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

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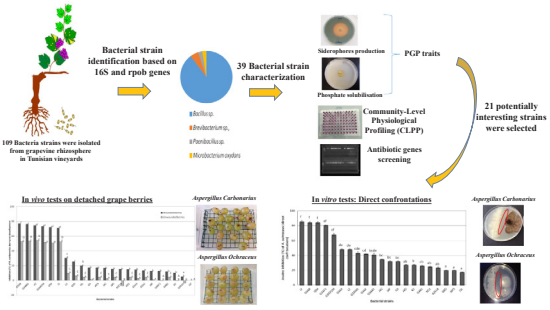
GRAPHICAL ABSTRACT



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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 *rpo*b 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

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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 microorganisms 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; Bejaoui 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; Zimmerli and Dick, 1996).

Although the application of fungicides remains one of the most powerful and cost-effective tools to reduce the incidence of fungal pathogens 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 defined 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 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 (Dimkic 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 rRNA and *rpo*B 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 sole-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 carbornius* 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. Rhisospheric 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 stirred for 15 min and filtrated, before being subjected to a dilution series (from 10^{-1} to 10^{-9}) 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 *rpo*B genes, respectively with the primers 799f and 1492r, and *rpo*Bf and *rpo*Br (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 are available at the Genbank database under accession numbers MH236385 to MH236421.

Table 1
Pairs of primers used.

Applications		Gene	Primer name	Sequence 5′3′	Amplicon size	Annealing T°C
Sequencing	Fungi	**ITS**	ITS1f	CTTGGTCATTTAGAGGAAGTA	650 bp	59 °C
			ITS4	A TCCTCCGCTTATTGATATGC		
	Bacteria	**16S rRNA**	799f	AACMGGATTAGATACCCKG	750 bp	52 °C
			1492r	GTTACCTTGTTACGACTT		
		***rpo*B**	*rpo*Bf	GACGATCATYTWGGAAACCG	350 bp	55 °C
			*rpo*Br	GGNGTYTCRATYGGACACAT		
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		

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 Chemchrome 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 in at least 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. The plates were incubated at 26 °C in the dark. Tetrazolium violet redox dye was used for each well as a colour indicator if added microorganisms utilized the substrates (Insam and Goberna, 2004). Absorbance or optical density (OD) was measured at a wavelength of 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 colour 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.).

2.2.5. Antagonistic and plant growth Promoting (PGP) traits screening

Detection of antibiotic genes. The 39 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) were searched for (Alvarez et al., 2011; Lin et al., 1998; Ramarathnam et al., 2007). PCR assays were performed in a Mastercycler Gradient Thermocycler (Eppendorf) in 30 µl reaction volume consisting of 3 µl of buffer (10X), 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) (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). PCR products were visualized by 2% TBE gel electrophoresis.

Siderophore production. Bacterial strains were tested for their ability to produce siderophores 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 colour 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 $0\,mm < ds \leq 5\,mm$, $5 < ds \leq 10\,mm$ and $ds > 10\,mm$. The experiment was made in triplicate and repeated three times.

Phosphate solubilisation. The 39 bacterial strains were further tested for their ability to solubilise phosphates on Pikovskaya agar medium (PVK) [$(NH_4)_2SO_4$, 0.5 g/l; yeast extract, 0.5 g/l; calcium phosphate ($Ca_3(PO_4)_2$), 5 g/l; KCl, 0.2 g/l; Magnesium sulphate ($MgSO_4{\cdot}7H_2O$), 0.1 g/l; glucose, 10 g/l; Agar, 15 g/l; $MnSO_4{\cdot}2H_2O$, 0.002 g/l; $FeSO_4{\cdot}7H_2O$ 0.002 g/l; pH = 7]. Bacterial plugs (5 mm diameter) from fresh bacterial cultures were placed on the centre of PVK 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 $5\,mm < dp \leq 15\,mm$, $15\,mm < dp \leq 30\,mm$ and $dp > 30\,mm$ (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, $(R_1–R_2)/R_1 \times 100$, 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 in control plates (Whipps, 1987).

