Endophytic bacteria with antagonistic traits inhabit the wood tissues of grapevines from Tunisian vineyards

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Highlights

- Specific bacteria inhabit the wood of Tunisian vines with esca-foliar symptoms.
- Nineteen strains were isolated and characterized for antagonistic traits.
- Bacterial strains have antibiotic genes and produced siderophores.
- Eleven strains were in vitro antagonists of the GTDs fungal pathogen N. parvum.
- B6 strain reduced the length of wood necrosis caused by N. parvum.

Graphical Abstract

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Abstract

Vineyards throughout the world, including Tunisia, are being attacked by Grapevine Trunk Diseases (GTDs) such as Esca and Botryosphaeria dieback. In this study, the bacterial microflora colonizing the non-necrotic and necrotic wood tissues of Tunisian mature grapevines (cv Muscat d’Italie) was investigated. Both types of tissues were studied in order to decipher microbial communities associated with them and to find a suitable BCA that can be applied to the Tunisian terroir. Single-Strand Conformation Polymorphism (SSCP) analyses showed that complex bacterial communities specifically colonized both types of wood tissues. The 19 most abundant cultivable strains, selected on their morphology, were isolated from plant samples and assigned to Pantoea, Pseudomonas, Curtobacterium and Bacillus species based on the 16S rRNA and rpoB genes. Biochemical and microbiological screenings revealed that those 19 strains (i) metabolized differently carbon sources, even within the same species, (ii) possessed antibiotic genes, (iii) produced siderophores and solubilized phosphates and (iv) had an in vitro antagonistic effect against 3 fungal pathogens (Lasiodiplodia theobromae, Neofusicoccum parvum and Schizolyphium commune) involved in GTDs. One strain, Bacillus subtillis “B6”, had a positive effect on young vines of a cultivar, Muscat d’Italie, frequently planted in Tunisia, by reducing the size of the wood necrosis caused by N. parvum, showing its potential to counteract infection caused by this GTDs agent.

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1. Introduction

Plants, like many other organisms, are colonized by bacteria inside or around them (Sessitsch et al., 2002; Berg et al., 2005; Compant et al., 2010). They colonize various plant tissues and organs: roots, leaves, flower clusters, seeds and fruits (Gray and Smith, 2005; Martins et al., 2013) and have been described as inhabitants of specific microenvironments of the phyllosphere, rhizosphere or other parts of the phytosphere, and the plant endosphere (James et al., 2002; Compant et al., 2010). Those microorganisms can be endophytic (Compant et al., 2011) as they are able to live inside plants tissues without causing diseases to their host (Iniguez et al., 2005). Via the vascular system they can move into plants from roots to leaves, flowers and fruits (Hardoin et al., 2008; Compant et al., 2011). Those bacteria have many important beneficial roles in the metabolism and physiology of the host plant. They can (i) stimulate the growth of plants by synthesizing plant growth hormones or enzymes (Hardoin et al., 2008; Bulgari et al., 2009; Compant et al., 2010, 2011; West et al., 2010); (ii) promote resistance of plants by inducing host-defense mechanisms (Iniguez et al., 2005; Miché et al., 2006; Rosenblueth and Martinez-Romero, 2006); (iii) control diseases by suppressing pathogens (Whipp, 2001) and/or (iv) solubilizing phosphates (Whipp, 2001; Hurek and Reinhold-Hurek, 2003) and producing siderophores (O'Sullivan and O'Gara, 1992).

Bacteria have been isolated from many crops, among which sugar beet, cotton (Misaghi and Donndeling, 1990), rice, soybean (Kulkinsky-Sobral et al., 2005), cucumber, potato and tomato (Sessitsch et al., 2005). They were also demonstrated to colonize the root system, phyllosphere and wood tissues of grapevine, Vitis vinifera (Compant et al., 2005a,b, 2008, 2011; Trotel-Azziz et al., 2008; West et al., 2010; Alfonzo et al., 2012; Marasco et al., 2013; Pinto et al., 2014; Bruez et al., 2015). Studies on bacteria inhabiting the wood tissues of grapevine were recently carried out by our group (Bruez et al., 2015; Haidar et al., 2016) but as many pieces of information are still lacking, our objective was thus to study for the first time, the bacterial microflora colonizing the wood of Tunisian mature grapevines and to select potential antagonistic strains against three fungal pathogens involved in two major Grapevine Trunk Diseases (GTDs), i.e. Esca and Botryosphaeria dieback. Two fungi, Lasiodiplodia pseudotheobromae and Schizaphylum commune, are involved in Esca and one, Neofusicoccum parvum, is involved in Botryosphaeria dieback. These GTDs affect the wood tissues of grapevines, a sampling was carried out in summer 2013, i.e. 12 necrotic (NW), 12 non-necrotic (HW) and 6 cordon ends (CE: thin cordon that can’t be cut longitudinally to determine the type of wood). All the wood samples were ground in liquid nitrogen with a TissueLyser II (Qiagen) and kept at −80 °C prior to DNA extraction.

