Isolation, identification and in vitro characterization of grapevine rhizobacteria to control ochratoxigenic Aspergillus spp. on grapes

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ABSTRACT

Aspergillus spp. are fungal pathogens that attack the grape and that are known for their secretion of mycotoxins, in particular, ochratoxin which is very toxic. A total of 39 bacterial strains isolated from the rhizosphere in Tunisian vineyards were identified using 16S rDNA and rpoB gene sequencing: 35 were Bacillus spp. strains, 2 were Brevibacterium spp., 1 was Paenibacillus sp. and 1 strain was Microbacterium oxydans. Biochemical and microbiological screenings revealed that those 39 strains (i) metabolized differently carbon sources, (ii) possessed antibiotic genes and (iii) produced siderophores. Based on their PGP traits, 21 strains were selected and tested in vitro for their antagonistic effect against two fungal pathogens, Aspergillus ochraceus and A. carbonarius. All the tested antagonists were able to reduce the growth of A. ochraceus, with Bacillus amyloliquefaciens being the most efficient; and A. carbonarius, in particular Bacillus pumilus. In vitro screening using detached berries showed the potential of B. pumilus strain G3AX for inhibiting contaminations by Aspergillus spp., that are OTA-producing fungi. At the berry surface, the efficacy of the bacterial strains strongly depended on the presence or absence of wounds.

1. Introduction

Grapevine is one of the most cultivated fruit crop species in the world. The world vineyard spreads over five continents and has a surface area of 7.5 million hectares (International Organization of Vine and Wine (OIVV) (2016)). In Tunisia, viticulture has begun in antiquity
as in other countries of the Mediterranean Basin, thanks to Phoenicians and Carthaginians. The vineyard sector occupies an important place in the national economy. It generates a volume of employments of about 1 million working days and financing in the trade balance by the annual export of 37,000 hl of wine to European countries (Interprofessional Grouping of Fruits (GIFRUIT) (2016)).

Currently, the Tunisian vineyard occupies 22,000 ha of which 12,000 ha are planted with table grape cultivars (Interprofessional Grouping of Fruits (GIFRUIT) (2016)). Vine plantations are concentrated in the North, the centre and the South, where planted areas spread quickly from one year to another (Interprofessional Grouping of Fruits (GIFRUIT) (2016)). The most cultivated vine cultivars in the Tunisian territory are Muscat d’Italie, Seedless Superior and Muscat d’Alexandrie. Despite its importance, domestic production of table grape represented 133,500 tons in 2016 (Agricultural Statistics Analysis (ASA) (2016))), but it remains dependent on weather conditions and disease resistance. Fungi are the main microbiorganisms responsible for losses in agriculture, 83% of plant diseases are due to fungi and 17% being caused by bacteria and viruses. Vine is known for its high sensitivity to fungal diseases and pests that seem to be the main cause of the decrease in production in the Tunisian vineyard. Aspergillus spp. are filamentous fungi attacking grape berries that can alter the hygienic quality of grapes. Contamination of grapes and grape products by Aspergillus spp. belonging to the Nigri section is known to occur very widely. The species Aspergillus niger, A. tubingensis and A. carbonarius are included within this section and during their growth, these fungi are able to produce mycotoxins including ochratoxin A (OTA) and fumonisin B2 (FB2) (Battilani and Pietri, 2002; Bejaou et al., 2006; Filali et al., 2001; Ospital et al., 1998; Somma et al., 2012). OTA is the most common mycotoxin detected in grapes and grape derived products, such as grape juices, wines and dried vine fruits (Aksoy et al., 2007; Visconti et al., 2008; Zimmeri and Dick, 1996).

Although the application of fungicides remains one of the most powerful and cost-effective tools to reduce the incidence of fungal pathogons in most crops (Munimbazi et al., 1997), the European Union has established a strict legislation concerning their use, due to the development of resistant fungal strains and the negative effects of fungicides on human health and the environment (De Costa and Bezerra, 2009). Maximum residue levels of pesticides were established for all foodstuffs intended for human or animal consumption in the European Union (European Commission, 2013). Recognizing the real danger of the presence of chemicals or fungicides in food for humans and animals (carcinogenic properties), farmers and consumers are increasingly turning to organic practices and the consumption of natural and healthy products (Mie et al., 2017).

