Biological Control 99 (2016) 28–37

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

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

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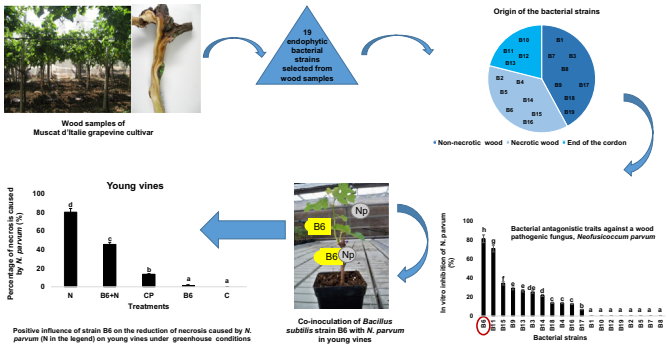
HIGHLIGHTS

- 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* in young vines.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 22 December 2015
Revised 11 April 2016
Accepted 13 April 2016
Available online 13 April 2016

Keywords:
Bacterial communities
Grapevine Trunk Diseases
Plant protection
Fingerprinting method
Wood necrosis

ABSTRACT

Vineyards throughout the world, including Tunisia, are being attacked by Grapevine Trunk Diseases (GTDs) such as Esca and Botryosphaeriae 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 *rpo*B 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 (*Lasidiodiplodia pseudotheobromae*, *Neofusicoccum parvum* and *Schizophyllum commune*) involved in GTDs. One strain, *Bacillus subtilis* "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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http://dx.doi.org/10.1016/j.biocontrol.2016.04.005
1049-9644/

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 (Hardoim 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 (Hardoim 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 Martínez-Romero, 2006); (iii) control diseases by suppressing pathogens (Whipps, 2001) and/or (iv) solubilizing phosphates (Whipps, 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 Donndelinger, 1990), rice, soybean (Kuklinsky-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-Aziz 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 Botryosphaeriae dieback. Two fungi, *Lasidiodiplodia pseudotheobromae* and *Schizophyllum commune*, are involved in Esca and one, *Neofusicoccum parvum*, is involved in Botryosphaeriae dieback. These GTDs affect the wood of grapevines and subsequently lead to plant death (Bertsch et al., 2013). They have become, over the last two decades, a subject of major concern for the wine industry in France (Bruez et al., 2013) and in many countries (Bertsch et al., 2013). For instance, besides reducing the plant life span, GTDs have also a negative impact on wine quality as demonstrated by Lorrain et al. (2012).

In that context, this study was conducted in order to investigate bacterial microflora associated with non-necrotic and necrotic wood tissues of mature Tunisian grapevines that showed external GTDs-foliar symptoms. Bacteria were selected and screened for their potential to act as BCAs. Different approaches were used: (i) the genetic structure of the bacterial communities was studied using the fingerprinting method, Single Strand Conformation Polymorphism (SSCP). (ii) From these bacterial communities, bacterial strains within both types of wood tissues were isolated and identified by sequencing the 16S rRNA and *rpo*B genes. (iii) The abilities of the selected bacteria in terms of control of plant pathogens: detection of antibiotics genes (4 fengycins coded A, B, D and E; 1 bacillomycin), degradation of different sole-carbon sources, production of siderophores and phosphate solubilization, and *in vitro* confrontations against three GTDs plant pathogenic fungi, *i.e. L. pseudotheobromae*, *N. parvum* and *S. commune*, were tested. One bacterial strain was then selected for an *in planta* assay on young vine of Muscat d'Italie, a cultivar frequently planted in Tunisia, to test its ability to reduce the development of wood necrosis caused by *N. parvum*.

2. Materials and methods

2.1. Plant material and sampling

In order to study the bacterial microflora inhabiting the wood tissues of Tunisian grapevines, a sampling was carried out in summer 2013 in four vineyards (Fig. 1A) located in the North of Tunisia. These vineyards consisted of mature vines (15–17-year old plants) of a table grape cultivar, Muscat d'Italie, grafted on rootstock P1103 and trellised as single vines on a pergola system (Fig. 1B). They were irrigated with a drip irrigation system and covered by nets (Fig. 1C) to protect the plants from hail.