DNA was extracted from 60-mg aliquots of woody tissues with the Indisorb Spin Plant mini Kit (Invitek) according to the manufacturer’s instructions. The DNA extracts were then quantified with a nanodrop (ND-1000, Thermoscientific, Labtech) and homogenized at a concentration of 10 ng µL−1.

A pair of primers recognizing the V5–V6 region of the 16S rRNA gene (799f/1115r) was used (Table 1). DNA was amplified by PCR in an Epgradient Mastercycler (Eppendorf) in a reaction mixture (25 µL final volume) consisting of 1 µL of DNA template (10 ng µL−1), 2.5 µL of 10× Pfu buffer (Agilent Technologies), 1 µL of dNTP (10 mM), 0.5 µL of each primer (20 µM), 2.5 µL of BSA (10 µg µL−1) (New England BioLabs), 0.5 µL of Pfu Turbo (Agilent Technologies) and 16.5 µL of sterile distilled water. The cycling conditions were as follows: enzyme activation at 95 °C for 2 min; 25 cycles of denaturation at 95 °C for 45 s, hybridization at 54 °C for 30 s and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR products were visualized by 2% TBE agarose gel electrophoresis prior to SSCP analysis.

SSCP analyses were performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) equipped with four 36-cm long capillaries. One microliter of a PCR product was mixed with 18.8 µL formamide Hi-Di (Applied Biosystems) and 0.2 µL standard internal DNA molecular weight marker Genescan 400 HD ROX (Applied Biosystems). The sample mixture was denatured at 95 °C for 5 min and immediately cooled on ice, and then loaded onto the instrument. The non-denaturing polymer consisted of 5.6% POP conformational analysis polymer (Applied Biosystems), 10% glycerol,
EDTA buffer 10× (Applied Biosystems) and water. The migration time was set to 2000 s, the voltage to 15 kV and the temperature was 32 °C.

Samples were co-migrated with the fluorescent size standard (Genescan-400 ROX) to allow comparison of migration profiles between samples. Patterns were aligned with StatFingerprints (version 2.0) and studied by Principal Components Analyses with R (version 3.1.3.). In the Vegan R-Package, the Anosim test using Bray-Curtis distance was employed to compare the data.

### 2.3. Isolation of bacteria from the wood tissues of mature grapevines

For each of the 10 vine cordons collected, 15 chips (around 5 mm in length) of wood tissues were randomly sampled. The wood fragments were surface disinfected by immersion in 70% ethanol for 1 min followed by 2.5% calcium hypochlorite solution for 3 min. Then, the samples were rinsed 3 times in sterile distilled water, dried on a sterile filter paper and 15 sterilized chips were plated onto R2A agar (Sigma) amended with 100 mg L⁻¹ cycloheximide (Sigma) for 48 h at 27 °C.

Sixty-nine bacterial strains were recovered from the 150 wood pieces collected. The 19 most abundant were selected based on morphological differences, subsequently purified onto R2A agar (Sigma) and characterized by using three biochemical tests following the standard procedures: (i) Gram staining, (ii) the catalase and (iii) oxidase activities, before being maintained on cryogenic storage beads (Cryosystème Protect, Dutscher) at −20 °C.

### 2.4. Identification of bacteria by sequencing the 16S rRNA and rpoB genes

Genomic DNA from the 19 selected bacterial strains was extracted from pure cultures grown in TSB (Tryptone Soy Broth, Conda) by using the commercial kit Invisorb Spin Plant Mini Kit
were performed in triplicate. The percentage of growth inhibition plates were made with only the mycelium plug. All experiments consisting of 3 GTDs pathogenic fungi, to compare the data. The 16S rRNA and rpoB sequences are available at the GenBank database respectively with the primers 799f and 1429r, and rpoBf and rpoBr (Table 1). For species level identification, sequences were compared with the GenBank database by using the Blastn program (Altschul et al., 1997).