Biological control is one of the most promising alternatives to unpopular synthetic fungicides, and research on postharvest biocontrol has increased in recent decades (Droby et al., 2009). The main characteristics of an ideal biocontrol agent were established by Wilson and Wisniewski (1989), and are related to biosafety, activity in a range of environments and against a variety of pathogens, and ease of management and use. Members of the genera Bacillus (Kumar et al., 2012; Ren et al., 2013), Pseudomonas (Cirvilleri et al., 2005; Zhou et al., 1999) and Pantoea (Nunes et al., 2002), among others, were shown to be effective in the biological control of mould rots. Bacillus, Pseudomonas and Streptomyces showed significant capacity for the biocontrol of bacteria (Bressan, 2003; Fravel, 2005). Bacteria of the genus Bacillus have ample propagation capacity for the production of secondary metabolites with antimicrobial activity, the main source of their antagonistic potential against pathogens in plant tissues (Rückert et al., 2011). Antimicrobial compounds with circular lipopeptide structures, produced by many strains of the genus Bacillus, demonstrated significant antifungal and antibacterial activity (Dimitic et al., 2013; Yu et al., 2002).

In that context, this study was conducted in order to investigate the potential of grapevine rhizobacteria as biocontrol agents against ochratoxigenic fungi, i.e. Aspergillus ochraceus and A. carbonarius. Different approaches were used: i) isolation and identification of bacterial candidates by sequencing the 16S RNA and rpoB genes. (ii) The abilities of the isolated bacteria in terms of control of plant pathogens: detection of antibiotic genes (4 fengycins encoded A, B, D and E; 1 bacillomycin), degradation of different coal-carbon sources, production of siderophores and phosphate solubilisation, were tested. (iii) Based on those criteria, the 21 best performing isolates were then selected to test their inhibitory effects in vitro against two ochratoxigenic fungi A. ochraceus and A. carbonarius, by dual confrontations onto agar plates and using a laboratory-scale detached berries test.

2. Materials and methods

2.1. Pathogenic fungal strains

From the fungi collection of the laboratory of Molecular Physiology of Plants in Biotechnology Center of Borj cedria, two of the most OTA-producing strains, i.e. ASP31 and ASP73, were selected for antagonistic assays. The ITS sequences of the two species (Aspergillus carbonarius and Aspergillus ochraceus) are available at the GenBank database under accession numbers MH249060 and MH249061.

2.2. Bacterial strains

2.2.1. Plant material and sampling

In order to study the bacterial microflora inhabiting the rhizosphere of tunisian grapevines, a sampling was carried out in summer 2013 in two vineyards located insahel region situated in the east central part of Tunisia (one is a biological plot, the other is a conventional one). These vineyards consisted of mature grapevines (10-years old plants) of the table grape cultivar, Rich Baba Sam. They were irrigated with a drip irrigation system. Rhiospheric soil samples (0–15 cm) were collected from 10 distant points of each plot: 20 in total.

2.2.2. Isolation of bacteria from the rhizosphere of Tunisian vineyards

Rhizobacteria were isolated according to the serial dilution technique, which consisted of mixing 8 g of each of the 20 soil samples, with 50 ml of a physiological saline solution (0.85% NaCl in distilled water). The solutions thus obtained were sterilized for 15 min and filtered, before being subjected to a dilution series (from 10−1 to 10−7) and inoculated on a Luria-Bertani culture medium (LB, 5 g of yeast extract, 10 g of peptone, 10 g of NaCl and 15 g of bacterial agar). Petri dishes were then incubated 24 h at 25°C.

After incubation, individualized colonies of different appearances were subcultured onto LB medium. The isolates thus obtained were purified by 3 successive subcultures on the same medium. A total of 109 bacterial strains were recovered from the soil samples collected. The 39 most abundant were selected based on morphological differences and subsequently purified onto LB agar and characterized.

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

Genomic DNA from the 39 selected bacterial strains was extracted from pellets obtained after centrifugation of pure cultures grown in Tryptone Soy Broth (TSB, Conda) by using the commercial kit Invisorb Spin Plant Mini Kit (Invitek) following the manufacturer’s instructions. The DNA extracts were quantified with a nanodrop (ND-1000, Thermoscientific, Labtech) and homogenized at a concentration of 20 ng/µl. DNA samples were sent to Beckman Coulter Genomics (Takeley, United Kingdom) for sequencing the 16S rRNA and rpoB genes, respectively with the primers 799f and 1492r, 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 are available at the Genbank database under accession numbers MH236385 to MH236421.
2.2.4. Community-level physiological profiling (CLPP) of rhizobacteria

Bacterial strains in suspension were quantified by fluorochrome staining (500 µl Chemsol B16 buffer + 2.5 µl fluorochrome Chemochrome V6 fluorescein acetate; Biomérieux, Marcy l’Etoile, France) followed by epifluorescent direct counts using an optical microscope (Model BH2, Olympus France, Rungis, France). A minimum of 300 cells was counted at least at 10 different fields of view and the average number of dyed cells per field was finally expressed as CFU/ml. The isolated bacteria were then distributed in 96-well Biolog™ EcoPlates (AWEL International) (150 µl/well) with a concentration of 10^6 CFU/ml. These 96-well plates contained 31 different carbon sources plus a control well in triplicate. To minimize the effect of differences in densities between plates, data were standardized as follows: the average well colour development (ACWD) was calculated for each plate; then, the blanked percentage of growth inhibition was calculated using the formula, R1 = (R2 - dp)/dp x 100, where R1 is the radial distance (mm) grown by pathogenic fungi in control plates and R2 is the radial distance (mm) grown by pathogenic fungi in the direction of the antagonist, and R2 is the radial distance (mm) grown by pathogenic fungi in control plates (Whipps, 1987).