Cordons were randomly collected from 10 mature vines that showed external GTDs-foliar symptoms in the four vineyards surveyed and cut longitudinally with an electric saw. From the inner part of each cordon, wood samples were taken from necrotic and/or from non-necrotic (apparently healthy) tissues, cut and stored before being processed for downstream analyses. For each wood tissues collected, the samples consisted of wood chips (around 5 mm in length) for microbiological analyses and approximately 5 g of tissue for molecular ones.

2.2. Analysis of the microbial communities colonizing the woody tissues by Single Strand Conformation Polymorphism (SSCP)

SSCP analyses of the bacterial communities were performed on the 30 wood samples taken from the vine cordons collected in Tunisia 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 TissueLyserII (Qiagen) and kept at −80 °C prior to DNA extraction.

DNA was extracted from 60-mg aliquots of woody tissues with the Indvisorb 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,

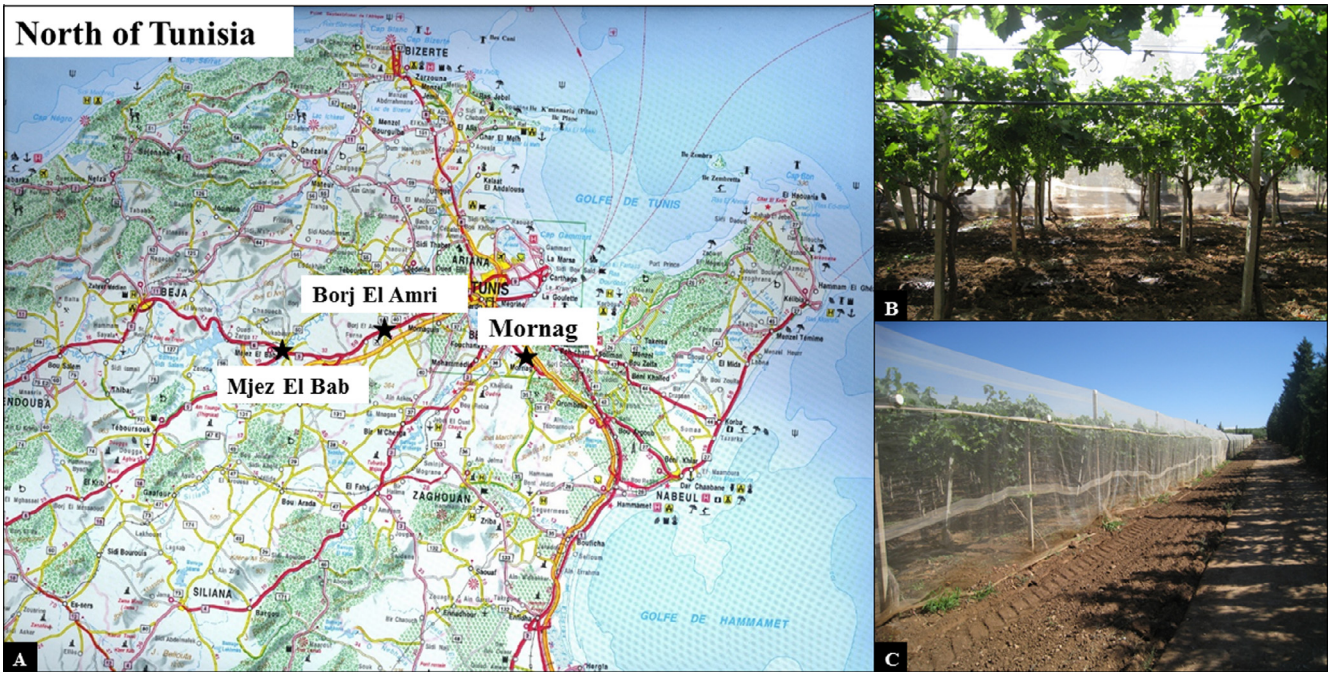

Fig. 1. Sampling sites in the North of Tunisia (A) with Muscat d'Italie cultivar trellised as single vines on a pergola system (B) with nets to protect plants from hail (C).

Table 1
Pairs of primers used.

