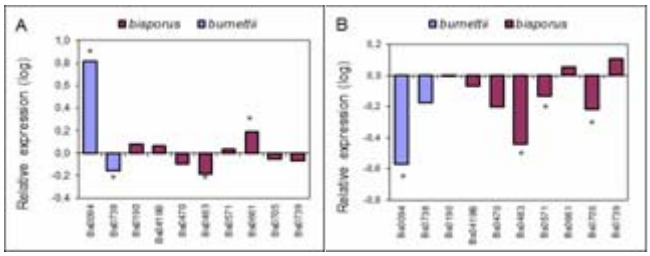

Fig.2: Relative expression of *028-1* (A) and *hspA* genes (B) in primordia, 25°C vs 17°C.

Effect of high temperature on vegetative mycelium. No significant regulation of both *028-1* and *hspA* genes was observed in the mycelium of the FHT- strains Bs0261 and Bs0679 after 12 h treatment at 32 °C (not shown). In contrast, both genes were significantly up-regulated in the mycelium of the three FHT+ strains but with lower ratios for *hspA* (Fig. 3).

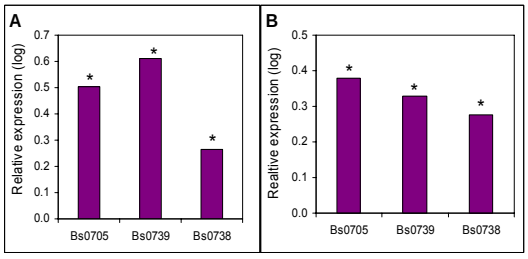

Fig.3: Relative expression of *028-1* (A) and *hspA* genes (B) in mycelium, 32°C for 12 h vs control.

Conclusion. The up-regulation of the *hspA* gene in primordia compared to sporophores was dependent neither on the fruiting temperature nor on the mushroom variety. Expression of the *028-1* gene varied slightly during mushroom maturation (sporophore vs primordium) at both temperatures. Regulations of *hspA* and *028-1* observed in primordia cultivated at high temperature did not correlate with mushroom yield showing that the genes seem not involved in the ability of *A. bisporus* to fruit at 25 °C. An increase of temperature to 32°C during incubation phase at 25 °C separated FHT+ and FHT- strains for *hspA* and *028-1* expression, but more individuals must be studied before to confirm the different response of FHT+ and FHT- strains and to conclude that both genes are involved, directly or not, in the adaptation of mycelium of FHT+ strains to high temperature.

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β-GLUCAN CONTENT FROM *Agaricus brasiliensis* CULTIVATED ON DIFFERENT SUCROSE CONCENTRATIONS MEDIUMS

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INTRODUCTION

The *Agaricus brasiliensis* has become an interesting mushroom for the scientific community attention due to its medicinal properties, specially its anti-tumor activity. The analysis of these properties has revealed that polysaccharides with β-(1-6) links called β-D-glucans in complexes with proteins corresponded to the fraction of higher anti-tumor activity.

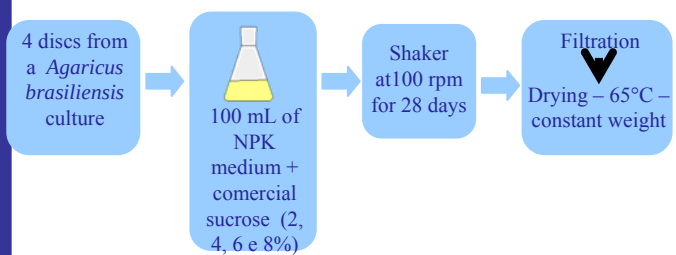
Although recent researches have been focusing the *A. brasiliensis* fruiting body, the mycelium cultivation has been revealed to be a strong source of antitumor substances. So it is necessary to establish the better and low cost cultivation conditions using alternative compounds as minerals and carbon sources, making sure that these conditions will not prejudice or decrease the production of the substances of interest.

OBJECTIVE

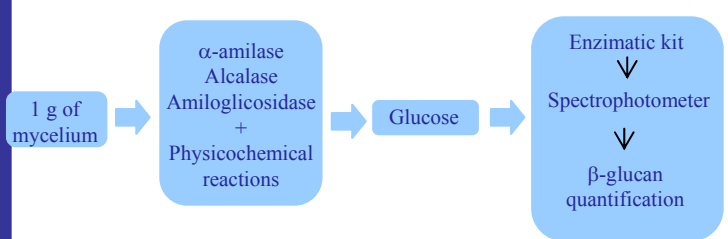
Determining the mycelia mass production and the β-glucan content into the dry mycelium of *Agaricus brasiliensis* cultivated in NPK medium with five different sucrose concentrations.

MATERIALS AND METHODS

• Mycelial mass production



• Extraction and analisys of the β-glucan content



Support: FAPEMIG, CNPq, CAPES

RESULTS

• The averages of the β-glucan content (g/100g) were compared by the Scott-Knott test at 5% of probability using the software Sisvar UFLA (Table 1).

Table 1 The β-glucan average content (g/100g) in each treatment.

Sucrose (%)	Dry weight (average) g/L	β-glucan (g/100g)
2	3,36	8.605250 a1
4	5,20	8.017000 a1
6	6,33	7.719750 a1
8	13,01	8.161250 a1

Averages followed by the same letter do not differ statistically between themselves, according to Scott-Knott test at 5% of probability.

• There was no significative difference between the β-glucan tenors in the tested sucrose concentrations;

• Higher sucrose concentration can be used for the obtention of higher mycelia mass without decrease the β-glucan production;

CONCLUSION

Higher sucrose concentrations can be used to obtain more mycelial mass causing no damage to the β-glucan production.

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Chemical characterization of the biomass of *Agaricus subrufescens* via ^{13}C CP/MAS NMR. Comparison with *A. bisporus*

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Introduction

The edible mushroom, *Agaricus subrufescens*, is a basidiomycete which has long been widely consumed in Brazil and Japan as healthy food because of its nutritional properties. Furthermore this mushroom is believed to prevent or to fight various diseases such as diabetes, hepatitis, hyperlipidemia and cancer although both the bioactive molecules it produces and their physiological roles are still not clear. Nevertheless, polysaccharides such as (1-6)-b-glucan, (1-3)-b-glucan, (1-4)-a-glucan or (1-2)-b-mannopyranosyl residues have been demonstrated to inhibit the growth of cancer cells and to increase the immune activity of patients.

Objectives

The objectives of this study were:
-to compare, by chemial way ,the fungal biomass from vegetative mycelium and fruiting bodies of fungal species *A. subrufescens* using ^{13}C solid-state NMR
-to investigate the interspecific chemical differences of vegetative mycelium biomass between *A. subrufescens* and *A. bisporus*.
-to identify markers indicating the presence of polysaccharides with β anomeric carbons

Material and Methods

Liquid cultures (malt extract 20g/L) of 18 strains of *Agaricus bisporus* and 13 strains of *Agaricus subrufescens* were performed at 25°C under shaking for 15 days. The vegetative mycelium was filtered, lyophilized and analyzed by ^{13}C solid-state NMR.
The fruiting bodies were produced by MycSA (INRA, Bordeaux). Briefly, 8kg of compost (Renaud SA, Pons, France) was inoculated by 2% of white spawn (rye seed) and incubated at 25°C and 85% of humidity during 20 days. Then a cover ground (1/3 ground limestone, 1/3 peat, 1/3 thin sand) was added. The previous culture conditions were maintained for 8 days (post-incubation period) and incubated at 25°C and 95% of humidity. The fruiting bodies were harvested young and size medium. They were lyophilized before NMR analyses.
NMR analysis: ^{13}C CP/MAS NMR spectra were obtained using a spectrophotometer Bruker DSX 400 MHz operating at 100.7 MHz. Samples (400 mg) were spun at 10 KHz at the magic angle. Contact times of 2 ms were applied with a pulse width of 2.8 µs and a recycle delay of 3 s. Chemical shift values were referenced to glycine signal (carbonyl C at 176.03 ppm). The non-parametric Mann-Whitney U-test was used to separate significantly different means (P<0.05) for each NMR signal C, Aromatic+Phenolic-C and Alkyl-C).

Chemical differences from the vegetative mycelium of *A. subrufescens* and *A. bisporus*

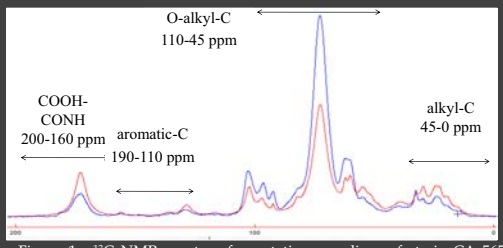

Figure 1 : ^{13}C NMR spectra of vegetative mycelium of strain CA 565 of *Agaricus subrufescens* (blue) and of strain Bs 514 of *Agaricus bisporus* (red).

The NMR spectra obtained from *Agaricus bisporus* and *Agaricus subrufescens* are qualitatively similar (Figure 1) but reveal differences in the relative intensities of each functional group : a higher relative intensity for the O-Alkyl-C region (polysaccharides) for *Agaricus subrufescens*, while higher relative intensities for both the COOH/CONH and the Alkyl-C regions (lipids) were found for *Agaricus bisporus* (Table1).

Interspecific comparison : no qualitative but quantitative differences

Chemical functions/ppm	Fungal species	Average of relative intensity
COOH-CONH	*A. subrufescens*	8.47+/-1.39*
160-200 ppm	*A. bisporus*	**12.02+/-0.77***
aromatic-C	*A. subrufescens*	2.39+/-3.63
110-160 ppm	*A. bisporus*	0.81+/-0.73
O-alkyl-C	*A. subrufescens*	**75.52+/-4.39***
45-110 ppm	*A. bisporus*	66.46+/-2.72*
alkyl-C	*A. subrufescens*	13.61+/-2.28*
0-45 ppm	*A. bisporus*	**17.90+/-1.19***

Table 1 :Relative intensities of chemical groups from ^{13}C NMR data of the vegetative mycelium from 18 strains of *Agaricus subrufescens* and 13 strains of *Agaricus bisporus*. The stars indicate significant differences between means (p<0.05) using the non-parametric U-test of Mann-Whitney.

These differences suggest that ***A. subrufescens*** **biomass contains more polysaccharides than *A. bisporus*** . This result is of importance since this fraction potentially contains polymers of medical interest.

Chemical differences in the biomass of the vegetative mycelium and the fruiting body

The results show qualitative and quantitative differences between the spectra of vegetative mycelia and fruiting bodies. Qualitative differences are mainly found in the polysaccharide moiety (Figure 2) .

Mycelium		Fruiting body	
ppm	%	ppm	%
96.97	3.08	X	X
92.54	0.46	X	X
X	X	69.14	8.96
X	X	67.63	0.75
X	X	63.48	1.2
60.85	7.14	X	X

Table 2 :Relative intensities of chemical groups from ^{13}C NMR data of vegetative mycelium and the fruiting body from 13 strains of *Agaricus subrufescens*. The stars indicate significant differences between means (p<0.05) using the non-parametric U-test of Mann-Whitney.