2.3.2. Volatile bacterial substances assays

Rhizobacteria were tested for their ability to produce volatile

Table 2
Description and *in vitro* screening results of the bacterial strains.

Strains	Cropping management	Molecular screening		Antagonistic[a] and PGP[b] traits						
		16S rDNA gene (GenBank ref %id)	rpoB gene (GenBank ref %id)	FeA	FeB	FeD	FeE	Bacc	Phosphate solubilisation	Siderophore production
KD1	Conv	*Brevibacterium frigoritolerans* (99%)	–	+	+	–	+	–	–	+ + +
I3L	Org	*Paenibacillus* sp. (97%)	–	+	+	+	+	–	–	+ +
G3AM1	Org	*Bacillus* sp. (97%)	*Bacillus* sp. (97%)	+	+	+	+	+	–	+
G3AX1	Org	*Bacillus siamensis*	*Bacillus velezensis* (100%)	+	+	+	+	–	–	+
G4B2	Org	*Bacillus mojavensis* (99%)	*Bacillus* sp.	+	+	+	+	+	–	+
G3AF2	Org	*Microbacterium oxydans* (99%)	–	+	–	–	–	–	–	+
J4F	Org	*Bacillus thuringiensis*	*Bacillus thuringiensis* (100%)	+	+	+	+	–	–	+
G3	Org	*Bacillus endophyticus*	*Bacillus safensis* (99%)	+	+	+	+	–	–	+
G5	Org	*Bacillus endophyticus*	*Bacillus safensis* (99%)	+	+	+	+	–	–	+
I1	Org	*Bacillus weihenstephanensis* (99%)	–	+	+	+	+	–	–	+
I2	Org	*Bacillus axarquiensis*	*Bacillus amyloliquefaciens* (100%)	+	+	+	+	–	–	+
I2M	Org	*Bacillus toyonensis*	*Bacillus velezensis* (100%)	+	+	+	+	–	–	+ +
J4C	Org	*Bacillus amyloliquefaciens*	*Bacillus amyloliquefaciens* (100%)	+	+	–	+	–	–	+ +
J4D	Org	*Bacillus amyloliquefaciens*	*Bacillus velezensis* (100%)	+	+	+	+	–	–	+ +
G5A1	Org	*Bacillus pumilus*	*Bacillus safensis* (100%)	+	+	+	–	–	–	+ +
G5D	Org	*Bacillus endophyticus* (99%)	*Bacillus* sp.	+	+	+	+	–	–	+
G3AX2	Org	*Bacillus subtilis*	*Bacillus safensis* (99%)	+	+	+	+	–	–	+
G3AX	Org	*Bacillus pumilus* (99%)	–	+	+	+	+	–	+	+
k2	Conv	*Bacillus* sp. (98%)	–	+	+	–	+	–	–	+ + +
I4	Org	*Bacillus amyloliquefaciens*	*Bacillus velezensis* (100%)	+	+	+	+	–	–	+
M4	Conv	–	*Bacillus endophyticus* (99%)	+	–	–	–	–	–	+
K4S	Conv	*Bacillus subtilis* (99%)	*Bacillus endophyticus* (99%)	+	+	+	+	–	–	+
L2	Conv	*Bacillus cereus*	*Bacillus amyloliquefaciens* (100%)	+	+	+	+	–	–	+ +
L3	Conv	*Bacillus pumilus* (99%)	–	+	+	–	–	–	–	+
J4F1X	Org	*Bacillus subtilis*	*Bacillus cereus* (100%)	+	–	–	+	–	–	+
K2E1	Conv	*Bacillus simplex* (99%)	–	–	–	–	–	–	–	+
I2C	Org	*Bacillus thuringiensis*	*Bacillus thuringiensis* (100%)	+	+	+	+	–	–	+
G4B1	Org	*Bacillus subtilis* (100%)	*Bacillus* sp.	+	–	+	+	+	–	+
G3AX1M	Org	*Bacillus atrophaeus*	*Bacillus velezensis* (100%)	–	–	+	+	–	+	+
G4A1	Org	*Bacillus subtilis* (99%)	*Bacillus* sp.	+	+	+	+	+	–	+ +
G3A3	Org	*Bacillus axarquiensis*	*Bacillus velezensis* (100%)	+	+	–	+	–	–	+ +
G5C	Org	*Bacillus endophyticus* (99%)	–	+	–	+	–	–	–	+
J1XM	Org	*Bacillus pumilus* (99%)	–	+	+	+	+	–	–	+
K3	Conv	*Bacillus* sp.	*Bacillus amyloliquefaciens* (100%)	+	–	–	–	–	–	+ + +
G3A2	Org	*Bacillus amyloliquefaciens* (99%)	*Bacillus* sp.	+	+	+	+	–	–	+ +
K2E1X	Conv	*Bacillus* sp. (98%)	–	+	+	–	+	–	+	+
G3AF1	Org	*Brevibacterium halotolerans* (99%)	–	+	+	+	+	–	–	+
J4FS	Org	*Bacillus amyloliquefaciens*	*Bacillus amyloliquefaciens* (100%)	+	+	+	+	–	–	+ +
G3A	Org	–	*Bacillus velezensis* (100%)	+	+	+	+	–	+	+