Applications		Gene	Primer name	Sequence 5′3′	Amplicon size	Annealing T °C
Antibiotics screening	Lipopeptide	Fengycin	FENAf	GACAGTGCTGCCTGATGAAA	900 bp	54 °C
			FENAr	GTCGGTGCATGAAATGTACG		
			FENBf	ATCCATGGTTAAAAACCAAAAT	950 bp	54 °C
			FENBr	ACGGATCCATGCTATTTGGCAGC		
			FENDf	TTTGGCAGCAGGAGAAGTTT	950 bp	53 °C
			FENDr	GCTGTCCGTTCTGCTTTTTC		
			FENEf	GCCAAAAAGAAACGAGCAG	950 bp	53 °C
			FENEr	GTCGGAGCTAACGCTGAAAC		
		Bacillomycin	BACCf	GAAGGACACGGCAGAGAGT	900 bp	60 °C
			BACCr	CGCTGATGACTGTTCATGC		
SSCP	Bacteria	16S rRNA	799f[b]	AACMGGATTAGATACCCKG	350 bp	54 °C
			1115r[a,b]	**6-FAM** AGGGTTGCGCTCGTTG		
Sequencing	Bacteria	16S rRNA	799f	AACMGGATTAGATACCCKG	750 bp	52 °C
			1492r	GTTACCTTGTTACGACTT		
		*rpo*B	*rpo*Bf	GACGATCATYTWGGAAACCG	350 bp	55 °C
			*rpo*Br	GGNGTYTCRATYGGACACAT		

[a] Fluorescently labelled primer 6-FAM (6-carboxyfluoresceine).
[b] Redford et al., 2010

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

(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 20 ng μL^{-1}.

DNA samples were sent to Beckman Coulter Genomics (Takeley, United Kingdom) for sequencing the 16S rRNA and *rpo*B genes, 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 *rpo*B sequences are available at the GenBank database respectively under accession numbers KT350548 to KT350554.

2.5. Detection of antibiotic genes in the bacterial strains

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 μL reaction volume consisting of 3 μL of buffer (10×), 1 μL of $MgCl_2$ (50 mM), 0.6 μL of dNTP (10 mM), 0.6 μL of each primer (Table 1), 3 μL of BSA (10 μg μL^{-1}) (New England BioLabs), 0.1 μL of Silver Star DNA polymerase (Eurogentec), 19.1 μL of sterile distilled water and 2 μL of DNA (20 ng μL^{-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™ Ecoplates (AWEL International) (150 μL/well with a concentration equivalent to 10^8–10^9 CFU mL^{-1} determined by comparison with a McFarland standard solution, bioMérieux® 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 °C in the dark. Optical density (OD) readings were measured 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 difference in densities 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 within 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 °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 26 °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, $(R_1 - R_2)/R_1 \times 100$, 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; Lugtemberg 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 μM Chrome Azurol S (Sigma) and 0.2 mM hexadecyltrimethylammonium 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^{+}, sid^{++} and sid^{+++} respectively when 0 mm < ds ⩽ 5 mm, 5 < ds ⩽ 10 mm and ds > 10 mm. The experiment was made in triplicate.

2.9. Phosphate solubilisation by bacteria

The 19 bacterial strains were screened for phosphate solubilisation on Pikovskaya agar medium (PVK) [$(NH_4)_2SO_4$, 0.5 g L^{-1}; yeast extract, 0.5 g L^{-1}; calcium phosphate ($Ca_3(PO4)_2$), 5 g L^{-1}; KCl, 0.2 g L^{-1}; Magnesium sulphate ($MgSO_4{\cdot}7H_2O$), 0.1 g L^{-1}; glucose, 10 g L^{-1}; Agar, 15 g L^{-1}; $MnSO_4{\cdot}2H_2O$, 0.002 g L^{-1}; $FeSO_4{\cdot}7H_2O$ 0.002 g L^{-1}; pH = 7]. Bacterial plugs (5 mm diameter) from fresh R2A pre-cultures of each isolate were placed on the center of PVK agar plates and incubated at 29 °C. Eight repetitions were made for each bacterial strain. The solubilisation zone was determined 10 and 15 days after inoculation by subtracting the diameter of bacterial colony from the diameter of total zone (dp). The strains were denoted $phos^{+}$, $phos^{++}$ and $phos^{+++}$ respectively when 5 mm < dp ⩽ 15 mm, 15 < dp ⩽ 30 mm and dp > 30 mm.