Chemical functions/ppm	Type of mycelium	Average of relative intensity
COOH-CONH	Vegetative mycelium	8.70+/-1.54*
160-200 ppm	Fruit body	**15.13+/-0.85***
aromatic-C	Vegetative mycelium	2.53+/-0.87
110-160 ppm	Fruit body	5.60+/-0.76
O-alkyl-C	Vegetative mycelium	**74.84+/-4.86***
45-110 ppm	Fruit body	55.91+/-2.72*
alkyl-C	Vegetative mycelium	13.92+/-2.54*
0-45 ppm	Fruit body	**23.36+/-1.34***

Vegetative mycelia *vs* fruiting bodies: **qualitative and quantitative** differences

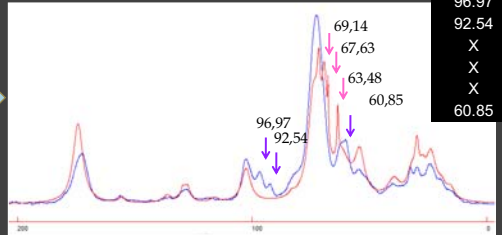

Figure 2 : ^{13}C NMR spectra of the vegetative mycelium biomass (blue) and of the fruiting body (red) from *Agaricus subrufescens*, strain CA 570 and relative intensities of chemical groups from ^{13}C NMR spectra of CA 565 of *Agaricus subrufescens* (blue) and of strain Bs 514 of *Agaricus bisporus* (red).

The fruiting bodies contain a higher percentage in COOH/CONH-C and alkyl-C i.e. lipids than the vegetative mycelia. On the other hand, **the vegetative mycelia show a higher level of O-Alkyl-C assigned to polysaccharides** (Table 2).

Conclusion and perspectives

Edible mushrooms can be considered as a source of active molecules and thus investigating whether certain factors (such as intra- and interspecies diversity or culture conditions) can enhance this potential is of importance. This study reveals that a high proportion of polysaccharides is found in *A. subrufescens* biomass. This warrants further investigations in order to purify certain polysaccharides of therapeutic interest from this fungus to clearly identify solid-state NMR chemical markers which would be helpful to test the effects of culture conditions on such polysaccharide concentrations.

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CONSERVE LIQUID OF MUSHROOM "CHAMPIGNON OF BRAZIL" (*Agaricus brasiliensis*)

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INTRODUCTION

Studies have demonstrated that the regular consumption of mushrooms or consumption of isolated bioactive constituents present in mushrooms is beneficial to health, therefore it might be considered as functional or nutritious product. *Agaricus brasiliensis* Wasser & Didukh, popularly known in Brazil as *Cogumelo do Sol* or champignon of Brazil (Amazonas & Siqueira, 2003) is consumed as concentrated extractor tea and popularly used against diabetes, atherosclerosis, hypercholesterolemia and heart disease. Moreover, the B- glucan isolated from *A. brasiliensis* has been described to exhibit antitumour and antimicrobial activities. The aim of this study was to evaluate different methodologies to conserve *A. brasiliensis* as alternative for consumption of this mushroom.

MATERIAL AND METHODS

The A. *brasiliensis* mushrooms conserve was accomplished using the methodology:

- **Solution 1: Described for Champignon** (Table 1)

Table 1. Concentration of different agents in the washing and preserve solutions, , used in the preparation of A. *brasiliensis* mushrooms conserve

	Solutions (g/L)		
Additive	Washing	Blanching	Conserve
Citric acid	1	5	1
Sodium bissulfite	4	1	-
NaCl	-	-	15

- ausence

- **Solution 2: Described by Siqueira (2004)**
- **Solution 3: Solution of citric acid and sodium chloride -1% citric acid/5% NaCl - citric acid 1%/10% NaCl**
- **Solution 4: Solution of white vinegar 70% and 5% NaCl**

Microbiological analysis

All *A. brasiliensis* mushrooms conserve that presented white color, were analyzed the microbiological population:

- Total coliforms and thermotolerant coliforms counts were done after two months of storage in room temperature protected from sunlight.

RESULTS AND DISCUSSION

The results showed that the mushrooms flavour is influenced by the methodology used for conservation This influence in the consumer's acceptability (data not show). Therefore for the good acceptability of the product is important to consider the visual aspect and conservation methodology of the product.

a)Conserve traditionally used for 'Champignon' was the best method for our mushroom conservation (solution 1):

Use of this procedure showed good color.

Elimination of sulfite during the bleaching process of the mushroom.

b) Analysis of the conserve of mushroom according to Siqueira (2004) (solution 2) and solution 3 (Solution of citric acid and sodium chloride -1% citric acid/5% NaCl - citric acid 1%/10% NaCl), were not efficient for the mushroom conservation.

c) Analysis of citric acid and sodium chloride solutions (Figure 1A) and d) Analysis of conserve in a solution of white vinegar and NaCl (Figure 1B)

Figure 1 Conserve mushroom *A. brasiliensis.* A – Citric acid and NaCl. B - Vinegar and NaCl

CONCLUSIONS

- For *A. brasiliensis* mushrooms conserves the best results were obtained using the same method as used for Champignon (solution 1);
- The vinegar solution (solution 4) was efficient for basidiocarps preservation;
- The solution 3 presented gas production and stewed cover.

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SUPPORT

CAPES CNPq FAPEMIG Fundação de Amparo à Pesquisa do Estado de Minas Gerais

SUBMERGED CULTIVATION OF NOVEL OXIDOREDUCTASE-PRODUCTING MACROMYCETES

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INTRODUCTION

White-rot fungi are able to degrade recalcitrant and heterogeneous lignin polymers using an unspecific oxidative system that involves extracellular peroxidases, oxidases and low molecular-mass compounds. Due to their chemical and catalytic features fungal lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase (Lac) are the three major lignin-degrading enzymes with great potential for biotechnological application. They may have significant value in many industrial processes, such as pulp bio-bleaching, biosensors, food industries, textile industries, soil bioremediation and in the production of complex polymers in synthetic chemistry. Activity of oxidoreductases has been found in various fungal species. Although there are lots of fungal laccases and peroxidases, have already been purified and characterized, nowadays it is still important to find novel strains – efficient producers of these enzymes.

Cultivation condition is an important aspect in requirements for biotechnological application of fungal strains. The strains of white-rot fungi used in this study were selected after screening of considerable number of macromycetes strains from various taxonomical and ecological groups for novel laccase producers.

The **AIM** of this work was to study the influence of cultivation temperature on the enzyme production and to optimize the composition of medium for high active laccase production by selected strains.

MATERIALS AND METHODS

Origin of the studied strains:

Three strains – *Antrodiella faginea* 1998, *Junghuhnia nitida* 2013 (*Steccherinaceae*) and *Polyporus ciliatus* 0626 (*Polyporaceae*) used in this study were obtained from the Komarov Botanical Institute Basidiomycetes Culture Collection (LE-BIN), St. Petersburg, Russia. Inoculum was grown statically on glucose–peptone (GP) medium during 10-15 days at 25ºC. Submerged cultivation of the strains was proceeded on circular shaker (180 r/min) in Erlenmeyer flasks at 25°, 30° and 35° C in the dark 19-24 days. Optimization of the cultivation medium was carried out using the strain *Junghuhnia nitida* 2013 by a 3^3 full factorial experimental design at 30°C.

Media:

GP (g/l): glucose – 10.0; peptone – 3.0; K_2HPO_4 – 0.4; KH_2PO_4 – 0.6; $CaCl_2$ – 0.05; $MgSO_4 \times 7H_2O$ – 0.5; $ZnSO_4 \times 7H_2O$ – 0.01; $FeSO_4 \times 7H_2O$ – 0.005; $CuS0_4 \times 5H_2O$ - 0.15 as Lac inductor

Enzyme activity:

Extracellular Lac activity was determined using syringaldazine [1], the activity was expressed as U · ml^{-1}. Lignin peroxidase (LiP) activity was measured using ABTS in the presence of 1 mM H_2O_2 [2], the activity was expressed as μM ml^{-1} · min^{-1}.

RESULTS

It was shown that cultivation temperature within the studied ambit had a great impact on the fungal enzymes production (Figs 1, 2). The optimal temperature for production both Lac and LiP was 30°C for all studied strains: Lac – 366.3, 154.4 and 82.9 U ml^{-1}, LiP – 0.328, 0.342 and 0.811 μM ml^{-1} min^{-1} for *Antrodiella faginea* 1998, *Junghuhnia nitida* 2013 and *Polyporus ciliatus* 0626, respectively. Induction of the enzymes by increasing the cultivation temperature from 25 to 30°C was 1.5-2.0 times for Lac and 1.2-1.5 times for LiP. Significant depression of the enzymes production was recorded for *Antrodiella faginea* 1998 and *Junghuhnia nitida* 2013 cultivated at 35°C (4.0 and 2.0 U ml^{-1} for Lac and 0.007 and 0.12 μM ml^{-1} min^{-1} for LiP, respectively), whereas *Polyporus ciliatus* 0626 demonstrated at 35°C only a slight decrease of activity (Lac 77.2 U ml^{-1} and LiP 0.722 μM ml^{-1} min^{-1}).

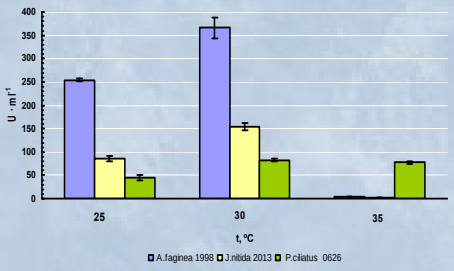


Fig. 1 Effect of temperature (°C) on the activity of laccase of the most active strains

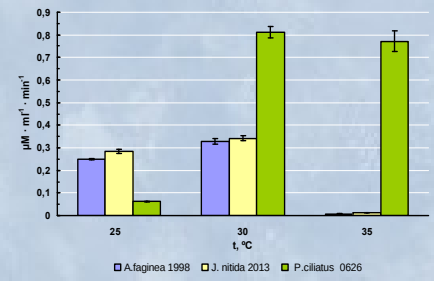
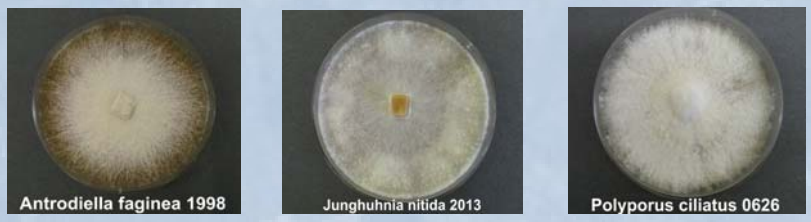


Fig. 2 Effect of temperature (°C) on the activity of lignin peroxidase of the most active strains



The other part of the study was optimization of the cultivation medium for increasing of laccase production by the strain *Junghuhnia nitida* 2013. Carbon, nitrogen and copper sources are main nutritional parameters for laccase production. They can regulate the level of gene transcription for laccase expression. Different concentrations of the following ingredients were evaluated to determine the highest level of the enzyme production: glucose (easiest carbon source), peptone (nitrogen source) and $CuSO_4$ (Lac inductor). Data analysis showed that increasing of carbon and copper concentrations to compare to the control ones (see in the methods) had positive effect on the enzyme production. As a result of the medium optimization, laccase production of the strain *Junghuhnia nitida* 2013 was increased in 1.5 times (Figs 3, 4). Thus, the best correlation of glucose: nitrogen: copper source was 15.5: 5.8: 0.25 g/l, respectively.