Phosphate solubilisation. The 39 bacterial strains were further tested for their ability to solubilise phosphates on Pikovskaya agar medium (PKV) ([NH₄]₂SO₄, 0.5 g/l; yeast extract, 0.5 g/l; calcium phosphate (Ca₃(PO₄)₂), 5 g/l; KCl, 0.2 g/l; Magnesium sulphate (MgSO₄·7H₂O), 0.1 g/l; glucose, 10 g/l; Agar, 15 g/l; MnSO₄·2H₂O, 0.002 g/l; FeSO₄·7H₂O 0.002 g/l; pH = 7). Bacterial plugs (5 mm diameter) from fresh bacterial cultures were placed on the centre of PKV Agar plates and incubated at 28°C. Five repetitions were made for each bacterial strain and the experiment was repeated three times. 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 0 mm ≤ dp ≤ 5 mm, 5 < dp ≤ 10 mm and dp > 10 mm. The experiment was conducted three times (Rezgui et al., 2016).

Based on the previously described characterization assays, the 21 most promising bacterial strains were evaluated for their biocontrol potential against *Aspergillus ochraceus* and *A. carbonarius*.

2.3. In vitro antagonism of bacteria against two ochratoxigenic fungi *A. ochraceus* and *A. carbonarius*

2.3.1. Direct confrontation assays

The antagonist activity of bacterial isolates was tested against *Aspergillus carbonarius* and *A. ochraceus*, i.e. 2 ochratoxigenic fungi frequently found in grapevine, using the dual culture technique described by Déniel et al. (2004). Bacterial strains were streaked at the edges of Petri plates containing Potato Dextrose Agar (PDA, Biokar diagnostics, France) and, after 48 h of incubation at 27°C, a 6 mm mycelial plug of each pathogenic fungus was placed in the centre of each plate. The plates were then incubated at 27°C for 5 days. All experiments were performed in triplicate and repeated three times. The percentage of growth inhibition was calculated using the formula, (R1-R2)/R1 x 100, where R1 is the radial distance (mm) grown by pathogenic fungi in the direction of the antagonist, and R2 is the radial distance (mm) grown by pathogenic fungi in control plates (Whipps, 1987).

2.3.2. Volatile bacterial substances assays

Rhizobacteria were tested for their ability to produce volatile

### Table 1

<table>
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<tr>
<th>Applications</th>
<th>Gene</th>
<th>Primer name</th>
<th>Sequence 5′/3′</th>
<th>Amplicon size</th>
<th>Annealing T°C</th>
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<td>Sequencing Fungi</td>
<td>ITS</td>
<td>ITS1f</td>
<td>CTGTCATTAGAGGAAGTA</td>
<td>650 bp</td>
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<td>ITS4</td>
<td></td>
<td>A TCTCCTATTAGGATATGC</td>
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<td>Bacteria 16S rRNA</td>
<td>799f</td>
<td></td>
<td>AACMMGATTAGATACCCCG</td>
<td>750 bp</td>
<td>52°C</td>
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<td></td>
<td>1492f</td>
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<td>GTACGGTGTAGACTC</td>
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<td>55°C</td>
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<td></td>
<td>rpoB</td>
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<td>GAGATCATTTYWGAAGAACCG</td>
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<tr>
<td></td>
<td>rpoB</td>
<td></td>
<td>GGNGTTYCTAGYGAGCACAT</td>
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<td>Antibiotics screening</td>
<td>Lipopeptide Fengycin</td>
<td>FENAf</td>
<td>GACAGTGCTGCTGATGAAAA</td>
<td>900 bp</td>
<td>54°C</td>
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<td>FENAv</td>
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<td></td>
<td></td>
<td>FENBr</td>
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<td>FENDr</td>
<td>TTGGCCAGCAGGAGAGTTT</td>
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<td></td>
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<td>900 bp</td>
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<td>BACCr</td>
<td></td>
<td>GCGTGTAGACGTCTGACATG</td>
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**Abbreviations:** ITS = internal transcribed spacer of 18S rRNA; PVK = Pikovskaya medium; PDA = Potato dextrose agar; BSA = bovine serum albumin; TBE = Tris/ Boric acid/ EDTA.
substances inhibiting fungal growth using the double plate technique. Strains of *Aspergillus ochraceus* and *A. carbonarius* were grown on Malt Extract Agar (MEA) for 7 days at 27 °C in the dark so that the colonies reached a sufficient size of 3.1 cm radius on average before being inverted over the Tryptic Soy Agar (TSA, Conda) streaked dishes subsequently incubated at 27 °C in the dark. 10 repetitions were made for each strain containing the bacterial strains to test and sealed with Para-Formaldehyde over the Tryptic Soy Agar (TSA, Conda) streaked dishes. Control plates had only each of the two bacterial strain and the experiment was repeated three times. The agar plates were incubated at 27 °C in the dark. Table 2