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

2.10.1. Grapevine material and experimental design

A total of 150 cuttings of cv. Muscat d'Italie originating from INRA experimental vineyards near Bordeaux, were used in the *N. parvum* wood inoculation bioassay. They were processed and prepared as described by Laveau et al. (2009) in a greenhouse in INRA precincts (Villenave d'Ornon, Bordeaux, France).

Five treatments, each applied on 30 plants, were tested: (i) C: control plants not inoculated with the fungal pathogen nor with the bacteria tested, (ii) CP: plants treated with sterile TSB medium and inoculated with a sterile malt agar plug, (iii) NP: plants inoculated with *N. parvum*, (iv) B6: plants inoculated with the antagonist *Bacillus subtilis* strain B6, (v) NP + B6: plants inoculated with both *N. parvum* and *B. subtilis* strain B6.

2.10.2. Strain of the pathogenic fungus, N. parvum, used to inoculate plants

The strain of *N. parvum* used in this assay was isolated in 2013 in a vineyard of the Mornag region, in Tunisia. It was stored at 4 °C on Malt Agar (MA) medium, and, 6–7 days before artificial inoculation, it was subcultured onto MA and incubated at 22 °C.

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-Wilks 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.

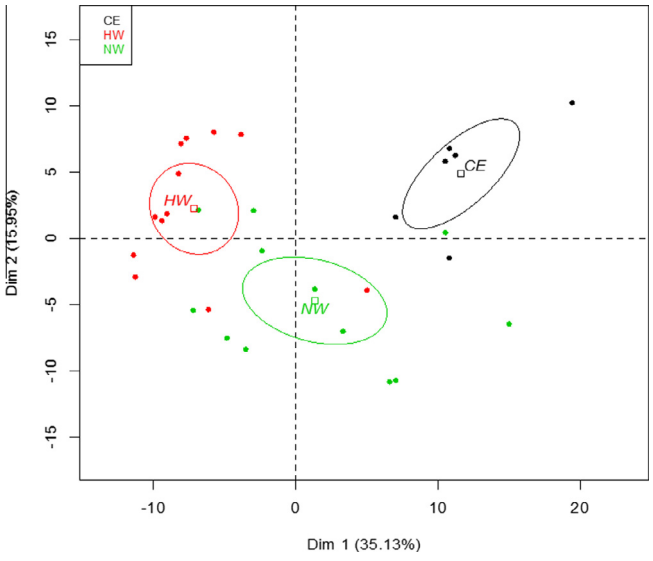

Fig. 2. Principal Component Analysis (PCA) of the bacterial communities inhabiting cordon end (CE), necrotic (NW) and non-necrotic tissues (HW) of grapevines sampled from vineyards in the north of Tunisia, based on SSCP profiles. The variation (%) explained by each PCA axis is given in brackets. Ellipses represent the 95% confidence intervals calculated for each community.

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 *rpo*B 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 amyloliquefaciens* (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).