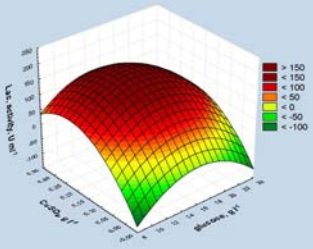


Fig. 3 Response surface plot showing the effect of carbon source and $CuSO_4$ on the production of laccase

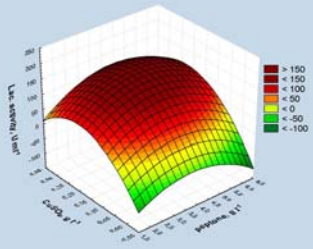


Fig. 4 Response surface plot showing the effect of nitrogen source and $CuSO_4$ on the production of laccase

Multivariate regression analysis was applied to get the following equation for laccase activity:

$$\text{Lac activity} = 181.079 + 50.790X_1 - 62.502X_1^2 - 56.821X_2^2 - 12.137X_1X_2^2 + 5.912X_1^2X_3 + 5.158X_3 - 35.342X_2X_3 - 32.188X_1X_2X_3 + 0.0508X_2^2X_3 - 38.382X_3^2 - 4.082X_1X_3^2 + 0.8892X_1^2X_2X_3^2 - 7.305X_2X_3^2 + 8.394X_1^2X_2^2 - 6.57X_2 + 6.199X_2^2X_3^2 + 3.367X_1X_2^2X_3^2$$

Where X_1 – glucose; X_2 – peptone; X_3 – $CuSO_4$

The value of the regression coefficient ($R^2 = 0.8856$) indicates that only 11.4% of the total variations are on explained by the model. The high correlation coefficient (88.6%) signifies a good correlation between the independent variables and the response.

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Profile of β-1,3 glucanase produced by isolates of *Trichoderma harzianum* grown on mycelial mass from basidiomycetes

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INTRODUCTION

Trichoderma harzianum is known for its excellence as biological control agent and to be a potent antagonistic of several species of phytopathogenic fungi. β-1,3-glucanase (Fig. 1) is an important cell wall-degrading enzyme involved in mycoparasitism by *Trichoderma* spp. during antagonism against phytopathogenic fungi [1]. The fungal wall is a complex structure composed typically of chitin, 1,3-β- and 1,6-β-glucan, mannan and proteins (Fig. 2) , although wall composition frequently varies markedly among species of fungi [2]. The aim of this study was to determine the profile of β-glucanase produced by three *T. harzianum* isolates grown on mycelial mass of basidiomycetes (*Pleurotus ostreatus, Pleurotus sajor-caju* and *Lentinula edodes*) as source of carbon and protein. Selective medium for the production of enzymes involved in breaking down of the cell wall from basidiomycetes were used. Because, there are commercial preparations for the production of fungi protoplasts, but theses preparations are not always efficient for some species of basidiomycetes, one example is the *Agaricus brasiliensis*, which is difficult to obtain protoplasts. Therefore, it is justified to seek alternative enzyme preparations.

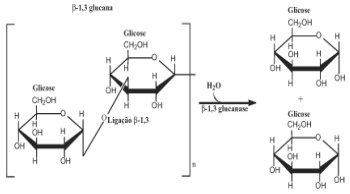

Fig. 1. Hidrolys 1,3-β-glucan by 1,3-β-glucanase
Source: Fleury & Sato, 2005, Química Nova.

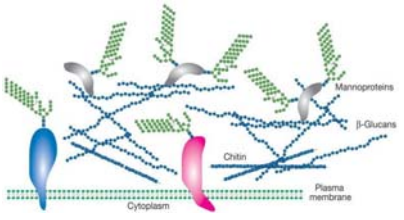

Fig. 2. illustration of the cell wall of fungi, showing the presence of glycoproteins and mannoproteins in the layer of the wall and an inner layer of different polysaccharides. The presence of different types of glucans and chitin varies between different fungal.
Source: Richard D Cummings and Tamara L Doering, Essentials of Glycobiology. 2nd edition, 2009.

METHODS

An aliquot (1 mL) of a spore suspension (10^6 spores/ml) of the different *T. harzianum* isolates (TC, T4 and T1051) were inoculated in Erlenmeyer flasks containing 50 mL of basic liquid medium with 1% of mycelial mass, were grown for 72h at 28°C, under agitation at 120 rpm. The culture supernatants were separated from the mycelium by filtration through filter paper (crude extract), and used for enzyme assays. β-glucanase activity was determined (in triplicate) by using a reaction system containing 100 mL of a laminarin solution (1%) dissolved in 50 mM sodium acetate buffer, pH 5.0 and 100 mL of crude extract. The reaction was carried on 30 min at 37°C and stopped by addition of 800 mL of 3,5-dinitrosalicylic acid reagent. Holocellulases activity were according Siqueira et al. (2011) [6]

RESULTS AND DISCUSSION

Pectinases activity showed, among holocellulases, most significant values, mainly *T. harzianum*_1051 and *T. harzianum*_4 grown on micelial *P. sajor-caju* (Fig. 3A). Hemicelulases activity (mannanases and xylanases) no significant differences in most of the tests and had values less expressive as than pectinases. Among the cellulases (endoglucanases, FPases, exoglucanases), only endoglucanases showed relevant results when compared with hemicellulases (Fig. 3A).

β-1,3-glucanase extracellular production was observed during growth of *T. harzianum* isolates in medium supplemented with 1.0% of mycelial mass from basidiomycetes. T4 result the most significant enzymatic activity, especially when grown on medium with *L. edodes* and *P. sajor-caju* as carbon and nitrogen sources, showing 219.3 and 179.0 IU.mg protein^{-1}, respectively (Fig 3B). In comparison with β-1,3-glucanase enzymatic activity from commercial preparation (Lyzing, Sigma), which presented 227.2 IU.mg protein^{-1} (data not showed), our results were satisfactory. In contrast, the other isolates showed minor specific activities. The *T. harzianum* isolate T1051 grown on basic medium containing chitin 0.5% as the carbon source showed β-1,3-glucanase activity of 0.3 U.mL^{-1} at 72h [4]. Likewise, under similar conditions, the TC isolate showed 3.0 U.mL^{-1}[3]. T4 grown on *L. edodes* showed higher specific activity (219.3 IU.mg protein^{-1}) than results reported by Ramada et al., 2010 [1] (mean 90.450 U.mg^{-1}).

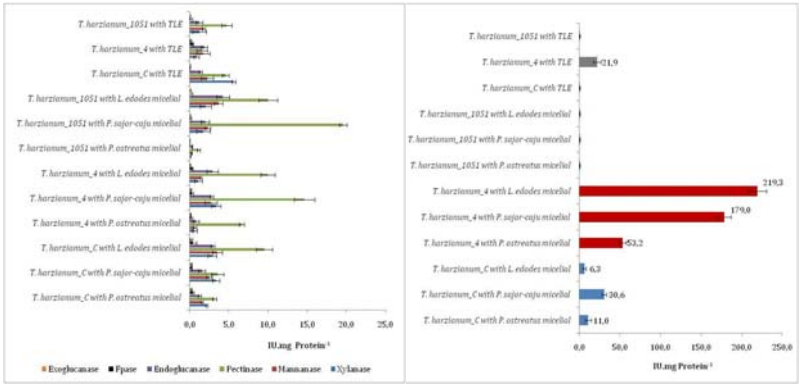

Fig. 3. A) Specific activity of the holocellulases from different strain *Trichoderma harzianum* grown on cell wall basidiomicetes carbon sources; B) Specific activity of the β-1,3 glucanase from different isolates *Trichoderma harzianum* grown on cell wall basidiomicetes carbon sources.

PERSPECTIVES

At present, Lysing Enzymes released 3.10^8 protoplasts mL^{-1} in experiments on mycelial mass of *Aspergillus ochraceus* [5], but not released when it was in cell-wall basidiomicetes by fusion protoplasts. For this reason, it is necessary to evaluate the potential of cell wall degrading-enzymes from T4 isolate in order to release protoplasts from *Agaricus brasiliensis*.

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ACKNOLEDGEMENTS

FAPEMIG Fundação de Amparo à Pesquisa do Estado de Minas Gerais
CAPES
CNPq Conselho Nacional de Desenvolvimento Científico e Tecnológico
fapdf Fundação de Apoio à Pesquisa do Distrito Federal
fapesb Fundação de Amparo à Pesquisa do Estado da Bahia

Waste to Remediation Resource:
Bioconversion of invasive *Phragmites australis* biomass to Oyster Mushrooms *Pleurotus ostreatus*

Kymberly Ra Draeger and Alma E. Rodriguez Estrada, University of Wisconsin Green Bay

Introduction

Like many wetland ecosystems throughout the world, the functional integrity of the bay of Green Bay, Wisconsin has been continually degraded because of human activities[3] (Fig. 1). Monospecific stands of the exotic common reed, *Phragmites australis,* proliferate where biodiversity once thrived[4]. The most effective means of eradicating the invasive *Phragmites* reed (essential to facilitate native ecosystem regeneration) has been to utilize two methods of control: chemical treatment and removal of the biomass[1].

Figure 1 – Location of Green Bay, Wisconsin. The site where *Phragmites australis* stems, leaves and inflorescence were harvested (for use as substrate for Oyster mushroom - *Pleurotus ostreatus* cultivation) is indicated with a star. Removal took place October, 2010. Map courtesy of USDA Farm Service Agency, 2008.

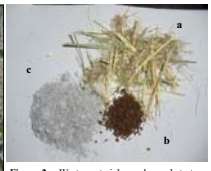

Figure 2 – Waste materials used as substrates and supplements for Oyster mushroom cultivation. a. Dried and chopped *Phragmites australis* biomass (reed grass), b. pelletized anaerobically digested dairy solids, and c. deink sludge from paper recycling.

To test the efficacy of waste resource utilization, the aboveground biomass of the invasive reed was used in the production of *Pleurotus ostreatus,* commonly known as Oyster Mushroom. Two additional wastes common to Green Bay, namely deink sludge from paper recycling [0, 30, and 50%] and anaerobically digested dairy solids [0, 1, and 2.5% supplementation], were also tested for value in balancing fungal nutritional demands[2] (Fig. 2). A total of nine substrate combinations and two genotypes (WC 923 – commercial isolate and WC 851 – wild isolate) were included in this study (Table 1).

Objective

Assess the bioconversion of *P. australis* biomass, as base substrate with and without supplementation, into *P. ostreatus* mushrooms, aiming to propose an alternative method for biomass disposal.

Materials and Methods

Table 1 – Experimental Design. Base substrate mixtures, identifier and percent of dairy solids supplementation. Nine different substrate formulations were tested with each genotype (WC 851 and WC 923) yielding a total of eighteen treatments. Ten replicates per treatment were included.