Org: organic cropping management, Conv: conventional cropping management, Bacc: bacillomycin, Fe: fengycin.
[a] Antagonistic traits: antibiotic genes, siderophore production.
[b] Plant Growth Promoting (PGP) trait: phosphate solubilisation.

substances inhibiting fungal growth using the double plate technique. Strains of *Aspergillus ochraceus* and *A. carbonarius* were grown on Malt Extact 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 containing the bacterial strains to test and sealed with Parafilm. This arrangement allowed the Petri plates inoculated with the *Aspergillus* strains not to be contaminated by the bacteria that may fall. Plates were incubated at 27 °C in the dark. 10 repetitions were made for each bacterial strain and the experiment was repeated three times. The fungal growth was measured after 5 and 7 days of incubation as compared to the control. The control plates had only each of the two *Aspergillus* species growing in them.

2.4. Grape berry rot bioassays with Aspergillus ochraceus and A. carbonarius

For *in vitro* grape berry rot bioassays, table grapes (cultivar: 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). Unwounded 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 were introduced into each wound site. The control treatments with wounded and unwounded berries consisted of: (i) UUC (untreated uninoculated control) untreated with the bacteria and uninoculated with the pathogen, (ii) UC (untreated control) inoculated with mycelium plugs of the pathogen only,

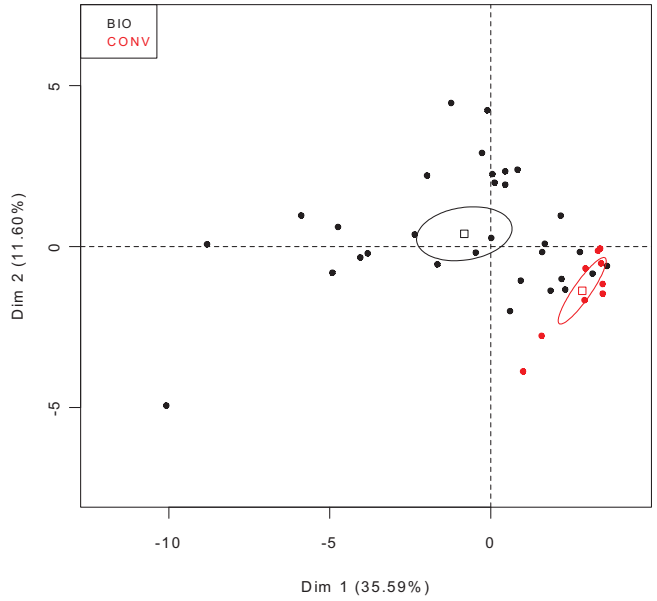

Fig. 1. Principal Component Analysis (PCA) of bacterial strains isolated from the rhizosphere of conventional and organic Tunisian vineyards based on their catabolic profile from Biolog^{TM} Ecoplates. Points represent means of 3 replicate samples. The variation (%) explained by each PCA axis is given in brackets. Biolog^{TM} Ecoplates were incubated 48 h at 27 °C in the dark.