The 16S rRNA and rpoB sequences were sequenced in triplicate. The percentage of growth inhibition was calculated using the formula, \((R_1 - R_2)/R_2 \times 100\), where \(R_1\) is the radial distance (mm) grown by pathogenic fungi in the direction of the antagonist, and \(R_2\) is the radial distance (mm) grown by pathogenic fungi (Whipps, 1987).

The ability to excrete siderophores, i.e. iron-complexing organic ligands known to provide a competitive advantage to microorganisms in iron-depleted environments (Duijff et al., 1994; Lugtenberg and Dekkers, 1999), was evaluated under Fe\(^{3+}\) limiting conditions by a plate assay adapted from Schwyn and Neilands (1987). Fresh cultures were plated onto CAS blue-agar [2.5% nutrient broth (NB, Conda), 1.5% agar, 0.1 M piperazin-1,4-bisethanesulfonic acid (PIPES), 10 \(\mu\)M Chrome Azurol S (Sigma) and 0.2 mM hexade-cytrtrimethylammonium bromide (HDTMA, Sigma)]. When Fe\(^{3+}\) was removed from the Chrome Azurol S complex by high-affinity bacterial siderophores, the color of plates changed from blue to orange.

Siderophore production was then measured after one week of incubation based on the size of the orange haloes (ds) formed around the colonies. The strains were denoted sid’t, sid” and sid”” respectively when \(0 \lt ds \lt 5 \text{ mm}\), \(5 \lt ds \lt 10 \text{ mm}\) and \(ds \gt 10 \text{ mm}\). The experiment was made in triplicate.

The 19 selected bacterial strains were screened for the production of lipopeptide (LP) antibiotics by using specific primers that amplify genes from the fengycin and iturin families. Four genes of 4 fengycin (A, B, D and E) and 1 of iturin (a bacillomycin gene) (Lin et al., 1998; Ramarathnam et al., 2007; Alvarez et al., 2011) were searched for. PCR assays were performed in a Mastercycler Gradient Thermocycler (Eppendorf) in 30 \(\mu\)L reaction volume consisting of 3 \(\mu\)L of buffer (10X), 1 \(\mu\)L of MgCl\(_2\) (50 mM), 0.6 \(\mu\)L of dNTP (10 mM), 0.6 \(\mu\)L of each primer (Table 1), 3 \(\mu\)L of BSA (10 \(\mu\)g \(\mu\)L\(^{-1}\)). PCR products were visualized by 2\% TBE gel electrophoresis.

2.6. Community-Level Physiological Profiling (CLPP) of bacteria

The isolated bacteria strains were distributed in 96-well Biolog\(^{TM}\) Ecoplates (AWEL International) (150 \(\mu\)L/well with a concentration equivalent to \(10^6\)–\(10^8\) CFU ML\(^{-1}\) determined by comparison with a McFarland standard solution, bioMérieux\(^{TM}\) SA).

These plates check simultaneously the metabolic reaction of the bacterial suspensions regarding 31 lyophilized substrates and a negative control present in triplicate on each plate. The plates were incubated at 27 \(^\circ\)C in the dark. Optical density (OD) readings were recorded for each plate at 590 nm with a microplate reader (Multimo microplate reader, Synergy HT, Biotek) after 24 h, 48 h and 5 days of incubation. To minimize the effect of differences between plates, data were standardized as follows: the average well color development (ACWD) was calculated for each plate; then, the blanked absorbance value of each well was divided by the ACWD of the corresponding plate to get a corrected OD value (Garland and Mills, 1991). All corrected OD values were set to fall between 0 and 2 (boundary limits) and were then used for Principal Components Analyses with R (version 3.1.3.). In the Vegan R Package, the Anosim test using Bray-Curtis distance was employed to compare the data.