Treatment[1]	Base Substrate Mixtures *Phragmites*/Paper (%)	Base Substrate ID	Dairy Solids %
1, 2	100% *Phragmites*/ 0% deink sludge	S1	0
3, 4	100% *Phragmites*/ 0% deink sludge	S1	1
5, 6	100% *Phragmites*/ 0% deink sludge	S1	2.5
7, 8	70% *Phragmites*/ 30% deink sludge	S2	0
9, 10	70% *Phragmites*/ 30% deink sludge	S2	1
11, 12	70% *Phragmites*/ 30% deink sludge	S2	2.5
13, 14	50% *Phragmites*/ 50% deink sludge	S3	0
15, 16	50% *Phragmites*/ 50% deink sludge	S3	1
17, 18	50% *Phragmites*/ 50% deink sludge	S3	2.5

[1] Odd numbers correspond to substrates inoculated with genotype WC 851 and even numbers to substrates inoculated with WC 923

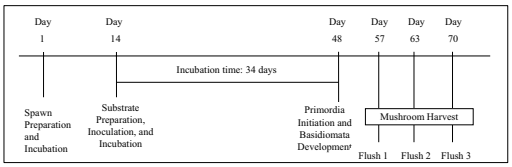

Figure 3 – Mushroom cultivation timeline from spawn preparation through mushrooms harvesting. Substrate incubation was done at 22 °C in darkness. Primordia initiation was triggered by decreasing the temperature to 15 °C, implementing a lighting cycle of 8 hours of light/16 hours of darkness, and increasing humidity to 90%.

Results

Figure 4 – Differences in morphology and color of primordia (a and b) and mature mushrooms (c and d) for genotype WC 851 (left) and genotype WC 923 (right).

All treatment combinations produced mushrooms (Fig. 4). Genotype and substrate composition were significant for all four parameters tested: yield (g/bag); biological efficiency (BE) (fresh mushrooms' weight per bag (g) /dry substrate weight * 100); number of mushrooms; and size (total yield/number of mushrooms) ($P<0.05$).

Isolate WC 851 produced the highest number of mushrooms (Fig. 5), yield (Fig. 6), and BE during the first flush on *Phragmites* base substrates supplemented with 30% and 50% deink sludge (S2 and S3, respectively) ($P<0.05$). Isolate WC 923 produced significantly larger mushrooms and higher yields during the second and third flushes on substrates S2 and S1 ($P<0.05$).

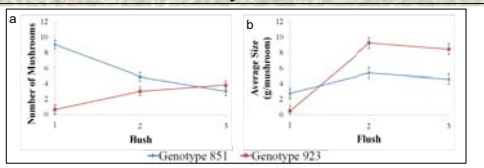

Figure 5 - Variation in number of mushrooms (a) and mushrooms' average size (b) (total weight (g)/number of mushrooms) produced by two *Pleurotus ostreatus* genotypes (WC 851 and WC 923) over three flushes. Error bars indicate standard errors.

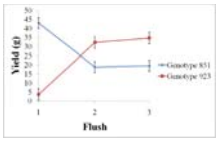

Figure 6 - *Pleurotus ostreatus* yield per genotype (WC 851 and WC 923) over three flushes. Error bars indicate standard errors.

There were no significant differences between genotypes with regard to total fresh mushroom yield or BE. Additionally, substrate enrichment with dairy solids had no statistically significant effects on yield, BE, number of mushrooms, or size, except as an interaction between substrate and genotype ($P<0.05$).

Conclusions

Genotypic variation of *P. ostreatus* resulted in two different production values: (1) WC 851 quickly colonized S3 and S2 substrates, producing 2.6 times more mushrooms than WC 923 genotype; (2) Genotype WC 923 employed a different energy investment strategy, defined by increased colonization time of S1 and S2 substrates, to yield 2.6 times larger mushrooms than WC 851 genotype during the second and third flushes.

Ultimately, the end result was the reduction of invasive reed grass biomass, recycled into an organic protein product. Additionally, cultivation of mushrooms on the biomass of plants grown in contaminated environments represents an opportunity to study translocation and accumulation of toxic compounds through the environment.

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ALTERNATIVE FRUITING SUBSTRATE FOR *AURICULARIA POLYTRICHA* (BLACK JELLY/ WOOD EAR MUSHROOM) IN MALAYSIA

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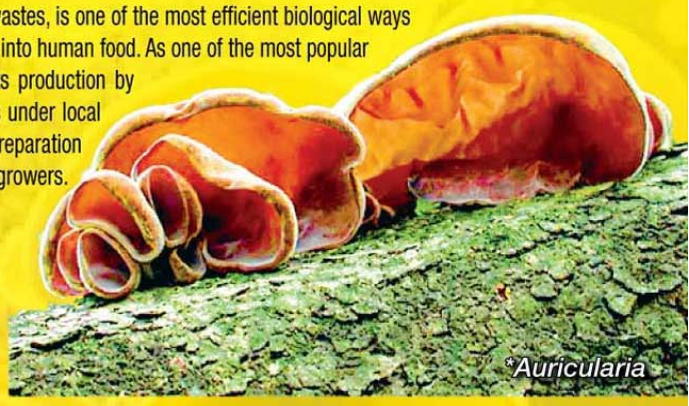
INTRODUCTION

Cultivation of edible mushrooms using agricultural residues or wastes, such as palm oil by-products / wastes, is one of the most efficient biological ways by which these residues can be recycled using a value-added process for conversion of these materials into human food. As one of the most popular culinary mushrooms in the world, *Auricularia polytricha* can be grown on a large scale. However, its production by Malaysian growers is still inefficient with low yield due to lack of research to domesticate the species under local conditions. This study aims to investigate the possible use of different types of oil palm wastes for the preparation of *A.polytricha* fruiting substrates to be used as alternative to sawdust in large scale cultivation by local growers.



MATERIALS & METHODS

To test the effect of different formulation of substrates to *A.polytricha* mycelia run, we chose palm pressed fibre (PPF), oil palm fronds (OPF) and empty fruit bunches (EFB) while sawdust was used as the primary substrate and also as control. A total of 16 substrate formulations were drawn and put into two groups, one with adjusted pH to 6.00±0.15 and another was not adjusted.

Substrates were mixed together with water to achieved 80% moisture content

15grams of each substrate formulations were put into glass petri dishes and then autoclaved for 60 minutes

7-days old mycelia plug were inoculated into the substrate

Incubated in the dark in growth chamber

RESULTS & DISCUSSION

Table 1. Growth rate (mm/day) and mycelia thickness for *A.polytricha* mycelia inoculated in different formulations of fruiting substrate

Substrate Formulations	Mean Growth Rate (mm/day)±SD		Mycelia thickness	
	pH not adjusted	pH adjusted to 6±0.15	pH not adjusted	pH adjusted to 6±0.15
A100% Sawdust	4±2b	5±1bc	+	+
100% PPF	6±1c	4±1b	++	+++
100% OPF	3±0a	2±0ab	++	++
100%	4±2b	3±1ab	+++	++
90%SD+10%PPF	5±0bc	5±1bc	++	+
90%SD+10%OPF	7±1c	6±1c	++	++
90%SD+10%EFB	6±1bc	5±1bc	++	+
80%SD+ 20%PPF	5±1bc	4±1bc	++	++
80%SD+20%OPF	4±2bc	5±1bc	++	++
80%SD+20%EFB	4±2b	4±1b	+	+
70%SD+30%PPF	5±2bc	5±1bc	++	++
70%SD+30%OPF	4±1b	5±1bc	++	++
70%SD+30%EFB	4±2b	4±1b	++	++
50%SD+50%PPF	4±2b	3±2ab	++	++
50%SD+50%OPF	4±2bc	4±1b	++	++
50%SD+50%EFB	5±1bc	5±1bc	+++	+++

Figure 1:

90%SD+10%OPF, pH not adjusted gave the highest mycelia extension rate (growth rate), 7mm/day

Figure 2:

100%OPF, pH not adjusted gave the lowest mycelia extension rate (growth rate), 3mm/day

1ANOVA analysis were performed using Minitab Statistical Software

2Each value is expressed as mean ± SD of five replicate analyses, brought to the nearest mm. Value with different small letters is significantly different at the level of 0.05 ($P<0.05$)

CONCLUSION

It is possible that oil palm wastes can be adopted and developed as alternative fruiting substrate for the production of *A.polytricha*, but further investigation will be carried out to optimize its potential.

ACKNOWLEDGEMENT

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Fruiting Substrate Formulation for the Large-scale Cultivation of *Schizophyllum commune* Fr. - a Medicinal-Culinary Mushroom

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ABSTRACT

Presently, *Schizophyllum commune* has not been cultivated on a large scale. It is only sold in the market during the wet / rainy season following a long dry spell when the mushroom starts to form sporophores naturally and collected. Hence, the supply is limited and seasonal. In this study, fruiting substrates were formulated from agroresidues consisting of sawdust, paddy straw and wastes resulting from palm oil processing i.e. empty fruit bunches (EFB) and palm parenchyma fibre (PPF). Mycelia growth occurred on all agroresidues studied with paddy straw exhibiting the best biological efficiency. Supplementation with nitrogen sources consisting of rice bran (20%) and brewery yeast (20%) further enhanced growth rate of mycelia with values between 5 – 6 mm/day. Sporophores were formed 3-5 days after full mycelia colonisation and slitting the sides of the bags. Up to four harvest can be obtained at intervals of 4-5 days. The maximum biological efficiency achieved was 40- 50%.

INTRODUCTION

Schizophyllum commune is a small, whitish mushroom with no stalk which grows on dead trees throughout the year and has a worldwide distribution. It grows in dense, often shelving clusters and is known as the Split-gill mushroom because of the longitudinally divided nature of the gills.

The leathery texture similar to meat, it's good nutritional composition and taste makes this mushroom or locally known as 'cendawan kukur' popularly consumed among the Malay community in Malaysia and Indonesia and people in Thailand, Philippines, Madagascar, Nigeria and Northeast India [1].

Besides being a delicacy, *S. commune* has actually been acknowledged for its medical importance [2]. Pharmacologically, *S. commune* is extremely important because it produces the polysaccharide schizophyllan (β 1-3, β1-6 D-glucan) which shows considerable medicinal properties [3]. This polysaccharide has been developed in Japan especially effective against cervical cancer. [4]. Our previous study showed that hot water extract of fruitbodies which mimics cooking of the mushroom exhibits good antioxidant potential as shown by the excellent β-carotene bleaching assay [5].

OBJECTIVE

To develop a cultivation technology utilizing abundant agricultural residues for the production of *S. commune* sporophores for consumption purpose.