(iii) MC (medium control) untreated and uninoculated controls sprayed only with sterile bacterial LB culture medium, (iv) FC (fungicide control) berries sprayed to runoff, using an EcoSpray sprayer (A 520) with a fungicide solution of Fluazinam (Sekoya, Syngenta France SAS, 50% a.i., 250 g a.i. 100 l) (Haidar et al., 2016).

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 (ANOVAs) and Tukey's post-hoc tests were done using the Rcmdr R-Package.

3. Results

3.1. Characterization of the bacterial strains isolated from the rhizosphere of Tunisian vineyards

Based on partial 16S rRNA and *rpo*B genes sequencing (Table 2), the majority of the bacterial strains belonged to the *Bacillus* genus (34 strains): *Bacillus velezensis* (7 strains), *B. 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. frigoritolerans* (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* G3AX1M 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 from1 to 5 mm (sid + strains).

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

The strains distribution on the principal plan 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 was considered, *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). The most efficient antagonistic bacteria were *Bacillus amyloliquefaciens* (I2) and *Bacillus* sp. (G3AM1) respectively against *A. carbonarius* (Fig. 4) and *A. ochraceus* (Fig. 5).

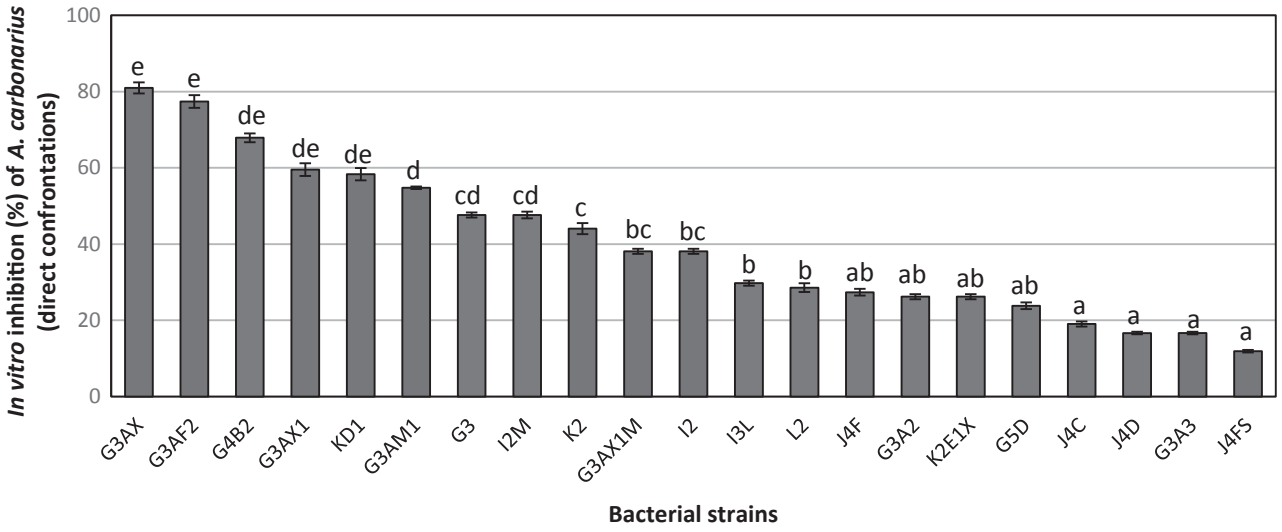

Fig. 2. Effect of the rhizobacterial isolates, applied as 24 h-bacterial cultures, on the growth of the ochratoxigenic fungus *Aspergillus carbonarius*. Mean values (3 replicates) sharing the same letters are not significantly different according to Kruskal-Wallis' non-parametric relative contrast effects post-hoc test at $P < 0.05$.