2.7. In vitro antagonism of bacteria against three GTDs pathogenic fungi

The antagonistic capacity of the 19 bacterial isolates against 3 GTDs pathogenic fungi, i.e. L. pseudotheobromae, N. parvum, and S. commune, was determined by employing dual culture technique. Bacterial strains were streaked at the edges of Petri plates containing PDA (Potato Dextrose Agar, Biokar diagnostics, France) and incubated at 28 \(^\circ\)C for 24 h. For each pathogenic fungus, a 6 mm mycelial plug was placed on the center of each plate. The plates were then incubated at 28 \(^\circ\)C for 5 days. For each fungus, control plates were made with only the mycelium plug. All experiments were performed in triplicate. The percentage of growth inhibition was calculated using the formula, \((R_1 - R_2)/R_2 \times 100\), where \(R_1\) is the radial distance (mm) grown by pathogenic fungi in the direction of the antagonist, and \(R_2\) is the radial distance (mm) grown by pathogenic fungi (Whipps, 1987).

2.8. Siderophore production by bacteria

The ability to excrete siderophores, i.e. iron-complexing organic ligands known to provide a competitive advantage to microorganisms in iron-depleted environments (Duijff et al., 1994; Lugtenberg and Dekkers, 1999), was evaluated under Fe\(^{3+}\) limiting conditions by a plate assay adapted from Schwyn and Neilands (1987). Fresh cultures were plated onto CAS blue-agar [2.5% nutrient broth (NB, Conda), 1.5% agar, 0.1 M piperazin-1,4-bisethanesulfonic acid (PIPES), 10 \(\mu\)M Chrome Azurol S (Sigma) and 0.2 mM hexadecytrtrimethylammonium bromide (HDTMA, Sigma)]. When Fe\(^{3+}\) was removed from the Chrome Azurol S complex by high-affinity bacterial siderophores, the color of plates changed from blue to orange.
2.10.3. The bacterial strain B6 used to protect plants

The bacterial strain tested for its potential antagonistic ability was a Bacillus subtilis strain B6 isolated from the inner trunk and selected based on the in vitro antagonism assay described above. Before the inoculation, the bacterial strain was grown at 28 °C for 24 h in TSB (Tryptocasein Soy Broth, Conda, Spain).

2.10.4. Bacterial strain B6 and N. parvum co-inoculation

Before the co-inoculation, the stem of each plant was surface-sterilized with 95% ethanol, then, the center of each stem cutting, below the upper bud, was artificially wounded by drilling a hole in the pith (4 mm diameter). The wound was then immediately drop-inoculated with 40 μL of the bacterial strain B6 suspension (10^8–10^9 CFU mL^-1 determined by comparison with a McFarland standard solution, bioMérieux® SA). Once the liquid was dried 20–40 min at air temperature, the hole was filled with a N. parvum mycelium plug excised from the margin of a fresh mycelial MA culture, with the mycelium facing the internal part of the stem. The treated wounds were then wrapped with a plastic film for being protected during all the incubation period.

2.10.5. Symptoms assessment

After an incubation period of 120 ± 5 days, the presence of external cankers was assessed visually on every cutting. Then, the bark was removed and the stem of each plant was cut longitudinally, and the percentage of the internal vascular lesions or necrosis in the cutting was recorded by dividing the size of necrosis downwards from the wound-inoculation hole “SN” by the size of the shoot “SS” [(SN/SS) * 100] (Fig. 5A).

2.10.6. Statistical analyses

All the statistical analyses were done using R statistical software, version 3.1.3. The data were first subjected to the Shapiro-Wilk and Levene’s tests to check the normality and equality of variances before being subsequently subjected to the non-parametric Kruskal-Wallis test and the relative contrast effects analysed by the nparcomp package (version 2.0). For SSCP and CLPP data, in the Vegan R-Package, the Anosim test using Bray-Curtis distance was employed to compare the data.

3. Results

3.1. Genetic structure of the bacterial communities inhabiting the wood tissues of Tunisian mature grapevines

A total of 30 SSCP profiles were generated from the wood samples collected in Tunisian vineyards. According to the number of peaks and the relative height of the baseline, the SSCP profiles revealed complex bacterial communities (data not shown). The distribution of the samples on the principal plan generated by the PCA analysis is represented in Fig. 2. PCA eigenvalues indicate that the first two principal components, Dim1 and Dim2, account for 51% of the total bacterial variability. Bacterial communities differed depending on the type of tissue they inhabited (P < 0.01), i.e. healthy (non-necrotic) (HW), necrotic (NW) and cordon ends (CE).