<table>
<thead>
<tr>
<th>Strains</th>
<th>Cropping management</th>
<th>Molecular screening</th>
<th>16S rDNA gene (GenBank ref %id)</th>
<th>rpoB gene (GenBank ref %id)</th>
<th>FeA</th>
<th>FeB</th>
<th>FeD</th>
<th>FeE</th>
<th>Bacc</th>
<th>Phosphate solubilisation</th>
<th>Siderophore production</th>
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<td>KD1</td>
<td>Conv</td>
<td><em>Brevibacterium frigorotolerans</em> (99%)</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+ + + +</td>
</tr>
<tr>
<td>J3L</td>
<td>Org</td>
<td><em>Paenibacillus sp.</em> (97%)</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G3AM1</td>
<td>Org</td>
<td><em>Bacillus sp.</em> (97%)</td>
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<td>–</td>
<td>+</td>
<td>+</td>
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<td>G3AX1</td>
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<td><em>Bacillus siamensis</em></td>
<td><em>Bacillus velezensis</em> (100%)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>G4B2</td>
<td>Org</td>
<td><em>Bacillus mojavensis</em> (99%)</td>
<td><em>Bacillus velezensis</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>–</td>
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<tr>
<td>G3AF2</td>
<td>Org</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>J4F</td>
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<td><em>Bacillus thuringiensis</em></td>
<td><em>Bacillus thuringiensis</em> (100%)</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>G3</td>
<td>Org</td>
<td><em>Bacillus endophyticus</em></td>
<td><em>Bacillus safensis</em> (99%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>G5</td>
<td>Org</td>
<td><em>Bacillus endophyticus</em></td>
<td><em>Bacillus safensis</em> (99%)</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>–</td>
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<tr>
<td>I1</td>
<td>Org</td>
<td><em>Bacillus weihenstephanensis</em> (99%)</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>I2</td>
<td>Org</td>
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<td><em>Bacillus amyloliquefaciens</em> (100%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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Antagonistic traits: antibiotic genes, siderophore production.

Plant Growth Promoting (PGP) trait: phosphate solubilisation.

For *in vitro* grape berry rot bioassays with *Aspergillus ochraceus* and *A. carbonarius*, Thompson Seedless originating from the supermarket were washed for 15 min under continuous tap-water flow. Then, they were surface disinfected via immersion in sodium hypochlorite solution (50 g/l; pH 7.2) for 10 min, rinsed three times with sterile distilled water and then left to dry at room temperature. Undamaged grape berries were selected visually and carefully cut off from the grape bunches, using scissors, with the pedicel attached.

The efficacy of bacteria against both *A. ochraceus* and *A. carbonarius* was tested on wounded and unwounded berries. The experimental design consisted of 20 berries per treatment (strain x pathogen x wounded-unwounded). Wounded berries were dipped in bacterial suspensions (LB medium). For wounded berries, three artificial wounds (1–1.5 mm in diameter) were made using a sterile pipette tip. Then, 10 µl of each bacterial strain suspension was introduced into each wound site. The control treatments with wounded and unwounded berries consisted of: (i) UUC (untreated unwounded control) untreated with the bacteria and uninoculated with the pathogen, (ii) UC (untreated control) inoculated with mycelium plugs of the pathogen only.
Humidi or inoculated with a mycelial plug (4 mm in diameter) of either culture, the centre of each wounded and unwounded fruit was immediately placed into a controlled growth incubator (Conviron CMP-5090; thogen inoculations, 20 berries were placed on a metallic grid in a plastic rotten berry surface area was visually scored, and the average rot severity was assessed at 8 dpi on wounded and unwounded berries. The experiment was repeated two times.