3.4. 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 repectively 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. Inhibtion 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

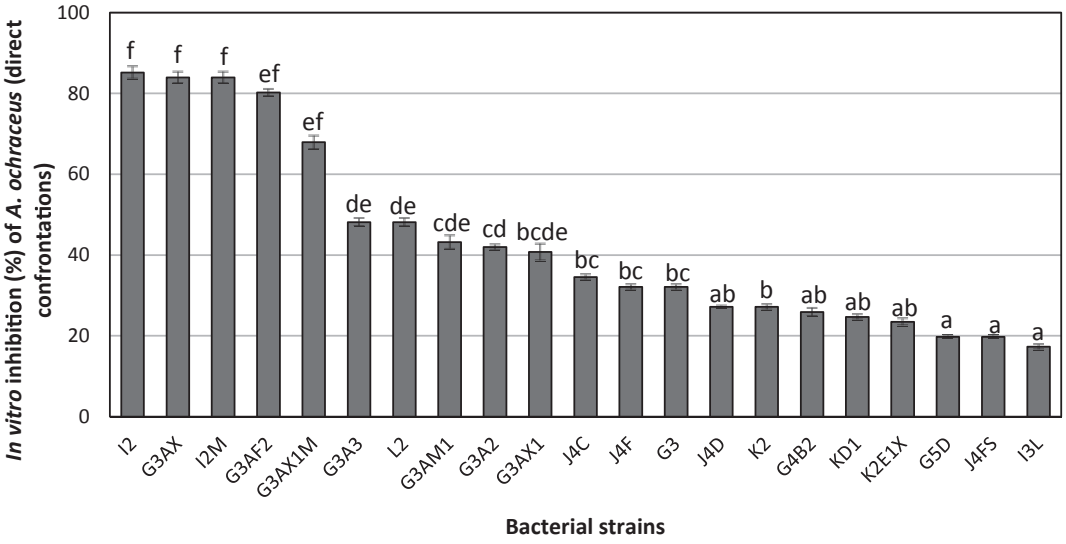

Fig. 3. Effect of the rhizobacterial isolates, applied as 24 h-bacterial cultures, on the growth of the ochratoxigenic fungus *Aspergillus ochraceus*. Mean values (3 replicates) sharing the same letters are not significantly different according to Kruskal-Wallis' non-parametric relative contrast effects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