3.2. Characterization of the bacterial strains isolated from the grapevine wood samples

Based on partial 16S rRNA and rpoB genes sequence similarity (Table 2), the bacterial strains belonged to Bacillus species (12 strains), i.e. Bacillus invictae (7 strains), Bacillus safensis (2 strains), Bacillus amylolyquefaciens (1 strain), Bacillus pumilus (1 strain) and Bacillus subtilis (1 strain). Other bacterial species were identified: Pantoea agglomerans (4 strains), Curtobacterium flaccumfaciens (1 strain) and Pseudomonas species (2 strains), i.e. Pseudomonas sp. (1 strain) and Pseudomonas fluorescens (1 strain).

The 5 antibiotic encoding genes were detected in only one strain, B. subtilis strain B6. Another strain, B. invictae strain B14, possessed the 4 fengycin genes while the other Bacillus strains expressed at least 1 out of 4 genes. Three out of 4 of the P. agglomerans strains had one of the 5 genes investigated. One isolate of P. fluorescens had one fengycin gene, whereas no gene detection was obtained for Pseudomonas strain B16. For C. flaccumfaciens strain B1, none of the 5 genes were detected (Table 2).

Regarding the PGP traits, i.e. (i) phosphate solubilisation and (ii) siderophores production, 13 strains demonstrated both of the characteristics evaluated (Table 2).

(i) All the bacterial strains produced siderophores: 15 strains developed small haloes, ranging from 1 to 5 mm (sid+ strains); and B. invictae strain B14 and Pseudomonas sp. strain B16 produced the greater halo zones, respectively 23 and 14 mm.

(ii) Thirteen strains developed sharp phosphate solubilisation zones, ranging from 5 to 30 mm and 9–37 mm, respectively after 10 and 15 days. Five strains, i.e. 3 Bacillus spp. (strains B2, B3, B4) and two P. agglomerans (strains B10, B17), produced zones greater than 20 mm. B. safensis B4 was the most efficient phosphate solubilizer (37 mm after 15 days).

3.3. Community-level physiological profiles of the isolated bacteria

The strains distribution on the principal plan generated by the PCA is represented in Fig. 3. PCA eigenvalues indicate that Dim1 and Dim 2 account for 50% of the total variability. In comparison with Bacillus spp. strains, those of P. agglomerans were more scattered on Dim1 (Fig. 3). No distinctive patterns in their use of carbon sources were obtained with the 19 bacterial strains (P > 0.05) (data not shown). The calculation of Average Well Color Development (AWCD) for each replicate showed that the 19 strains were able to transform at least 7 carbon sources after 24 h, 48 h and 120 h incubation. P. agglomerans B19 was the strain that metabolized the most carbon sources after 120 h incubation (data not shown). No differences were observed for bacteria isolated in healthy or necrotic wood tissues (P > 0.05) (data not shown).
3.4. In vitro bacterial antagonism against three GTDs pathogenic fungi

The 19 bacterial strains showed variable inhibition percentages for the three fungal pathogens tested, i.e. *L. pseudotheobromae* (*P* < 0.01), *N. parvum* (*P* < 0.01), and *S. commune* (*P* < 0.01). They ranged from 2.5% to 81.5%, the mean being 24.9% (Table 3). Of the 19 bacteria screened for their antagonistic ability, 6 strains had an inhibitory effect on the 3 fungal pathogens (strains B4, B6, B11, B15, B16 and B18), 11 strains only inhibited *N. parvum* (Fig. 4), 9 strains inhibited *L. pseudotheobromae* and 18 strains inhibited *S. commune*. The most efficient antagonistic bacteria for the 3 pathogenic fungi was *B. subtilis* B6 (Table 3).

3.5. Assessment of plant protection induced by bacterial strain B6 against *N. parvum* attack

All the stems of Muscat d'Italie cultivar co-inoculated with *N. parvum* and the bacterial strain B6, showed internal necrotic lesions. For the control cuttings inoculated with the pathogen *N. parvum* only, the percentage of necrosis was 80% at the end of the 120 ± 5 day-incubation period. *B. subtilis* strain B6 significantly reduced the percentage of necrosis (*P* < 0.05) caused by *N. parvum* by 35% when compared with control cuttings inoculated by *N. parvum* only (Fig. 5B and C). A slight lesion, due to the drilling effect, was observed after the inoculation of stems with bacteria in the absence of the fungal pathogen and in control cuttings “CP” inoculated with a sterile MA plug (Fig. 5C).