In order to allow bacteria to better colonize the berries before pathogen inoculations, 20 berries were placed on a metallic grid in plastic boxes (19 × 13 × 4 cm) filled with 100 ml of sterile distilled water and placed into a controlled growth incubator (Conviron CMP-5090; Winnipeg, Manitoba, Canada) at 28 °C in the dark for 24 h. After incubation, the centre of each wounded and unwounded fruit was inoculated with a mycelial plug (4 mm in diameter) of either A. ochraceus or A. carbonarius with the mycelial side facing the berry surface. The humidified boxes for A. ochraceus and A. carbonarius were then replaced in the growth chambers at 28 °C. For each berry, the percentage of rotten berry surface area was visually scored, and the average rot severity of each treatment was calculated as described by Haidar et al. (2016). Development of A. carbonarius and A. ochraceus rot severity was assessed at 8 dpi on wounded and unwounded berries. The experiment was repeated two times.

### 2.5. 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 nonparametric Kruskal-Wallis test and the relative contrast effects analysed by the nparcomp package (version 2.0). For CLPP data, in the Vegan R-Package, the Anosim test using Bray-Curtis distance was employed to compare the data (Rezgui et al., 2016). For the grape berries rot bioassays, analyses of variance (ANOVs) and Tukey’s post-hoc tests were done using the Rcmdr R-Package.

### 3. Results

#### 3.1. Characterisation of the bacterial strains isolated from the rhizosphere of Tunisian vineyards

Based on partial 16S rRNA and rpB genes sequencing (Table 2), the majority of the bacterial strains belonged to the Bacillus genus (34 strains): Bacillus velezensis (7 strains), Bacillus amyloliquefaciens (6 strains), B. endophyticus (4 strains), B. safensis (4 strains), B. pumilus (3 strains), B. subtilis (3 strains), B. thuringiensis (2 strains), B. mojavensis (1 strain), B. weihenstephanensis (1 strain), B. simplex (1 strain), B. cereus (1 strain) and 3 strains of Bacillus sp. Other bacterial species were identified: Brevibacterium species (2 strains), i.e. Brevibacterium halotolerans (1 strain) and B. frigortolerans (1 strain), Microbacterium oxydans (1 strain) and Paenibacillus sp. (1 strain).

The 5 genes coding for the screened antibiotics were detected in three strains: Bacillus sp. G3AM1, B. mojavensis G4B2, and B. subtilis G4A1. Twenty-one strains possessed the 4 fengycin genes (19 Bacillus spp., Paenibacillus spp. and Brevibacterium halotolerans) while the other strains expressed at least 1 out of 4 genes. For the strain of B. simplex K2E1, none of the 5 genes were detected (Table 2).

Regarding the PGP traits, i.e. phosphate solubilisation and siderophore production, only 4 strains out of 39 demonstrated both of the characteristics evaluated: B. pumilus G3AX, B. velezensis G3AX1 and G3A, and Bacillus sp. K2E1X (Table 2). None of the other strains were able to solubilize phosphates whereas all the bacteria produced siderophores. Three strains produced the greater halo zones, ranging from 14.5 to 18 mm (sid ++ + strains) and 23 strains developed small haloes ranging from 1 to 5 mm (sid + strains).

#### 3.2. Community-level physiological profiles (CLPP) of the isolated rhizobacteria

The strains distribution on the principal plane generated by the PCA is represented in Fig. 1. PCA eigenvalues indicate that the first two axes, Dim 1 and Dim 2, explain 47.2% of the total variability. Globally, no distinctive pattern in the use of carbon sources was observed between the 39 bacterial strains isolated from the rhizosphere of Tunisian vineyards (data not shown) except when the type of cropping management, i.e. organic or conventional (Fig. 1). Bacterial species metabolized differently carbohydrates and amino acids depending on the farming system, i.e. organic or conventional.

#### 3.3. In vitro antagonism of bacteria against two ochratoxigenic fungi

##### 3.3.1. Direct confrontations

The 21 selected bacterial strains showed variable inhibition percentages for the growth of the two fungal pathogens tested, i.e. A. carbonarius (P < 0.01) and A. ochraceus (P < 0.01). They ranged from 11.9% to 80.9% and from 17.2% to 85.2% respectively for A. carbonarius (Fig. 2) and A. ochraceus (Fig. 3). The most efficient antagonistic bacteria were Bacillus pumilus (G3AX) and Bacillus amyloliquefaciens (I2) respectively against A. carbonarius (Fig. 2) and A. ochraceus (Fig. 3).

##### 3.3.2. Volatile bacterial substances

All the 21 bacterial isolates tested showed strong fungal growth inhibition activity via volatiles. Variable inhibition percentages for the growth of the two fungal pathogens tested were obtained: they ranged from 20 to 93.3% and from 8.3 to 97.6% respectively for A. carbonarius (Fig. 4) and A. ochraceus (Fig. 5).