MATERIALS & METHODS

SPAWN PREPARATION

Preparation of Mycelia by Liquid Fermentation

Alginate immobilized mycelia

FORMULATION AND PREPARATION OF FRUITING SUBSTRATES

a) Effect of nitrogen — Addition of 20% RB & BY
b) Effect of 0.4% woodceramics (Life Light, LL)

- SD 100%
- PS 100%
- EFB 100%
- PPF 100%
- SD 100% + RB 20% + BY 20%
- PS 100% + RB 20% + BY 20%
- EFB 100% + RB 20% + BY 20%
- PPF 100% +RB 20% + BY 20%
- SD 100% + RB 20% + BY 20% + LL
- PS 100% + RB 20% + BY 20% + LL
- EFB 100% + RB 20% + BY 20% + LL
- PPF 100% +RB 20% + BY 20% +LL

Inoculation of sterilized substrate

Colonization of the substrate
- Measurement of mycelia growth rate

Fruiting in the growing house
- Days for sporophore formation from inoculation

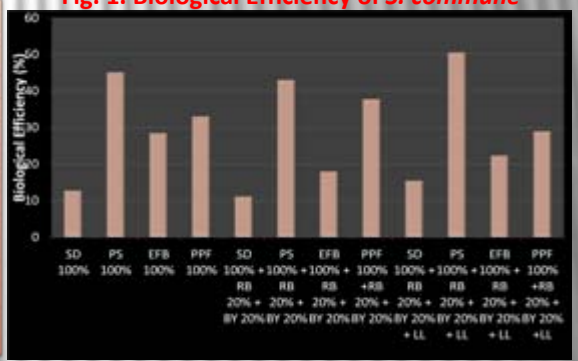
Harvesting of fruiting bodies
- Average weight of fruit body / harvest
- No. of harvest (min-max)
- Biological efficiency (%)

RESULTS & DISCUSSION

Table 1. Growth and Production of *S. commune* sporophores

Formulation	Mycelial Growth Rate * (mm/day)	Days for sporophore formation from inoculation	Average weight of fruitbody /harvest (g)	No of harvest per bag (min-max)	pH of agroresidues at 80% moisture content	C:N ratio
SD 100%	5 ± 0.0	30	3.77	2–4	4.62	95:1
PS 100%	4 ± 0.1	30	13.35	2–3	5.42	111:1
EFB 100%	4 ± 0.2	34	9.85	2–3	4.36	249:1
PPF 100%	4 ± 0.2	30	10.69	2–4	4.31	144:1
SD 100% + RB 20% + BY 20%	6 ± 0.3	28	8.09	1–2	5.30	63:1
PS 100% + RB 20% + BY 20%	5 ± 0.2	26	18.85	1–2	5.26	66:1
EFB 100% + RB 20% + BY 20%	6 ± 0.1	26	8.95	2–3	4.89	96:1
PPF 100% +RB 20% + BY 20%	5 ± 0.3	26	16.86	2–3	4.89	78:1
SD 100% + RB 20% + BY 20% + LL	5 ± 0.1	28	14.89	1–2	5.66	–
PS 100% + RB 20% + BY 20% + LL	5 ± 0.3	28	39.55	1	5.90	–
EFB 100% + RB 20% + BY 20% + LL	5 ± 0.2	28	16.76	1–2	5.34	–
PPF 100% +RB 20% + BY 20% +LL	5 ± 0.2	28	21.98	1–3	4.88	–

Fig. 1. Biological Efficiency of *S. commune*



- Utilizing PS (100%) as fruiting substrate gave the highest BE of 53 % among all the agroresidues tested similar to the cultivation of *Pleurotus sajor-caju* [6].
- The addition of rice bran (20%) and brewery yeast (20%) showed insignificant enhancement of mycelia growth rate in EFB and SD compared to other agroresidues. In addition the BE was not enhanced except for PPF.
- PS, EFB and PPF supplemented with RB 20% and BY 20% had the shortest time of 26 days for sporophore formation from inoculation while EFB 100% recorded the longest time taken for sporophore formation (34 days).
- Woodceramic added to the PS fruiting substrate enhanced the sporophores production per harvest and hence, biological efficiency
- Based on the average weight of fruitbody per harvest and biological efficiency, PS 100% + RB 20% + BY 20% + LL was the best formulation for large-scale culivation *S. commune* for food.

ACKNOWLEDGEMENT

The authors would like to thank University of Malaya for Grant RG036/09SUS for the support.

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Population Dynamics and Characterization of Microorganisms Present in Phase II of Composting for Producing *Agaricus brasiliensis*

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Introduction

Composting is a biological degradation process of organic matter, performed by microorganisms in controlled conditions. The study of microbial population and the factors which affect its activity can generate valuable information for the improvement of the composting technique and increasing of the productivity in the cultivation of mushrooms in Brazil. Temperature is one of the most important factors that determine the diversity and metabolic activity in all process.

Objective

The objective of this work was the morphological characterization of the microbiota, and determine the population of bacteria, actinobacteria and fungi, depending on the temperature variation during phase II of composting.

Materials and Methods

→Analysis and isolation were made from sugarcane bagasse and *coast-cross* grass based compost (1:1 ratio);
→Five samples were collected at intervals of two or three days between them, between the 17th and 28th day of composting;
→The morphological characterization for actinobacteria and *Thermoactinomyces* was performed by the method of cultured on nutrient agar blocks;
→Fungi were characterized according to their macroscopic and microscopic characteristics;
→Temperatures up to 70°C was applied between 22th and 23th for observing the behavior of microorganisms at temperatures above the recommended for the literature;

Results and Discussion

The population of bacteria had the most oscillation as a function of temperature, while that the population of actinobacteria remained almost constant (Figure 1). The fungal population was smaller, however, it tended to increase throughout the phase II, with a predominance of fungi of the genus *Scytalidium* and *Thermomyces*. Yeast had little expression, ranging from 0.4 to 0.7% in each sample.

Phase II period	Temperature		UFC/g
17º day	61,6º C	Bacteria	$6,58x10^8$
		Actinobacteria	$1,4x10^9$
		Fungi	$1,6x10^4$
20º day	54,6ºC	Bacteria	$1,16x10^9$
		Actinobacteria	$1,08x10^9$
		Fungi	$2,5x10^4$
24º day	45ºC	Bacteria	$7,32x10^8$
		Actinobacteria	$1,41x10^9$
		Fungi	$3,09x10^4$
26º day	60,5ºC	Bacteria	$1,18x10^9$
		Actinobacteria	$1,12x10^9$
		Fungi	$1,0x10^5$
28º day	39ºC	Bacteria	$1,16x10^8$
		Actinobacteria	$1,82x10^9$
		Fungi	$8,34x10^5$

Table 1. Microbial population dynamic during composting phase II.

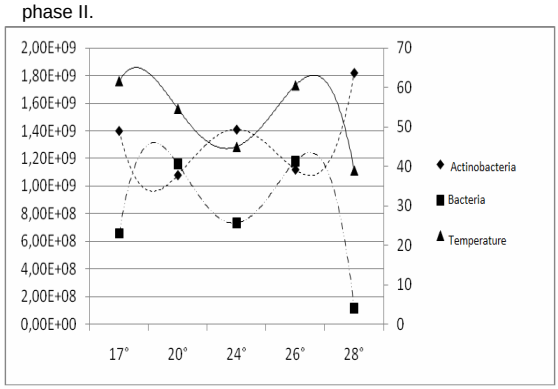

Figure 1. Dynamics of Bacteria and Actinobacteria population in relation to temperature during composting phase II.

Conclusions

Thermophilic fungi and actinobacteria are microbial agents considered the most important in the composting process, it can be concluded that microbiota found it is according to the literature and the use of temperatures above 60°C did not affect the microbial population of the compost during phase II.

Support:

FAPEMIG
CNPq Conselho Nacional de Desenvolvimento Científico e Tecnológico
CAPES

Agaricus brasiliensis mycelial growth in compost inoculated with the fungus *Scytalidium thermophilum*

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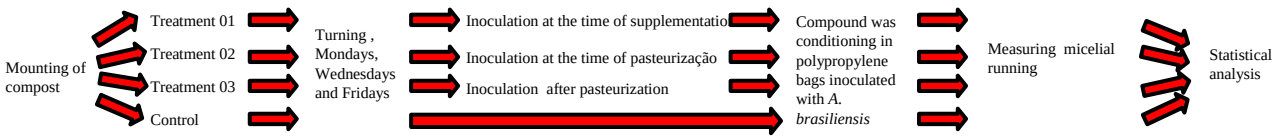
INTRODUCTION

Most of the growing of mushrooms of the genus *Agaricus* in Brazil is performed by small farmers with low invest capacity. Economically, the best option for these growers is to produce their own compost for cultivation, however, the conventional manufacturing process requires an appropriate infrastructure for Phase II composting. Long Composting followed by steam pasteurization can be an alternative because it needs to low level of investment, but the quality of the compost obtained is lower than with the traditional methodology. This work was aimed at testing inoculation of fungus *Scytalidium thermophilum* during long composting in order to obtain a better quality of compost.

MATERIAL AND METHODS

In all treatments of bagasse from sugar cane and grass coast-cross were utilized as basal substrate. After mounting stacks of the compost, was turned over every two days for two weeks. In the fourth turn over the substrate it was supplemented with wheat bran, urea, gypsum, lime and superphosphate. At the end of the process, the compost was pasteurized with water vapor in two cycles of 12 hours, and turned over after the first cycle for substrate homogenization. We used a spore suspension of *Scytalidium thermophilum* at a concentration of 10^6 spores per g of dry compost at different times of the process: T1- at the time of supplementation, T2, at the time of pasteurization, after pasteurization T3-T4 and-control. The compost was conditioned in polypropylene bags, inoculated with *A. brasiliensis* CS1 at a rate of 2% and incubated at room temperature for 25 days. Mycelium growth was recorded each day and expressed as mm per day. The statistical design was randomized blocks with 14 repetitions.



RESULTS

The inoculation of compost with *Scytalidium thermophilum* had a significant effect on mycelial growth of *A. brasiliensis*, when performed before the end of the pasteurization.

Table 1- Mycelial growth rate for each treatment

Treatment	Average	
Inoculation at the time of supplementation	0,9791 mm	a
Inoculation after pasteurization	0,8018 mm	a
Inoculation at the time of pasteurização	0,4680 mm	b
Control	0,4625 mm	b

The averages followed by same letter don't differ at level of 5% of probability in agreement with the test of Scott-Knott

Figure 01- Colonized compost

CONCLUSION

These results indicate the potential for using *Scytalidium thermophilum* to produce composts pasteurized with steam that are efficient for supporting favoring the vegetative mycelial growth of *A. brasiliensis*. The inoculation with *S. thermophilum* can be made in the moment of supplementation or after compost pasterurization. However, it is necessary to evaluate the productivity of mushrooms in each treatments to confirm the potential use of this technique in the production of the compost for the cultivation *Agaricus brasiliensis* mushroom

FINANCIAL SUPPORT: FAPEMIG, CAPES

ALTERNATIVE SUBSTRATES TO CASING LAYER AFFECT THE PRODUCTIVITY OF *Agaricus brasiliensis* MUSHROOM

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INTRODUCTION

The search for alternative substrates used as casing layer for the production of mushrooms of the genus *Agaricus* in Brazil is an important challenge, because of the absence of local peat moss and environmental restrictions for extraction. Nowadays, we use alternative types of soil horizon B as casing layer, however, the variations from of the different types of soil are very high from region to region. Thus, the use of soil as casing layer does not allow to achieve the uniformity necessary for the cultivation to be made on an industrial scale, showing the need for alternatives to their use.

MATERIAL AND METHODS

In this study, the Plantmax® substrate has been evaluated as a material of casing layer or in combination with Rhodic Hapludox in different concentrations, and the treatments were established as follows; i- Plantmax® 100%, ii- Plantmax® 80% + Rhodic Hapludox 20%, iii- Plantmax® 60% + Rhodic Hapludox 40%, iv- Plantmax® 40% + Rhodic Hapludox 60%, v- Plantmax® 20% + Rhodic Hapludox 80%, vi- Rhodic Hapludox 100% (Figure 1). The experimental design was completely randomized blocks with six replicates. The evaluated variables were: biological efficiency (BE= [weight of fresh mushrooms/weight of dried compost] x 100), Productivity = [weight of fresh mushrooms/weight of wet compost] x 100), an average weight of mushroom.