4. Discussion

To our knowledge, this report is the first that describes by molecular, microbiological and biochemical approaches that diverse bacterial communities inhabit the wood tissues of Tunisian grapevines, some of these bacteria being endowed with antagonistic activities against one GTDs pathogenic fungus, i.e. *N. parvum*.
Necrotic and non-necrotic tissues were present in cordon of grapevines showing external GTDs-foliar symptoms. This observation is in line with Maher et al. (2012) and Bruez et al. (2014, 2015) who sampled esca-symptomatic or asymptomatic grapevines in French vineyards. Molecular fingerprinting analyses (SSCP) revealed that complex and diverse bacterial communities inhabit the inner wood tissues of Tunisian grapevines. These communities are tissue-dependent since they differed in the healthy and necrotic wood tissues. The same type of information was reported by Chabot et al. (1993, 1996a,b) showing that two strains of Bacillus subtilis strain B4 was the most efficient to solubilize phosphate. The ability of bacterial strains to act as fungal antagonists was shown they have a wide spectrum of antifungal activity (Alfonzo et al., 2012) and they can protect grapevines (Trotel-Aziz et al., 2008; Haidar et al., 2016) or tomato plants from pathogenic attacks (Sadfi-Zouaoui et al., 2008). Consequently, our results support these earlier reports by showing that bacterial strains inhabiting the wood tissues of Tunisian mature grapevines have antagonistic traits (e.g., antibiotic genes, siderophore production, carbon source use) or are able to control a pathogen involved in GTDs.

If we focus on these 3 antagonistic traits, it can be mentioned that:

(i) All the Bacillus isolated strains expressed differentially at least one of the 5 antibiotic genes investigated, with only the strain B. subtilis B6 having the 5 encoding genes. These results agree with Jacques et al. (1999) on B. subtilis and Koumoutsi et al. (2004) on B. amyloliquefaciens, to produce fengycins. According to Hofemeister et al. (2004), Koumoutsi et al. (2004) and Jourdan et al. (2009), the function of the fengycin and iturin families is related to antimicrobial properties. For instance, they display specific fungitoxic action against filamentous fungi (Nishikori et al., 1986). Ongena and Jaques (2007) reported that iturin family has a strong in vitro antifungal action against a wide variety of yeasts and fungi.

(ii) For siderophore production, Pseudomonas sp. strain B16 was the highest producer. Strains from this genus are frequently reported as siderophore producers, as shown by Cabrefiga et al. (2007) and Renault et al. (2007). Siderophores are known to play a role in the control of some plant diseases and in the suppression of several phytopathogens (Duffy and Défago, 1999; Whippes, 2001).

(iii) As regards the global metabolism of the isolated bacteria and their ability to compete for nutrients, the carbohydrates group was the most intensively metabolized by the 19 strains after 24 h, 48 h and 120 h, with P. agglomerans strain B19 being the highest metabolizer.

Based on the contribution of phosphate-solubilizing bacteria in plant nutrition (Goldstein, 1986) and plant growth performance (Rodriguez and Fraga, 1999), it was also shown that the bacteria isolated from the wood of grapevines have another trait, phosphate solubilisation, that can be useful to select a bacterial strain. For instance, B. safensis strain B4 was the most efficient to solubilize phosphate. This beneficial effect on plants has been reported many times; for example, Chabot et al. (1993, 1996a,b) showed that two strains of Rhizobium leguminosarum, capable of mineral phosphate solubilization, were able to stimulate the growth of maize and lettuce.

The ability of bacterial strains to act as fungal antagonists was assessed in a dual-culture assay. The isolated bacteria were used to control 3 plant pathogenic fungi involved in GTDs, i.e. N. parvum, S. commune and L. pseudoeurobium. In the in vitro assay, the greater inhibition zone of the 3 fungal pathogens was obtained with the B. subtilis strain B6. This strain was chosen for the in planta assay. In another experiment, Alfonzo et al. (2008) reported that B. subtilis strains inhibit the in vitro growth of 3 other pathogenic fungi involved in Esca, i.e. Phaeoacremonium aleophilum, Phaeomniella chlamydospora and Fomitiporia mediterranea. So, B. subtilis strains seems to be relevant BCA candidates to control pathogens of GTDs.