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Fig. 1. Principal Component Analysis (PCA) of bacterial strains isolated from the rhizosphere of conventional and organic Tunisian vineyards 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.
3.4. Grape berry rot bioassays with Aspergillus ochraceus and A. carbonarius

Severity reduction results are shown in Figs. 6 and 7. The untreated controls UC (berries inoculated with the pathogen only) in both bioassays showed notable Aspergillus rot symptoms. In the A. carbonarius bioassay, overall rot severity reached 77% and 54% with the untreated control (UC) treatments respectively on wounded and unwounded berries (data not shown). In the A. ochraceus bioassay, the overall rot severity values reached with the untreated controls (UC) were 51% on wounded berries and 12% on the unwounded fruit test (data not shown). In both bioassays, grape berries for uninoculated UUC and MC controls were always asymptomatic during the entire incubation period. The controls FC treated with a fungicide (Fluazinam) applied at the registered dosage, significantly reduced A. carbonarius and A. ochraceus symptoms respectively by 74% and 47% on wounded berries and by 54% and 12% on unwounded berries.

3.4.1. Inhibition of A. carbonarius berry rot development by bacterial strains

The inhibition rates of 21 bacterial strains for controlling A. carbonarius rot development at the surface of detached grape berries are shown in Fig. 6. On wounded berries, 20 strains out of the 21 tested, significantly protected grape berries from A. carbonarius rot compared with the untreated control (UC) inoculated with the pathogen only. Their inhibition levels ranged from 10% to 77%. However, 5 bacterial strains were characterized by inhibition rates higher than 70%. The highest value corresponds to the strain G3AX (B. pumilus). On unwounded berries, only 5 strains had significant inhibition levels compared to the UC control (P > 0.05), the highest value being exhibited by Bacillus sp. G3AM1 (54%). Furthermore, pretreatment with two strains (K2E1X and G3AX1) tended to increase A. carbonarius symptoms on unwounded fruits (this was not significant at P = 0.05 compared to the corresponding untreated control UC).

3.4.2. Inhibition of A. ochraceus berry rot development by bacterial strains

The results for the reduction of A. ochraceus rot lesions are shown in Fig. 7. On wounded berries, only 9 strains significantly reduced the development of A. ochraceus rot compared with the untreated control (UC) inoculated with the pathogen only. Their inhibition levels ranged from 22% from 50% with Bacillus pumilus G3AX being the most efficient
strain. On unwounded berries, only 5 out of the 21 strains tested significantly reduced *A. ochraceus* rot symptoms. The highest inhibition level was 12%. Five strains (I3L, L2, G3AF2, J4C and J4F) tended to increase *A. ochraceus* symptoms on unwounded fruit (this was not significant at P = 0.05 compared to the corresponding untreated control UC).

4. Discussion

The aim of this study was to isolate efficient bacterial inoculants having the ability to control or at least to reduce the pernicious effects of two ochratoxigenic pathogens on grapevine berries, *Aspergillus ochraceus* and *Aspergillus carbonarius*. To our knowledge, this is the first report describing by molecular, microbiological and biochemical approaches rhizobacterial strains that inhabit the soils of organic and conventional Tunisian vineyards, some of these being endowed with antagonistic abilities.

The molecular characterization of the sampled bacteria was performed by 16S rDNA sequencing and showed that these isolates belonged to the genera *Bacillus* (35 strains), *Brevibacterium* (1 *B. frigoritolerans* and 1 *B. halotolerans*), *Microbacterium* (1 *M. oxydans*) and *Paenibacillus* sp. (1 strain). As some strains of *Bacillus* spp. could not be identified based on the 16S rDNA gene, sequencing of the *rpoB* gene was undertaken leading to the identification of 11 *Bacillus* species: *B. velezensis* (7 strains), *B. amyloliquefaciens* (6 strains), *B. endophyticus* (4 strains), *B. safensis* (4 strains), *B. pumilus* (3 strains), *B. subtilis* (2 strains).
strains), B. thuringiensis (2 strains), B. cereus (1 strain), B. mojavensis (1 strain), B. simplex (1 strain) and B. weihenstephanensis (1 strain) (Table 2). Some of those are described in the literature as frequent and common colonizers of grapevine organs and tissues, i.e. flowers, berries, leaves, seeds, roots and vessels (Compant et al., 2011; Marasco et al., 2013; Pinto et al., 2014; Rezgui et al., 2016; West et al., 2010).

It is known that microorganisms associated with plants, in particular Bacillus spp., can promote their growth and development through a number of mechanisms, among which the growth inhibition of phytopathogenic microorganisms (Beneduzi et al., 2012; Choudhary and Johri (2009)). Our results support these previous reports by showing that the bacterial strains inhabiting the soils of Tunisian vineyards possess antagonistic traits, i.e. antibiotics encoding genes, production of siderophores, metabolization of carbon sources, and plant growth promoting traits, i.e. solubilisation of phosphate.