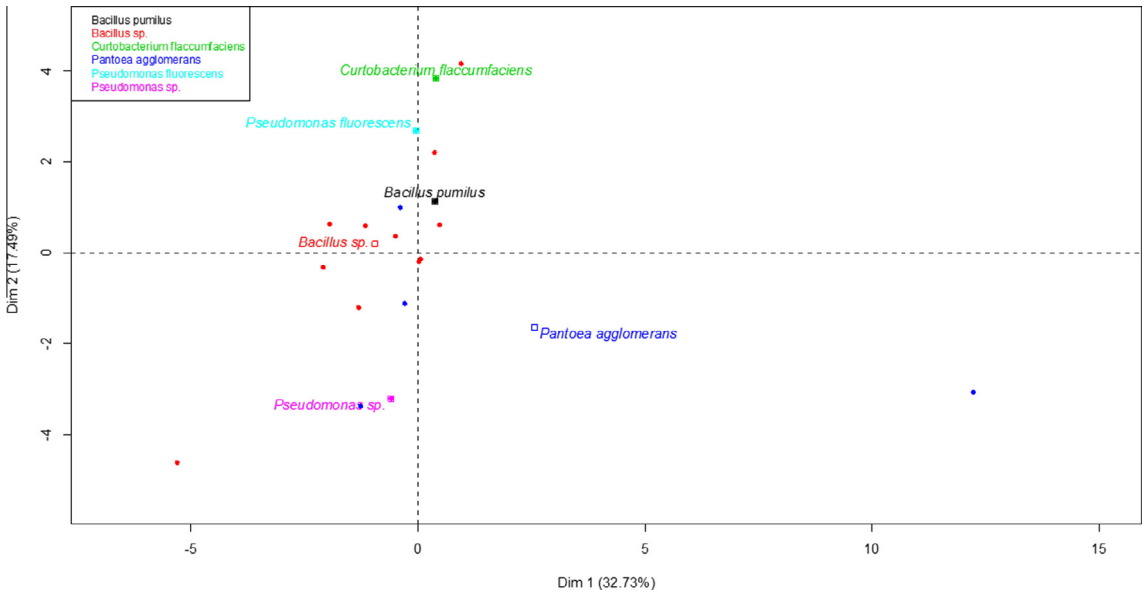

Fig. 3. Principal Component Analysis (PCA) of bacterial strains isolated from Tunisian grapevine wood tissues based on their catabolic profile from Biolog Ecoplates™. Points represent means of 3 replicate samples. The variation (%) explained by each PCA axis is given in brackets. Biolog Ecoplates™ were incubated 48 h at 27 °C in the dark.

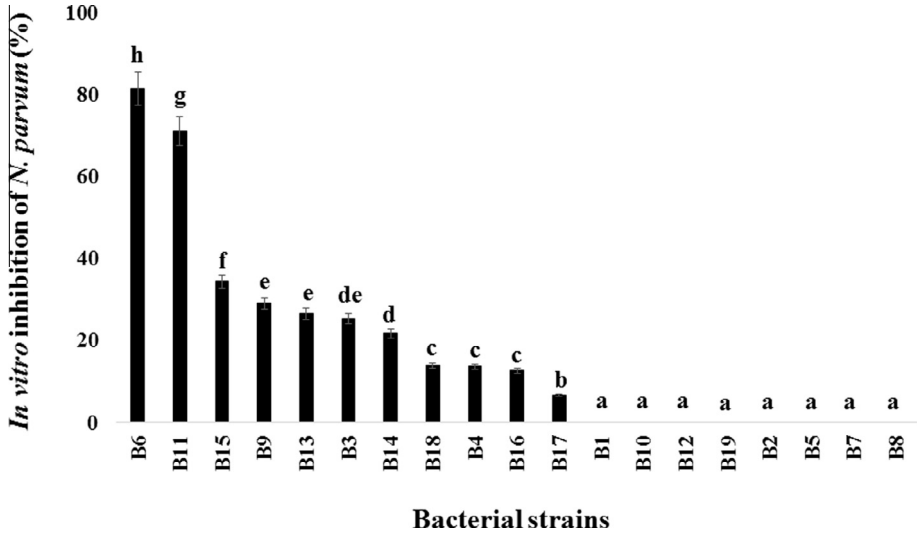

Fig. 4. Effect of the endophytic bacterial isolates, applied as 24 h-bacterial cultures, on the growth of the plant pathogenic fungus *N. parvum*. Mean values (3 replicates) sharing the same letters are not significantly different according to Kruskal-Wallis' non-parametric relative contrast effects post hoc test at $P < 0.05$.

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*.