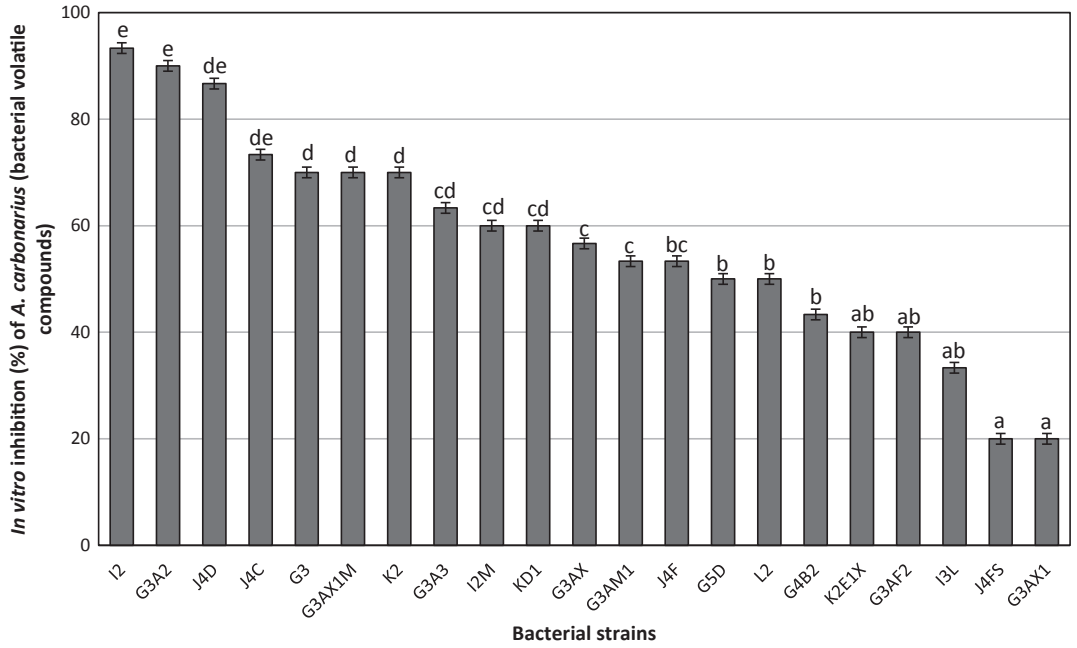

Fig. 4. Effect of the bacterial volatile compounds, using the double plate technique, on the growth of the ochratoxigenic fungus *Aspergillus carbonarius*. Mean values (3 replicates) sharing the same letters are not significantly different according to Kruskal-Wallis' non-parametric relative contrast effects post-hoc test at $P < 0.05$.

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 *rpo*B 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

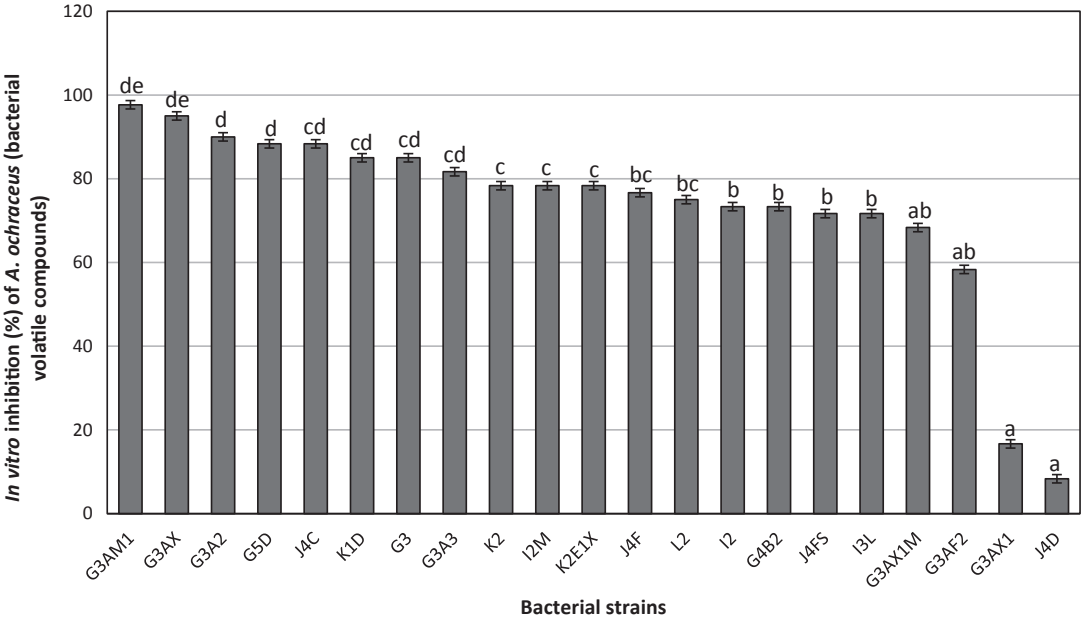

Fig. 5. Effect of the bacterial volatiles compounds, using the double plate technique, on the growth of the ochratoxigenic fungus *Aspergillus ochraceus*. Mean values (3 replicates) sharing the same letters are not significantly different according to Kruskal-Wallis' non-parametric relative contrast effects post-hoc test at $P < 0.05$.