RESULTS

The highest mushrooms yields were with soil as casing layer (7,36% productivity) or soil containing 20% Plantmax® (10.12% productivity), whose differences were not significant. We also observed a falling productivity with higher concentrations of Plantmax, reaching 2.21 % when we used 100 % Plantmax® (Table 01). However, this substrate has a low water retention capacity, which might have affected productivity.

Table 01:Results of Biological Efficiency and Productivity

	Treatments	B.E.	Prod.
5-	Plantmax® 20% + Rhodic Hapludox 80%	16,87 A	10,12 A
6-	Rhodic Hapludox 100%	12,26 B	7,36 B
4-	Plantmax® 40% + Rhodic Hapludox 60%,	10,37 B	6,22 B
3-	Plantmax® 60% + Rhodic Hapludox 40%	7,96 C	4,78 C
2-	Plantmax® 80% + Rhodic Hapludox 20%,	5,06 C	3,04 C
1-	Plantmax® 100%,	3,68 C	2,21 C

* The Means followed by the same letter do not differ in the level of 5% probabilityaccording to Tukey's test.

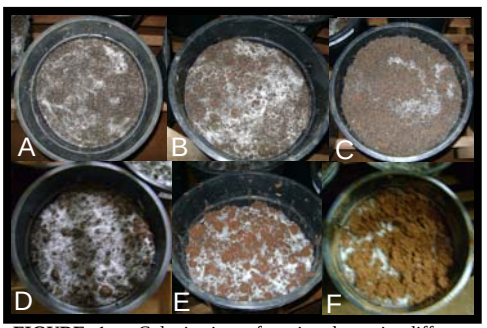

FIGURE 1 – Colonization of casing layer in different treatments
A – Plantmax® 100%, B- Plantmax® 80% + Rhodic Hapludox 20%, C-Plantmax® 60% + Rhodic Hapludox 40%, D- Plantmax® 40% + Rhodic Hapludox 60%, E- Plantmax® 20% + Rhodic Hapludox 80%, F- Rhodic Hapludox 100%.

FIGURE 2 – Fruiting bodies of *Agaricus brasiliensis* in different treatments; A – Plantmax® 100%, ii- Plantmax® 80% + Rhodic Hapludox 20%, iii- Plantmax® 60% + Rhodic Hapludox 40%, iv- Plantmax® 40% + Rhodic Hapludox 60%, v- Plantmax® 20% + Rhodic Hapludox 80%, vi- Rhodic Hapludox 100%.

CONCLUSION

Based on these results, it is suggested that further studies on the mix of substrate with materials having higher water retention capacities and also using different kinds of management, are need for increasing yield of mushrooms.

FINANCIAL SUPPORT: FAPEMIG, CAPES

Effect of substrate supplementation on mycelial growth of six strains of *Lentinula edodes* and *Pleurotus eryngii*

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Introduction

Ligninolytic fungi, such as *Lentinula edodes* (Berk.), Pegler are capable of degrading different types of residues, from agriculture, agro-industry, agro-forestry and other activities or logging, through their enzymes for the obtaining of compounds such as carbon and nitrogen, among other nutrients for their growth. This work aimed to evaluate the mycelial growth speed of six strains of *L. edodes* (Le1, Le2, Le3, Le4, Le5 e Le 6), the Shiitake mushroom, and one strain of *Pleurotus eryngii* (Pe), as an effect of the supplementation of the sawdust based substrate with wheat bran.

Material and Methods

Initially we compared the mycelial growth and the biomass production among the strains in Petri dishes containing PDA culture medium (Potato, Dextrose and Agar), where a 4 mm disk was placed on the surface of the PDA in the central area of a dish. The dishes containing the 6 strains of *L. edodes* and a strain of *P. eryngii*, with 10 repetitions were conditioned in a germination chamber at a controlled temperature of 25ºC with of 12 hour photoperiod. Every 48 hours mycelial growth readings were taken (mm/day). Soon afterwards, the dry weight was determined, obtaining the speed of the mycelial growth and the biomass production of each strain. For the substrate supplementation test we have used 3 treatments: Treatment 1: Sawdust 80%, Wheat bran 20%; Treatment 2: Sawdust 80%, Rice bran 12,9%, Wheat bran 1.94%, Cotton meal 1.94%, Coarse cornmeal 1.94%; Treatment 3: 80% sawdust and 20% sugar. The moisture of each substrate was adjusted to 60%, and then the substrate was conditioned in glass flasks with filter windows for gas exchanges. The flasks were autoclaved for 2 hours at 121ºC with. Five 4 mm diameter disks containing the strains grown in PDA were distributed equidistant on the surface of the substrate. Every 48 hours, for 10 days, readings were taken to verify the growth of the mycelium, obtaining the mycelial growth speed in mm/day. The design used was randomized block, using the Scott-Knott test (1974) for comparison of the averages.

Results and Discussion

The results of the mycelial run in Petri dishes showed that the strain with the highest growth speed, Le 5, had a lower biomass production than Le 1, which had the lowest growth speed even if it presented the highest biomass production (table 1).

Table 1 – Mycelial run and dry weight of the strains.

Strain	PDA	Rice	Dry weight
Le1	0,44 D	0,378 C	0,168 A
Le2	0,424 D	0,384 C	0,013 E
Le3	0,438 D	0,29 C	0,027 D
Le4	0,476 C	0,496 B	0,029 D
Le5	0,922 A	0,93 A	0,16 B
Le6	0,376 E	0,546 B	0,029 D
Pe	0,802 B	0,608 B	0,15 C
CV	3,86	24,23	5,34

*Same capital letters in the same column do not differ statistically by the Scott-Knott test (5%).

The mycelial growth in the sawdust based substrate was different from that presented in PDA using the same strains, where the strain that grew with higher speed was Le 4 in the treatment 1, and this way it demonstrated to be the most efficient in relation to the other treatments (table 2). On the other hand, the treatment 3 did not present mycelial growth, suggesting that the addition of sugar does not supply the nutritional needs of the fungus. On the other hand, the treatment 3 did not present mycelial growth, suggesting that the addition of sugar does not supply the nutritional needs of the fungus.

Table 2 – Substrate supplementation test.

	Strains						
Treatment	Le	Le2	Le3	Le4	Le5	Le6	Pe
T1	0,3167 d A	0,447 b A	0,367 d A	0,567 a A	0,42 c A	0,467 b A	0,489 b A
T2	0,325 b A	0,325 b B	0,297 b B	0,271 b B	0,422 a A	0,38 a B	0,452 a A
T3	-	-	-	-	-	-	-
CV	14,48						

*Same capital letters in the same column do not differ statistically by the Scott-Knott test (5%). **Same lowercase letters in the same line do not differ statistically by the Scott-Knott test (5%).

Conclusion

The results presented in this work demonstrate the best way to cultivation of strains of L. edodes in vitro and an alternative way to produce the substrate of cultivation.

INSECTICIDE BIOACCUMULATION BY *Agaricus brasiliensis*

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INTRODUCTION

Several agro-industrial residues can be used in the production of mushroom substrate. The major activities in the Brazil are agro-industrial and residues are frequently wasted. Wasted materials include coffee residues, cereal straws, sugarcane bagasse, native and fast gowing grasses and several types of industrial residues such as sawdust and manure. The substrate formulations used in Brazil for production *A. brasiliensis* includes sugarcane bagasse. However, it is possible that many wastes containing toxic substances, for example, insecticides, could reach the edible mushroom and produce adverse effects on human health. Fipronil has been applied to sugar cane plantations at stand establishment in Brazil field pest control. Could *A. brasiliensis* bioaccumulate this insecticide? Since heavy metal concentrations in mushroom are, commonly, considerably higher than those in agricultural crop plants, vegetables and fruit. This suggests that mushrooms possess a very effective mechanism that enables them readily to take up some heavy metals and others subtances from the ecosystem. The objective of this study was to evaluate the effect of the insecticide fipronil on the productivity of *Agaricus brasiliensis* and its bioaccumulation.

MATERIAL AND METHODS

Four weeks composting after vapor pasteurization was used for *Agaricus brasiliensis* cultivation. The compost containing sugarcane bagasse and *coast-cross* hay, containing 1.5% nitrogen. The experiment was conducted in two stages. In the first, fipronil was added in the casing layer in concentrations of 0, 2, 4 and 8 parts per million (ppm) in order to verify mushroom bioaccumulation. In the second stage, fipronil was added to substrate after pasteurization, in final concentrations of 0, 4, 8, 16 and 32 ppm, representing, respectively, treatments T1, T2, T3, T4 and T5. Analyses of quantification fipronil were performed by gas-liquid chromatography (HP 6890), using a HP-5 capillary column. N_2 fluxes were 2.3 and 30.0 mL/min, for column. In cases of synthetic air and H_2, fluxes were 60 and 3 mL/min, respectively. Injector and detector temperatures were 260 and 300ºC, respectively. With analytic pattern it was used fipronil obtained at BASF® (98,2% purity), the experiment was made in 6 replications. For the statistical analysis of the results Scott-Knott test for the means comparison at 5% probability were applied using SISVAR UFLA.

RESULTS AND DISCUSSIONS

Table 1. Total mean productivity of *Agaricus brasiliensis* mushroom in compost containing fipronil at concentrations of 0, 4, 8, 16 and 32 ppm.

Tratamento	Produtividade (%)
T1	8,55 b
T2	3,87 a
T3	5,09 a
T4	3,41 a
T5	4,42 a

Means followed by same letter do not differ by Scott-Knott test at 5% probability.

Table 2. Bioaccumulation of fipronil in compost of *Agaricus brasiliensis*.

Tratamento	Teor de fipronil (ppm)
T1	< 0.01
T2	< 0.01
T3	< 0.01
T4	< 0.01
T5	0.01

Means followed by same letter do not differ by Scott-Knott test at 5% probability.

Table 3. Total means productivity of *Agaricus brasiliensis* with casing layer fipronil at concentrations 0, 2, 4 e 8ppm.

Tratamento	Produtividade (%)
T1	0.83a
T2	2.80a
T3	1.94a
T4	1.97a

Means followed by same letter do not differ by Scott-Knott test at 5% probability.

Table 4. Bioaccumulation of fipronil in casing layer of *Agaricus brasiliensis* in concentration of 0, 2, 4 and 8 ppm.

Tratamento	Teor de fipronil (ppm)
T1	< 0.01
T2	0.03
T3	0.08
T4	0.26

Means followed by same letter do not differ by Scott-Knott test at 5% probability.

CONCLUSIONS

The insecticide fipronil significantly altered the productivity of *Agaricus brasiliensis* when it was applied to the compost of cultivation, however the mushroom showed no bioaccumulation of the insecticide.

The productivity of the mushroom *A. brasiliensis* was very low, even for the control treatment, in the experiment of application of fipronil in the casing layer.

The mushroom showed bioaccumulation of the fipronil when it was applied in casing layer. Higher fipronil concentrations resulted in higher accumulation by the mushroom.