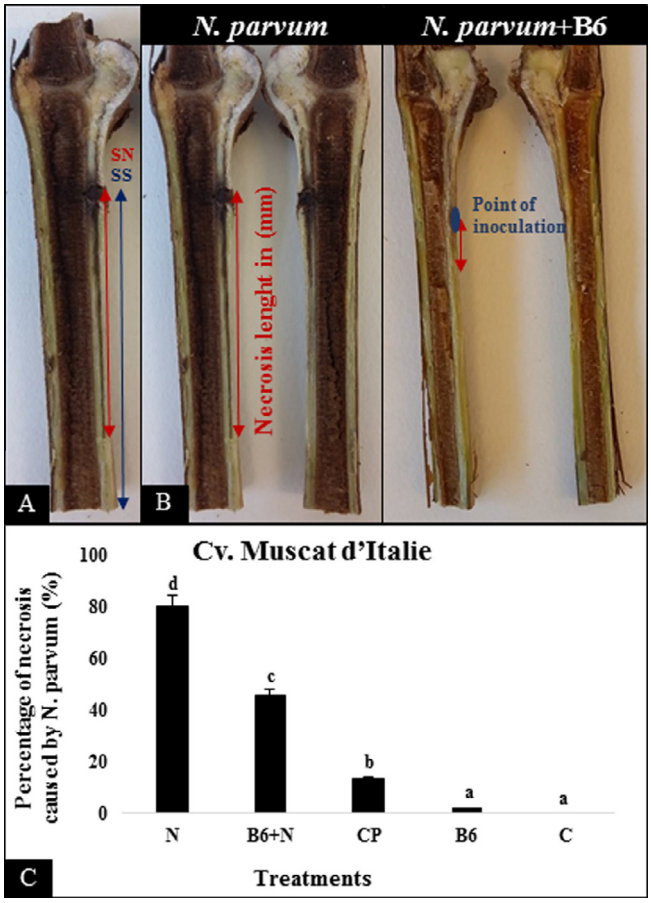

Fig. 5. Effect of the *Bacillus subtilis* strain B6 on the necrosis caused by *N. parvum* on young vines of cv. Muscat d'Italie under greenhouse conditions. (A) Percentage of necrosis measurement: [(SN/SS) * 100] with SN, the size of necrosis downwards from the wound-inoculation hole and SS, the size of the shoot. (B) Examples of internal necrosis observed. (C) Percentage values are the mean of 30 measures per treatment. Mean values sharing the same letters are not significantly different according to Kruskal-Wallis' non-parametric relative contrast effects post hoc test at $P < 0.05$. C: control cuttings not inoculated with the fungus, CP: control cuttings inoculated with sterile malt agar plugs.

Necrotic and non-necrotic tissues were present in cordons 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 Bruez et al. (2015) with a cultivar, Cabernet Sauvignon, planted in France. All these result support the point that whatever the cultivar and the country of sampling, identifying various bacterial communities in the necrotic and non-necrotic wood tissues is a common trait of these communities.

For the 19 most abundant bacteria selected on their morphology, sequencing of the 16S rRNA gene led to the identification of 4 genera: *Bacillus* (12 strains), *Pantoea* (4 strains), *Pseudomonas* (2 strains) and *Curtobacterium* (1 strain). Five *Bacillus* species: *B. amyloliquefaciens*, *B. invictae*, *B. pumilus*, *B. safensis* and *B. subtilis* have been identified after sequencing of the *rpo*B gene. In the literature (West et al., 2010; Compant et al., 2011; Marasco et al., 2013; Pinto et al., 2014), those bacteria are described as frequent and common colonizers of grapevine organs and tissues, *i.e.* flowers, berries, leaves, seeds, roots and vessels. Similarly, for the *Pseudomonas* spp. and *P. agglomerans*, Compant et al. (2011) and Rolli et al. (2015) reported they are inhabitants of grapevines organs and tissue.

As regards the biological properties of bacterial species, such as *B. amyloliquefaciens* isolated in the present study, reports have 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 activity 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; Whipps, 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. pseudotheobromae*. 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*, *Phaemoniella 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.