All the isolated Bacillus spp. strains expressed differentially at least...
one of the 5 antibiotic genes investigated (4 fengycins and 1 bacillo-
ymycin), with only three strains of Bacillus, i.e. Bacillus sp. G3AM1, B.
mojavensis G4B2 and B. subtilis G4A1, having the 5 encoding genes. It-
urine (bacillomycin) and fengycin families were reported to have a strong
in vitro antifungal action against a wide variety of yeasts and fungi (Li et al., 2016). Fengycins are biologically active lipopeptides
produced by several Bacillus subtilis strains, known to develop anti-
fungal activity against filamentous fungi likely by making the plasma
membrane of the target cell more permeable (Deleu et al., 2008; Jourdan et al., 2009).

Many reports indicated that several antifungal mechanisms of
Bacillus species contribute to phytopathogen antagonism, as side-
rophores, which are important natural iron chelators representing a
novel class of antibiotics with considerable therapeutic potential
(Plhacek et al., 2016). Our results indicated that all the isolated bac-
teria produced siderophores; the most productive was the Brevi-
bacterium frigoritolerans strain KDI (Table 2). Strains from this genus are
frequently reported as siderophore producers, as shown by Noordman
et al. (2006) and Pham et al. (2017).

The metabolism of the bacterial strains isolated from two Tunisian
vineyards was also studied with the method of Biolog Ecoplate. The
results showed that the rhizobacteria metabolized carbon sources in the
same way, but metabolized differently carbohydrates and amino acids
depending on the farming system, i.e. organic or conventional (47.2% of
the total variability; Fig. 1). In the same context, Rezgui et al. (2016)
reported that the carbohydrates group was the most intensively meta-
bolized by their 19 strains, with Pantoea agglomerans being the highest
metabolizer.

Regarding the solubilization of phosphates, previous studies (Kang
et al., 2014; Matos et al., 2017) showed that Bacillus strains, e.g. B.
pumilus and B. megaterium, exhibited a strong ability to solubilize
phosphates. In this study, of the 39 isolates assessed, only 3 strains
belonging to the genus Bacillus were able to do this in very small
amounts: B. pumilus (G3AX), B. velezensis (G3AX1M) and Bacillus sp.
(K2E1X). Phosphorus is, after nitrogen, the second important key ele-
ment as a mineral nutrient in terms of quantitative plant requirement.
It plays significant role in increasing root ramification and strength
by imparting vitality and disease resistance capacity to plant (Sharma
et al., 2013).

Based on the criteria described above, 21 strains were selected for in
vitro experiments to test their ability as fungal antagonists against two
common pathogens of table grapes causing black rot and OTA con-
tamination in grapes, i.e. A. carbonarius and A. ochraceus (Allam et al.,
2011; Atoui et al., 2006; Leong et al., 2008; Magnoli et al., 2003).

In the direct confrontation tests, the largest zones of inhibition of
A. carbonarius and A. ochraceus were respectively obtained with B. pumilus
strain G3AX (81% inhibition, Fig. 2) and B. amyloliquefaciens strain I2
(85% inhibition, Fig. 3). Similar results were observed concerning the
inhibition of different pathogens by Bacillus strains both in vitro and in
vivo (Gordillo et al., 2009; Haidar et al., 2016; Rezgui et al., 2016). In
particular, B. pumilus and B. amyloliquefaciens were reported as being
successful in controlling respectively Fusarium wilt on tomatoes with
beneficial effect on plant growth (Heidarzadeh and Ravari, 2015); and
soft and brown rots caused by Erwinia carotovora and Monilinia fructicola
on vegetables and stone fruits (Liu et al., 2011; Zhao et al., 2013).

Furthermore, Bacillus and Paenibacillus species were shown to ex-
hibit antibacterial and/or antifungal activity through the emission of
volatile organic compounds (VOCs) (Berrada et al., 2012; Cernava,
2012; Rybakova et al., 2015). Several VOCs can reduce fungal growth,
impair fungal spores and hyphae, and/or promote plant growth (Kai
et al., 2007; Weisskopf, 2015). In our study, all the bacterial strains
produced VOCs: the greatest mycelial inhibitions were observed with B.
amyloliquefaciens strain I2 and Bacillus sp. G3AM1 against A. carbonarius
(93% inhibition, Fig. 4) and A. ochraceus respectively (97% inhibition,
Fig. 5).