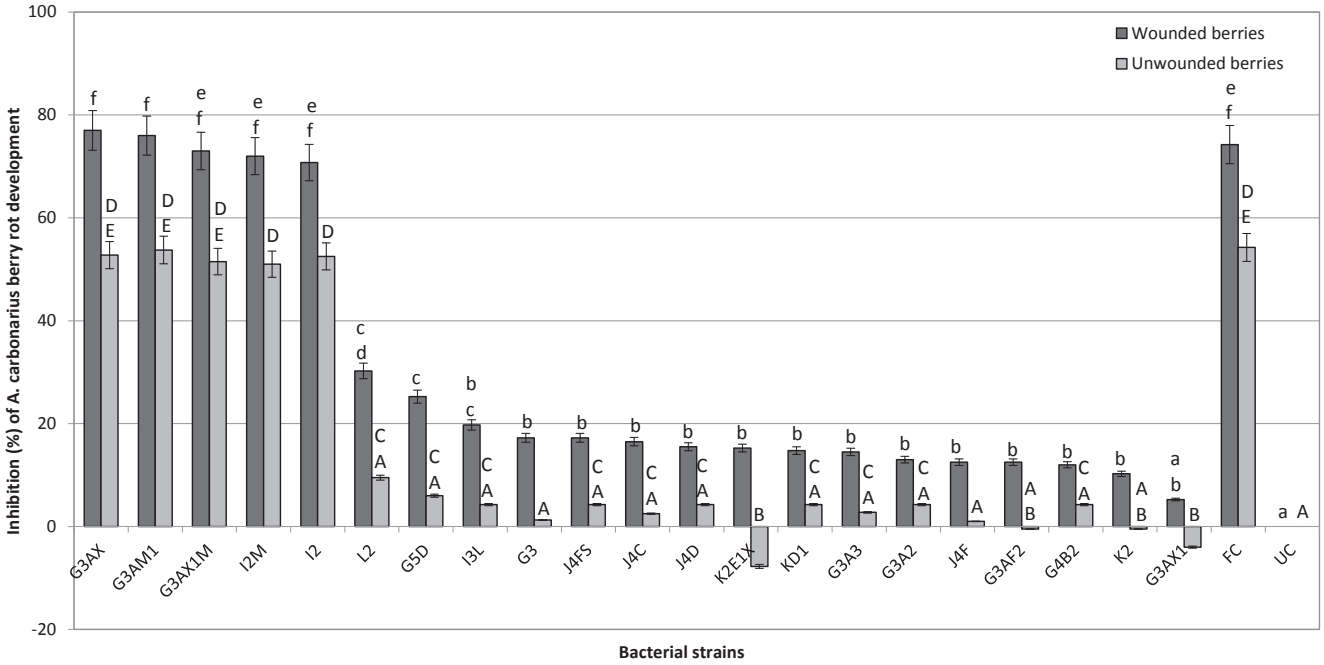

Fig. 6. Effect of the rhizobacterial isolates on the rot development of the ochratoxigenic fungus *Aspergillus carbonarius* on wounded (dark grey bars) and unwounded (light grey bars) berries. Mean values (20 replicates) sharing the same uppercase or lowercase letters are not significantly different according to the Tukey test after ANOVA at $P < 0.05$ (one ANOVA per type of berries, wounded or unwounded). UUC: Untreated Uninoculated Control, UC: Untreated Control (*i.e. A. carbonarius* only), MC: Medium Control (*i.e.* sterile LB medium), FC: Fungicide Control (*i.e.* fluazinam).