SUPPORT

CAPES
FAPEMIG Fundação de Amparo à Pesquisa do Estado de Minas Gerais
CNPq

Characterisation of *Trichoderma aggressivum* Infection in Bulk Phase III Mushroom systems

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Introduction

Trichoderma aggressivum is a fast growing filamentous fungus which causes Compost Green Mould in *Agaricus bisporus* cultivation, a problem for mushroom producers worldwide[1].

During severe outbreaks in the 1980s and 1990s contamination of Phase 2 compost at spawning was identified as the primary cause of Compost Green Mould[2].

In the technologically advanced Bulk Phase 3 systems compost is spawn run in large tunnels before being bulk handled and transported to the grower. While earlier research indicates that fully spawn run compost is not very susceptible to infection by *T. aggressivum*[3] no research has being carried out into the epidemiology of *T. aggressivum* in Bulk Phase 3 mushroom systems.

This study looks at the effect of mixing *T. aggressivum*-infected Phase 3 compost into a larger volume of healthy Phase 3 compost to determine if such a method of infection can cause crop loss.

Material and Methods

Fungal strains

Agaricus bisporus strain Sylvan A15
Trichoderma aggressivum f. europaeum strain FM10

Production of Inoculum

Pre-spawned Phase 2 compost was inoculated with *T. aggressivum* on spawn grains and then spawn run under standard conditions. This produced *T. aggressivum*-infected Phase 3 compost which was then used as inoculum for the subsequent experiments (Fig.1). The fungal load of *T. aggressivum* in the inoculum was determined by the Most Probable Number (MPN) method [4]at the end of spawn run.

Cropping Experiments

18kg plots of Phase 3 compost were inoculated with *T. aggressivum* at rates of 10%, 1%, 0.1%, 0.01% with a control group remaining un-inoculated. Half of the plots were supplemented with a nitrogen based supplement at a rate of 0.5%. Four replicate plots were prepared for each treatment combination for a total of 40 plots.

The crop was cased and incubated under standard mushroom growing conditions. Mushrooms were harvested as closed cups and weighed. The experiment was repeated twice.

Results

Inoculum used to infect the Phase 3 compost was heavily colonized with *T. aggressivum* (Fig. 1). The fungal load in the inoculum was estimated at 2.6 x 10^8 and 1.39 x 10^9 propagules per gram (Experiment 1 and Experiment 2, respectively).

Infection of Bulk Phase 3 compost using *T. aggressivum*-infected Phase 3 compost was effective (Fig. 2). Yield reduction was between 9% and 100% in the first flush depending on crop and infection rate (Fig. 3). There was a highly significant correlation between infection rate and yield loss ($P<0.001$).

Mushroom yields were significantly lower in Experiment 2 than in Experiment 1 ($P<0.001$) but there was no significant difference between the yield from the control plots in these trials ($P=0.11$).

There was no significant difference in yield between the supplemented and un-supplemented treatments in either trial ($P=0.99$).

Discussion

The mixing of *Trichoderma aggressivum*-infected Phase 3 compost into healthy spawn-run Phase 3 compost caused an economically significant reduction in yield. The well established *Agaricus* mycelium was not enough to prevent the proliferation of *T. aggressivum* and the loss of mushroom production. This highlights the potential problem of localised areas of heavily infected Phase 3 compost being mixed through the larger volume of Phase 3 compost during tunnel emptying, bulk handling and transport.

The reason for the differential effect of infection rate in the 2 cropping experiments is unclear. While the fungal load in the inoculum for Experiment 2 was higher than for Experiment 1 the reduction in yield was disproportionately severe. One explanation for this is that in Experiment 2 the compost was mixed more thoroughly to reflect the level of mixing that occurs during Phase 3 tunnel emptying and transportation. Trials are currently underway to investigate whether a greater level of mixing causes a more severe loss of yield with the same inoculum.

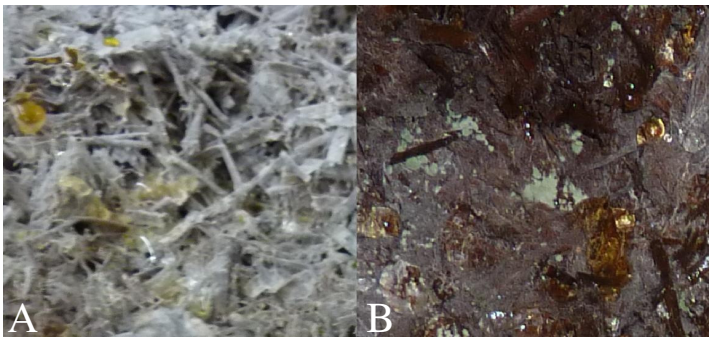

Figure 1: Healthy Spawn run Phase 3 compost (A) and Phase 3 compost infected with *T. aggressivum* prior to spawn run (B).

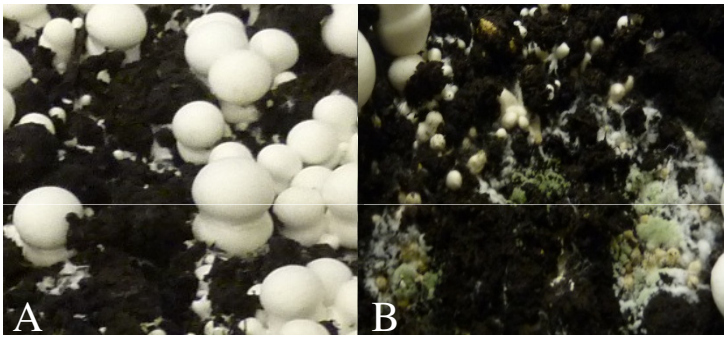

Figure 2: Mushrooms on casing surface of control plot at first flush (A). Fewer fruiting bodies and Green Mould symptoms on infected plot at first flush (B).

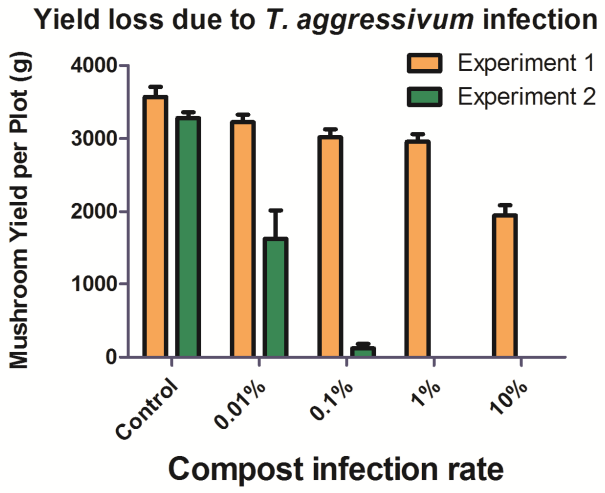

Figure 3: Mushroom yield vs. infection rate. The results are the average of 8 replicates for both trials

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The effect of UV radiation on the pathogenic fungi of cultivated mushrooms

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INTRODUCTION

Agaricus bisporus (LANGE/IMBACH) is the most commonly cultivated edible mushroom of the world. Hungarian production of this species takes place mainly in limestone cellars and in no longer used stables. As most of these facilities are not properly air-conditioned, and the growing conditions are not always optimal, it is difficult to face the hygienic regulations.

Two pathogenic fungi – *Verticillium (Lecanicillium) fungicola var. fungicola* PREUSS and *Mycogone perniciosa* MAGNUS – are the most serious diseases occurring during the growing period. In order to prevent their appearance, growers disinfect the rooms, sterilize the equipment, minimize human contact with the growing materials and use certain chemicals against mushroom flies which act as vectors of diseases. Years of experience prove that in the traditional growing houses these mushroom protecting procedures are not effective enough by themselves; additional methods should be brought into the production.

The sterilizing ability of UV radiation is well known, UV lamps are already used in laboratories for sterile work. In addition to the current mushroom protecting technologies, applying UV irradiation in the growing areas can enhance effectiveness. It could be used to sterilize materials like casing soil, which is often the source of the dry and wet bubble disease, or equipment which come contact with the mushroom culture.

MATERIALS AND METHODS

In our experiment the effect of UV light on the in vitro tissue culture of the two diseases were examined. The aim of the study was to determine which UV light range is more effective against *Verticillium fungicola var. fungicola* and *Mycogone perniciosa*, and to define the optimal irradiation time period against the two pathogens.

Two wavelengths of the UV range were used: UVB (312 nm) and UVC (245 nm). The Vilbert Lourmat-115M type lamps were set up in a laboratory on a special stand, where the distance of the radiation source and the tissue culture can be adjusted.

The pathogens were inoculated on 9 cm Petri-dishes. After 16 days incubation, the widest and narrowest diameters of the tissue cultures were measured with caliper.

Picture 1. *Verticillium fungicola var. fungicola* and *Mycogone perniciosa* tissue cultures before UV treatments

Both pathogens were irradiated in 3 repeats, on the two wavelengths. 6 different time periods (5, 10, 15, 20, 25 and 30 minutes) were applied in 3 repeats, this way the tissue cultures got 15 to 90 minutes irradiation. Diameters of the treated and untreated (control) cultures were measured before the first, and after the second and third treatments.

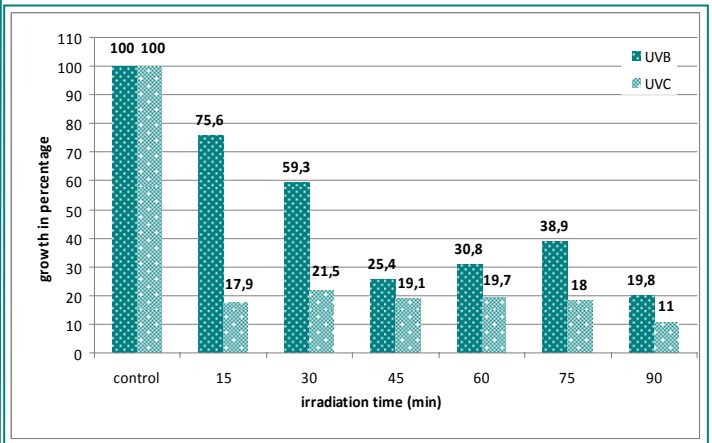

Figure 1. Growth of *Verticillium fungicola var. fungicola* tissue cultures after UV irradiation

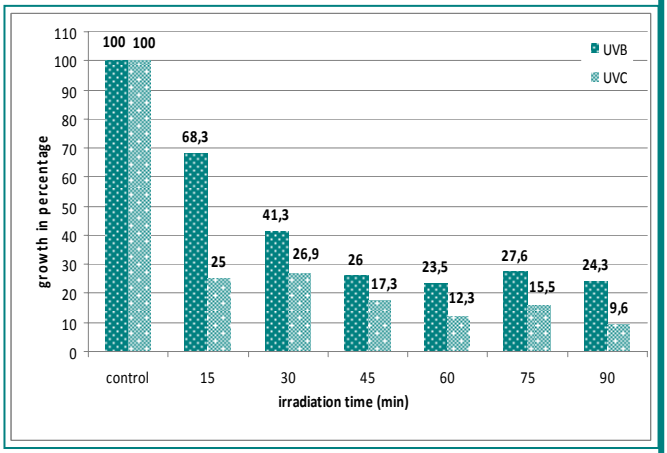

Figure 2. Growth of *Mycogone perniciosa* tissue cultures after UV irradiation

RESULTS AND CONCLUSIONS

The changes in growth intensity of the tissue cultures after UV treatment are demonstrated on *Figure 1.* and *2*. Data shows that in case of both pathogens (irradiated on both wavelengths and for any time period), UV treatment caused significant setback in tissue-growth. UVC irradiation proved to be more efficient, as it caused 73,1-90,4% decline, while the UVB treated tissues grew only 24,4-80,2% less, than the untreated control. The two pathogens reacted the same; the rate of setback in tissue growth was similar.