The greenhouse assay on cv. Muscat d’Italie cuttings showed that, B. subtilis strain B6, reduced by 35%, the size of necrosis when
Table 2
Description and in vitro screening results of the bacterial strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Tissue</th>
<th>Biochemical screening</th>
<th>Molecular screening</th>
<th>Antagonistic* and PGPb traits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gram Cat Oxi</td>
<td>16S rDNA gene (GeneBank ref, %id)</td>
<td>Bacc FeA FeB FeD FeE Siderophore production Phosphate solubilisation</td>
</tr>
<tr>
<td>B1</td>
<td>HW</td>
<td>+ + –</td>
<td>Curtobacterium flaccumfaciens (JN689331.1, 100%)</td>
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</tr>
<tr>
<td>B2</td>
<td>NW</td>
<td>+ + +</td>
<td>Bacillus sp. (JX831701.99%)</td>
<td>– + – – – ++ ++</td>
</tr>
<tr>
<td>B3</td>
<td>HW</td>
<td>+ + +</td>
<td>Bacillus sp. (JX831817.99%)</td>
<td>+ + – – + ++</td>
</tr>
<tr>
<td>B4</td>
<td>NW</td>
<td>+ + +</td>
<td>Bacillus sp. (JX831817.99%)</td>
<td>+ + – – – + ++</td>
</tr>
<tr>
<td>B5</td>
<td>NW</td>
<td>– + +</td>
<td>Pseudomonas fluorescens (KCB10843.1, 99%)</td>
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<tr>
<td>B6</td>
<td>NW</td>
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<td>Bacillus sp. (CP002905.1, 96%)</td>
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<td>HW</td>
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<td>B9</td>
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<td>Bacillus sp. (JX831701.99%)</td>
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<tr>
<td>B10</td>
<td>CE</td>
<td>– + –</td>
<td>Pantoea agglomerans (FJ57814.1, 99%)</td>
<td>– – + – + +</td>
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<tr>
<td>B11</td>
<td>CE</td>
<td>+ + +</td>
<td>Bacillus sp. (CP003332.1, 99%)</td>
<td>+ + + + + + ++</td>
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<tr>
<td>B12</td>
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<td>Pantoea agglomerans (FJ57814.1, 99%)</td>
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<tr>
<td>B13</td>
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<td>Bacillus sp. (FJ57814.1, 99%)</td>
<td>+ + + + + + ++</td>
</tr>
<tr>
<td>B14</td>
<td>NW</td>
<td>+ + +</td>
<td>Bacillus pumilus (JX831701.99%)</td>
<td>+ + + + ++ ++</td>
</tr>
<tr>
<td>B15</td>
<td>NW</td>
<td>+ + +</td>
<td>Bacillus sp. (JX831701.99%)</td>
<td>+ + + + ++ ++</td>
</tr>
<tr>
<td>B16</td>
<td>NW</td>
<td>– + –</td>
<td>Pseudomonas sp. (JX183170.1, 99%)</td>
<td>– – – – + +</td>
</tr>
<tr>
<td>B17</td>
<td>NW</td>
<td>+ + –</td>
<td>Pantoea agglomerans (FJ357814.1, 99%)</td>
<td>– – – – ++ ++</td>
</tr>
<tr>
<td>B18</td>
<td>NW</td>
<td>+ + +</td>
<td>Bacillus sp. (FJ57814.1, 99%)</td>
<td>+ + – – + +</td>
</tr>
<tr>
<td>B19</td>
<td>NW</td>
<td>+ + –</td>
<td>Pantoea agglomerans (FJ57814.1, 99%)</td>
<td>– – – – + +</td>
</tr>
</tbody>
</table>