Berry rot, known as Aspergillus rot or black rot, is caused by
Aspergilli whose presence is very common in vineyards (Bejaoui et al.,
2006; Perrone et al., 2006; Tsitsigiannis et al., 2012). In order thus to
directly observe the antagonistic effect of our 21 bacterial strains on
such ochratoxinogenic pathogens, we carried out experiments on
wounded and unwounded grape berries. These comparative in vivo
screening tests showed clear differences in the ranking of the efficacy
of bacterial strains against both fungal pathogens, i.e. A. carbonarius
and A. ochraceus, depended on the pathogen considered and on the presence
or absence of wounds at the fruit surface.

In our biotests, results of pathogenicity were positive in 100% of the
cases; all the berries (wounded or not) inoculated developed rot
symptoms. However, the overall rot severity was lower when A.
ocraceus was inoculated and when unwounded berries were used. The
degree of virulence of A. carbonarius was 77% on wounded berries and
54% on unwounded berries (Fig. 6). Concerning A. ochraceus, its viru-
ence was lower, i.e. 51% and 12% respectively for wounded and un-
wounded berries (Fig. 7). Such differences in the degree of infection
between wounded and unwounded berries might be explained by the
fact that Aspergillus spp. infect grape berries through wounds, con-
fiming the report of Onivins (2005) observing that burst berries by
water supply after a dry period are very favorable to the development of
pathogenic fungi, as well as wounds caused by insects, birds, hail, stalk
dehydration, advanced maturity. Berry wounds may also provide nu-
trients that are likely required by phytopathogenic fungi for initiating
the pathogenic process (Haidar et al., 2016; Onivins, 2005).

On wounded berries, 5 strains provided important levels of pro-
tection greater than 70% for A. carbonarius and 42% for A. ochraceus: B.
pumilus G3AX, Bacillus sp. G3AM1, B. velezensis G3AX1M and 12M, and
B. amyloliquefaciens I2 (Figs. 6 and 7). On unwounded berries, those
same five strains revealed lower levels of inhibition; they were slightly
higher than 50% with A. carbonarius, but they did not exceed 12% with
A. ochraceus. Similarly to fungal pathogenic processes that likely re-
quire specific nutrients to be initiated, differences in nutrient avail-
ability between wounded and unwounded grape berries might be the
main factor accounting for the differences in the antagonistic effects of
the tested bacterial strains on grape berries. Despite such inhibitory
differences, these biossays confirmed the efficacy of Bacillus strains in
controlling pathogenic fungi on different plant hosts (Heidarzadeh and
Ravari, 2015; Mardanova et al., 2017; Palazzini et al., 2016; Pantelides
et al., 2015; Tsitsigiannis et al., 2012; Yuan et al., 2012). Indeed, in our
bioassays, in the presence of the bacterial strains, the disease incidence
was null and/or the lesions induced by both pathogenic Aspergilli dis-
appeared or were reduced even if the antagonistic effects were different
depending on the ochratoxigenic fungus considered. As an example, the
B. amyloliquefaciens strain I2 reduced A. carbonarius mycelial growth by
30% but increased A. ochraceus virulence by 2% on wounded grape
berries (Figs. 6 and 7). Moreover, we observed for the first time that
two strains, i.e. Bacillus sp. K2E1X and B. velezensis G3AX1, increased A.
ocraceus virulence on unwounded grape berries while five strains, i.e.
Paenibacillus sp. 13L, B. amyloliquefaciens L2 and J4C, B. thuringiensis J4F
and Microbacterium oxydans G3AF2, increased the one of A. ochraceus
on wounded berries. Haidar et al. (2016) observed similar phenomena
with strains of B. pumilus and Xanthomonas sp. in berry biocontrol as-
says of Botrytis cinerea.

In accordance with the in vitro tests, i.e. direct confrontations and
volatils, B. pumilus strain G3AX was the most effective strain in con-
trrolling both ochratoxinogenic Aspergilli. The same strain reduced the
levels of rot expression by 77% and 53% for A. carbonarius, and by 50%
and 12% for A. ochraceus, respectively on wounded and unwounded
berries.

5. Conclusions

The present study showed that various bacterial strains colonize the
rhizosphere of Tunisian vineyards. Our results demonstrate that B. pu-
milus strain G3AX has great potential as a biocontrol agent against
Aspergillus diseases on grapevine. Further research should be carried out in the vineyards to test the ability of that strain, but also of other isolated bacteria that have excellent antagonist activity (Bacillus spp. G3AM1 and Bacillus velesensis I2M), to control infections by Aspergillus carbonarius and Aspergillus ochraceus and other fungi involved in grape berry rots. The better protection results on grape berries could be obtained as a surface treatment before or/and during grape storage period when fruits were immersed in a liquid culture containing strong antagonistic bacteria to avoid disease caused by Aspergillus spp..

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