Due to the data, both UV ranges are capable of significant inhibition of tissue growth. With correct application (proper irradiation time and distance), UV irradiation could function as an additional technique in mushroom protection, by preventing diseases in growing houses and cellars. In order to be able to develop this technique, we are planning an in vivo trial in our experimental mushroom growing room.

The results presented here are an output from research fund TÁMOP-4.2.1./B-09/1/KMR 2010-0005.

COMPARATIVE EVALUATION OF BIOMASS PRODUCTION OF *AGARICUS BISPORUS* STRAINS IN LIQUID CULTURE

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INTRODUCTION

The adaptation of liquid culture to the production of fungi mycelia offers the possibility of its application in the mushroom spawn industry where the production process is currently based on the solid state fermentation of cereal grain [1]. Two the most important advantages of adapting the liquid culture to the spawn production process are: an increase in the level of process control and a reduction in the duration of the production cycle time [1, 2]. It has been cited that in a liquid medium not only a higher productive capacity of biomass is maintained, but also the genetic, physiological and morphological properties of the strains are maintained too [3]. A comparative evaluation of biomass production of *Agaricus bisporus* in liquid culture under two growth conditions was carried out, with the aim to estimate the biomass production capacity and the physiological adaptation of the strains tested, through the phenols estimation in the culture medium.

MATERIALS AND METHODS

Fungal strains

Five commercial strains of *A. bisporus* were studied. All cultures are of different sources: HAI 24-Ukraine, Delta Amycel-USA, Phoenix-USA, Mis-Pag 365-Spain and GUR-45-Spain. Replicates of the strains were deposited in the Culture Collection of the Instituto de Ecología and recorded as IE (Xalapa, Mexico).

Liquid culture evaluation

In order to observe if different alternating periods of agitation affect biomass production, initially only one strain of *Agaricus* was studied (HAI 24). Four treatments were tested: 70 rpm-120 min day^{-1}, 70 rpm-60 min day^{-1}, 35 rpm-120 min day^{-1} and 35 rpm-60 min day^{-1}. A mycelia plug (8mm Ø) from the peripheral of a 15 d plate provided the inoculum for the production of liquid cultures. The fungus was cultivated in a media prepared with an infusion of commercial compost (1000 ml infusion, 10 g glucose) method modified from INRA-Station de Recherches sur les Champignons, Bordeaux; 10 ml of medium in a 40 ml flask was used. Each sterile culture was inoculated with three plugs and was grown at 25°C during 20 d; five flask were prepared per strain. Mycelial dry weights were determined by filtration through pre-weighed Whatman No. 1 filter papers (125 mm Ø). Filter papers were oven dried at 100°C to a constant weight for the mycelial dry weight determination.

One agitation condition was selected and all strains were cultured in the same liquid culture media under two growth conditions: stationary culture (SC) and agitated culture (AC). Biomass production of mycelia, phenolic content in liquid culture media and pH were measured during all incubation period (20 d). The total phenol content was estimated according to the procedure outlined by Box [4].

Statistical analysis

The data was analyzed using a two-way analysis of variance (ANOVA). The mean values of five replicates were separated by Tukey's multiple range test (α<0.05). All statistical analyses were performed using Statistica package 7.0 for Windows.

RESULTS AND DISCUSSIONS

Effect of alternating periods of agitation in biomass production

In the preliminary test, the treatment of 35 rpm-120 min day^{-1} was better with a growth mycelium rate of 1.52 mg ml^{-1} day^{-1}. The increase of the level of agitation to 70 rpm resulted in a decrease in growth rate (Fig. 1).

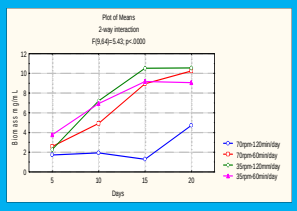

Figure 1. Effect of different alternating periods of agitation on the growth of *Agaricus bisporus* strain (HAI 24) in liquid culture medium.

When the mycelia production of *A. bisporus* strains in AC and SC conditions was compared, it was observed that agitation affected the biomass production. A growth rate of 2.08 mg ml^{-1} day^{-1} was recorded under static conditions compared to 1.93 mg ml^{-1} day^{-1} at an agitation level of 35 rpm (Fig. 2) In AC condition, statistical differences (p<0.05) were observed in three of the five evaluated strains, while in SC no significant differences between all strains was presented.

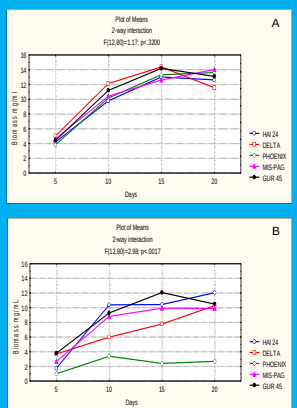

Figure 2. Biomass production of *Agaricus bisporus* strains under stationary (A) and agitated culture (B).

Phenolic content in liquid media decresed after AC in all strains, while this ocurred less in SC. In general, phenol concentration decreced by 0.27 mmol l^{-1} in AC, by 0.24 mmol l^{-1} in SC. No significant differences were observed for all strains in both growth conditions (p>0.05), namely the phenolic content tended to be related to incubation period (Fig. 3). However, it has been cited that a rapid reduction in phenolic concentration accelerate colonization (this doesn´t happened in the present work), and could be associated to a decrease of the risk of invation by mold contaminants, among other advantages [5]. During the incubation period mycelial growth did not tend to be related to pH. No correlation between the biomass production and phenolic content was observed.

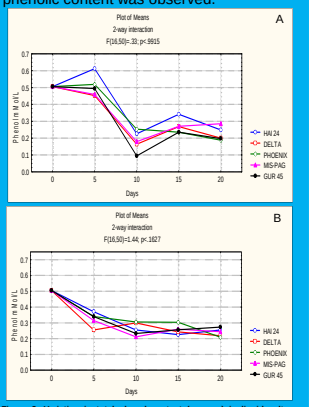

Figure 3. Variation in total phenol content (average) in liquid culture media during mycelial growth of *Agaricus bisporus* strains under stationary (A) and agitated culture (B).

CONCLUSIONS

The results suggest that the low growth rate observed in the agitated culture during the first 10 d of incubation were likely due to the different ability of the strain to utilize nutrients available in the medium, which impedes an efficient biomass over production; the vegetative growth during incubation period was more related to the decrease of phenol of the medium than to an increase in the biomass and a damage of the hyphal cells by disturbing their physical or metabolic balance resulting in viability loss.

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Comparative study of mycelial growth and dry weight of *Agaricus bisporus* and *Agaricus brasiliensis*

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INTRODUCTION

Agaricus bisporus is the most globally produced and consumed mushroom, because their strains present high yield [1]. Its growth is unstable in most of the culture media traditionally used, such as PDA and Agar Malt. Besides the irregular growth, it is common to observe a degeneration of the micelial growth over the course of time, until the culture becomes inactive. The *Agaricus brasiliensis* is a mushroom native to Brazil, however, its commercial cultivation is recent. As such, the need to development appropriate technologies is growing [2]. Various studies are conducted with the purpose of optimizing mushroom production, with the development of new technologies, which include from the obtaining of the mycelium to the appropriate cultivation substrate [4]. The objective of the study was to compare the micelial growth with the biomass produced of *A. bisporus* and *A. brasiliensis.*

MATERIAL AND METHODS

Mycelial growth run:
Rice Agar Dextrose Yeast Extract (RAGE), Malt Extract Agar (MEA), Potato Dextrose Agar (PDA), Rich Medium (RM), Rice Agar (RA), Wheat Flour Agar (WFA) and Wheat Bran Agar (WBA).

↓

Dry weight (vacuum pump and pressure)

↓

Randomized block design, 4x7 factorial
Scott-Knott test (5%)

↓

Mycelial growth and dry weight of the ABI-6 was compared with the CS10 experimental strain *(A. brasiliensis)* cultivated in RAGE medium.

RESULTS AND DISCUSSION

Higher growth was obtained with the strain ABI-6 cultivated in medium supplemented with glucose and yeast extract (RAGE: 3.6 mm/day) when compared with the other strains (table 1).

Table 1 – Micelial growth in different medias.

Culture media	ABI-2		ABI-3		ABI-5		ABI-6	
	Growth	Dry weight	Growth	Dry weight	Growth	Dry weight	Growth	Dry weight
RAGE	2,0	92,0Cd	2,1	142,0Ab	2,0	131,0Bc	**3,6**	**203,0Aa**
MA	1,2	54,0Eb	1,5	60,0Ea	1,0	43,0Ec	1,0	2,0Gd
PDAE	2,0	162,0Aa	1,2	100,0Bc	1,5	130,0Bb	3,5	160,0Ca
MC	1,0	80,0Dc	1,1	88,0Cb	1,5	93,0Ca	0,7	12,0Fd
RA	2,0	19,0Fb	1,0	4,0Fc	1,0	4,0Fc	3,3	93,0Ea
WFA	1,8	139,0Bc	1,2	100,0Bd	2,2	200,0Aa	2,8	158,0Db
WBA	1,5	13,0Gd	1,0	70,0Dc	1,0	77,0Db	3,2	182,0Ba
VC	**7,02**	**1,13**	**7,74**	**1,02**	**6,98**	**1,57**	**7,55**	**1,45**

Means within the same column followed by the capital same letter and means within the same line followed by the small same letter do not differ significantly to Scott-Knott test (P% 0.05).
*Growth is measured in mm/day; ** Dry weight is in g.

After 10 days of incubation at 25ºC, micelial growth values as well as the dry weight allowed to affirm that, statistically, better growth and biomass production were observed with the strain CS10 (4.1 mm/day; 0.221g) when compared with the strain ABI-6 (3.6 mm/day; 0.203g) (figure 1). Research conducted with the micelial growth of *A. bisporus* in culture medium based on compost extracts obtained averages between 1.64 and 2.75 mm/day, after 12 days of development [3]. Studies carried out at our laboratory demonstrate an average of growth of CS10 in PDA medium of 3.9 mm/day. This suggests that RAGE medium can be used for the biomass production of *A. bisporus* and *A. brasiliensis* strains.

Figure 1- A: *A. brasiliensis* mycelial growth; B: Colony morphology of CS-10; C: *A. bisporus* mycelial growth; D: Colony morphology of ABI-6.

CONCLUSIONS

RAGE medium can be an alternative for the *in vitro* cultivation of *A. bisporus* and *A. brasiliensis* strains;

The use of cultivation media capable to produce fungal biomass in a short span of time is fundamental for the production of edible fungi;

The results obtained in the work considering the two appraised parameters, the micelial growth speed presents positive correlation with the biomass produced, in other words, the higher the growth vigor, the higher the biomass production.

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