Table 3

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>N. parvum GI (%) (±SE)</th>
<th>L. pseudotheobromae GI (%) (±SE)</th>
<th>S. commune GI (%) (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0.0 ± 0.0 a</td>
<td>2.5 ± 0.4 ab</td>
<td>14.9 ± 0.7 de</td>
</tr>
<tr>
<td>B2</td>
<td>0.0 ± 0.0 a</td>
<td>33.9 ± 2.7 d</td>
<td>13.6 ± 1.2 de</td>
</tr>
<tr>
<td>B3</td>
<td>25.3 ± 2.1 e</td>
<td>0.0 ± 0.0 a</td>
<td>12.1 ± 0.2 ce</td>
</tr>
<tr>
<td>B4</td>
<td>13.7 ± 0.2 c</td>
<td>7.0 ± 0.8 b</td>
<td>5.8 ± 0.3 b</td>
</tr>
<tr>
<td>B5</td>
<td>0.0 ± 0.0 a</td>
<td>0.0 ± 0.0 a</td>
<td>7.7 ± 0.3 bc</td>
</tr>
<tr>
<td>B6</td>
<td>81.5 ± 0.7 b</td>
<td>70.8 ± 3.8 f</td>
<td>67.6 ± 1.7 b</td>
</tr>
<tr>
<td>B7</td>
<td>0.0 ± 0.0 a</td>
<td>0.0 ± 0.0 a</td>
<td>16.0 ± 1.0 e</td>
</tr>
<tr>
<td>B8</td>
<td>0.0 ± 0.0 a</td>
<td>0.0 ± 0.0 a</td>
<td>10.7 ± 1.4 cd</td>
</tr>
<tr>
<td>B9</td>
<td>29.1 ± 2.7 e</td>
<td>0.0 ± 0.0 a</td>
<td>36.7 ± 2.7 g</td>
</tr>
<tr>
<td>B10</td>
<td>0.0 ± 0.0 a</td>
<td>0.0 ± 0.0 a</td>
<td>3.7 ± 0.6 ab</td>
</tr>
<tr>
<td>B11</td>
<td>71.2 ± 1.8 g</td>
<td>62.3 ± 2.0 e</td>
<td>33.2 ± 2.3 g</td>
</tr>
<tr>
<td>B12</td>
<td>0.0 ± 0.0 a</td>
<td>0.0 ± 0.0 a</td>
<td>3.3 ± 0.2 ab</td>
</tr>
<tr>
<td>B13</td>
<td>26.6 ± 1.3 e</td>
<td>0.0 ± 0.0 a</td>
<td>7.5 ± 0.5 bc</td>
</tr>
<tr>
<td>B14</td>
<td>21.7 ± 0.4 d</td>
<td>5.1 ± 0.6 b</td>
<td>0.0 ± 0.0 a</td>
</tr>
<tr>
<td>B15</td>
<td>34.4 ± 1.7 f</td>
<td>28.2 ± 1.3 c</td>
<td>12.6 ± 1.0 de</td>
</tr>
<tr>
<td>B16</td>
<td>12.7 ± 0.4 c</td>
<td>3.8 ± 0.4 ab</td>
<td>10.7 ± 2.0 cd</td>
</tr>
<tr>
<td>B17</td>
<td>6.7 ± 0.2 b</td>
<td>0.0 ± 0.0 a</td>
<td>15.1 ± 0.9 de</td>
</tr>
<tr>
<td>B18</td>
<td>13.9 ± 1.9 c</td>
<td>34.8 ± 0.4 d</td>
<td>21.6 ± 1.8 f</td>
</tr>
<tr>
<td>B19</td>
<td>0.0 ± 0.0 a</td>
<td>0.0 ± 0.0 a</td>
<td>5.3 ± 0.5 b</td>
</tr>
</tbody>
</table>

* Percentages of mycelial growth inhibition were determined after 5 days of incubation using Whipp’s formula (1987). Mean values sharing the same letters are not significantly different according to Kruskal-Wallis’ non-parametric relative contrast effects post hoc test at P < 0.05.

it was co-inoculated in planta with the pathogen N. parvum. This finding is in agreement with previous reports on the capacity of Bacillus strains to control plant diseases (Emmert and Handelsman, 1999; Collins et al., 2003; Toure et al., 2004; Trottel-Aziz et al., 2008; Essgaier et al., 2014) and is of the utmost importance because N. parvum is a GTDs fungal pathogen for which no efficient treatments are currently available.

To conclude, our study showed that various bacterial strains colonize the wood tissues of grapevine planted in Tunisia. The bacterial strain B. subtilis B6 was the most interesting bacterium to control wood necrosis caused by N. parvum on the table grape cultivar Muscat d’Italie, the most widespread cultivar planted in Tunisia. Further research should be carried out in the vineyards to test the ability of the strain B6, but also of other isolated bacteria that have interesting BCA traits, to control infections by N. parvum and the other fungi involved in GTDs.

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References