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

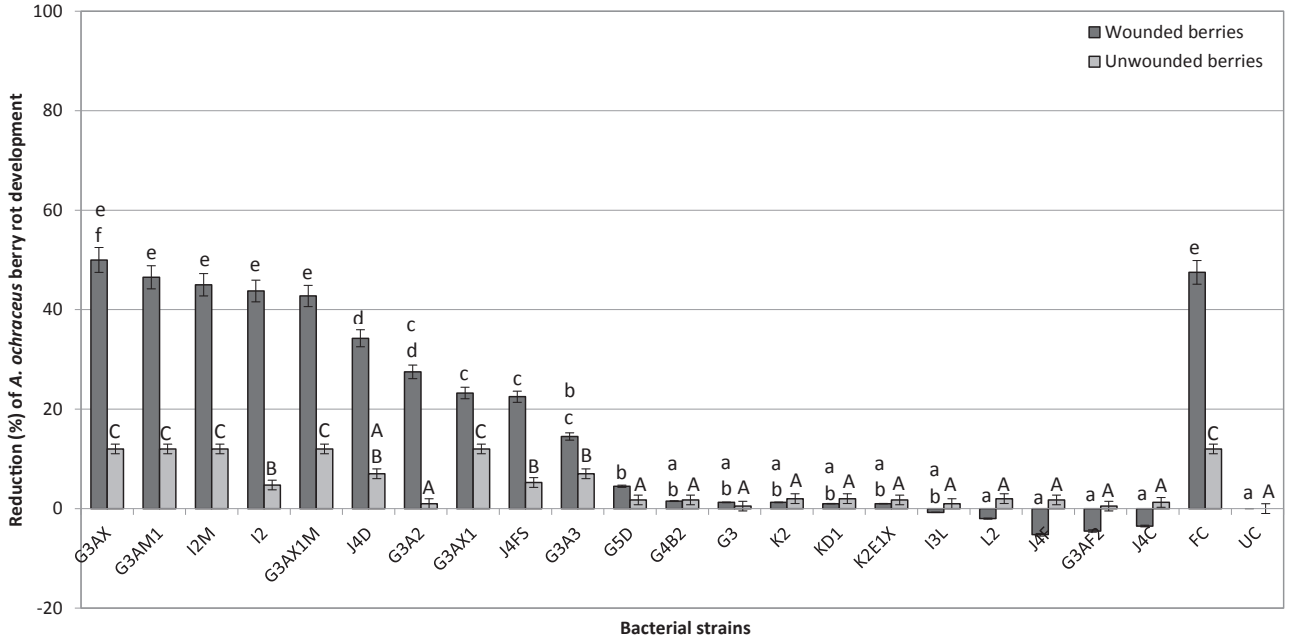

Fig. 7. Effect of the rhizobacterial isolates on the rot development of the ochratoxigenic fungus *Aspergillus ochraceus* on wounded (dark grey bars) and unwounded (light grey bars) berries. Mean values (20 replicates) sharing the same uppercase or lowercase letters are not significantly different according to the Tukey test after ANOVA at $P < 0.05$ (one ANOVA per type of berries, wounded or unwounded). UUC: Untreated Uninoculated Control, UC: Untreated Control (*i.e. A. ochraceus* only), MC: Medium Control (*i.e.* sterile LB medium), FC: Fungicide Control (*i.e.* fluazinam).

one of the 5 antibiotic genes investigated (4 fengycins and 1 bacillomycin), with only three strains of *Bacillus*, *i.e. Bacillus* sp. G3AM1, *B. mojavensis* G4B2 and *B. subtilis* G4A1, having the 5 encoding genes. Iturine (bacillomicine) 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 antifungal 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 siderophores, which are important natural iron chelators representing a novel class of antibiotics with considerable therapeutic potential (Pluhacek et al., 2016). Our results indicated that all the isolated bacteria produced siderophores; the most productive was the *Brevibacterium frigoritolerans* strain KD1 (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 metabolized 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 element 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 contamination 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 exhibit 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, 2013). 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. ochraceus* 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 virulence was lower, *i.e.* 51% and 12% respectively for wounded and unwounded 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, confirming 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 nutrients 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 protection greater than 70% for *A. carbonarius* and 42% for *A. ochraceus: B. pumilus* G3AX, *Bacillus* sp. G3AM1, *B. velezensis* G3AX1M and I2M, 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 require specific nutrients to be initiated, differences in nutrient availability 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* disappeared or were reduced even if the antagonistic effects were different depending on the ochratoxigenic fungus considered. As an example, the *B. amyloliquefaciens* strain L2 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. carbonarius* virulence on unwounded grape berries while five strains, *i.e. Paenibacillus* sp. I3L, *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 assays 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 controlling 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. pumilus* 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 velezensis* 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..

CRediT authorship contribution statement

Mayssa Arfaoui: Investigation, Methodology, Formal analysis, Software, Writing - original draft. **Jessica Vallance:** Conceptualization, Formal analysis, Validation, Supervision, Writing - review & editing. **Emilie Bruez:** Resources, Formal analysis. **Awatef Rezgui:** Methodology, Resources. **Imen Melki:** Resources. **Samir Chebil:** Conceptualization, Resources. **Najla Sadfi-Zouaoui:** Supervision, Funding acquisition, Project administration. **Patrice Rey:** Supervision, Funding acquisition, Project administration.

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