Strain	Tissue	Biochemical screening			Molecular screening		Antagonistic[a] and PGP[b] traits						
		Gram	Cat	Oxi	16S rDNA gene (GeneBank ref, %id)	*rpo*B gene (GeneBank ref, % id)	Bacc	FeA	FeB	FeD	FeE	Siderophore production	Phosphate solubilisation
B1	HW	+	+	–	*Curtobacterium flaccumfaciens* (JN689331.1, 100%)	–	–	–	–	–	–	+	–
B2	NW	+	+	+	*Bacillus* sp.	*Bacillus invictae* (JX183170.1, 99%)	–	+	–	–	–	++	++
B3	HW	+	+	+	*Bacillus* sp.	*Bacillus safensis* (JX183187.1, 99%)	+	+	–	–	+	+	++
B4	NW	+	+	+	*Bacillus sp.*	*Bacillus safensis* (JX183187.1, 99%)	+	+	–	–	–	+	+++
B5	NW	–	+	+	*Pseudomonas fluorescens* (KC810843.1, 99%)	–	–	+	–	–	–	+	+
B6	**NW**	**+**	**+**	**+**	***Bacillus* sp.**	***Bacillus subtilis* (CP002905.1, 96%)**	**+**	**+**	**+**	**+**	**+**	**+**	–
B7	HW	+	+	+	*Bacillus* sp.	*Bacillus invictae* (JX183170.1, 99%)	+	–	+	–	–	+	+
B8	HW	+	–	–	*Bacillus* sp.	*Bacillus pumilus* (AB098578.1, 99%)	–	+	–	–	–	+	+
B9	HW	+	–	+	*Bacillus* sp.	*Bacillus invictae* (JX183170.1, 99%)	+	+	+	–	–	+	+
B10	CE	–	+	–	*Pantoea agglomerans* (FJ357814.1, 99%)	–	–	–	+	–	–	+	++
B11	CE	+	+	+	*Bacillus* sp.	*Bacillus amyloliquefaciens* (CP003332.1, 99%)	+	+	–	–	+	++	–
B12	CE	–	+	–	*Pantoea agglomerans* (FJ357814.1, 99%)	–	–	–	+	–	–	+	++
B13	CE	+	+	+	*Bacillus* sp.	*Bacillus invictae* (JX183170.1, 99%)	–	+	+	+	–	+	++
B14	NW	+	+	+	*Bacillus pumilus*	*Bacillus invictae* (JX183170.1, 99%)	–	+	+	+	+	+++	–
B15	NW	+	+	+	*Bacillus* sp.	*Bacillus invictae* (JX183170.1, 99%)	+	+	+	–	+	+	++
B16	NW	–	+	+	*Pseudomonas* sp.	–	–	–	–	–	–	+++	–
B17	HW	–	+	–	*Pantoea agglomerans* (FJ357814.1, 99%)	–	–	–	–	–	–	+	++
B18	HW	+	+	+	*Bacillus* sp.	*Bacillus invictae* (JX183170.1, 99%)	–	+	+	–	–	+	+
B19	HW	–	+	–	*Pantoea agglomerans* (FJ357814.1, 99%)	–	+	–	–	–	–	+	–

HW: non-necrotic wood, NW: necrotic wood, CE: cordon end, Cat: catalase, Oxi: oxidase, Bacc: bacillomycin, Fe: fengycin.
[a] Antagonistic traits: antibiotic genes, siderophore production.
[b] Plant Growth Promoting (PGP) trait: phosphate solubilisation.

Table 3
Effect of bacterial isolates on the *in vitro* growth of three fungi: *Neofusicoccum parvum*, *Lasidiodiplodia pseudotheobromae* and *Schizophyllum commune* (% mycelial inhibition).

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

* Percentages of mycelial growth inhibition were determined after 5 days of incubation using Whipps 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; Trotel-Aziz et al., 2008; Essghaier 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.

Acknowledgments

The authors are grateful to the program PHC-Utique France-Tunisie managed by CMCU (project 13G0924), French Ministry in charge of Agriculture (Casdar program V1302), Poupelain Foundation and International Organisation of Vine and Wine (OIV). We are grateful to Javid Ojaghi (Genetic Resources Institute of Azerbaijan

National Academy of Sciences) for his help on statistical analyses, Mokhtar Dridi (Laboratoire de Protection des Végétaux, Institut National de la Recherche Agronomique de Tunisie (INRAT) 2049, Ariana, Tunisia) for his help on sampling vines. We are also indebted to the INRA UMR-SAVE technical staff, Gilles Taris and Sébastien Gambier, for their valuable help for the greenhouse and laboratory experiments. The authors also thank the reviewers for helpful comments to improve the manuscript.

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