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Antifungal lipopeptides from *Bacillus* strains isolated from rhizosphere of *Citrus* trees

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ABSTRACT

The microbial ecology within the rhizosphere of *Tylenchulus semipenetrans*-infected *Citrus* L. trees was examined. Sixty bacterial strains were obtained from the roots of infected trees and from the eggs of *T. semipenetrans*. Among these strains some were obtained from the agar plates of two nematophagous fungi, *Dactylellina gephyropaga* (Drechsler) Ying Yang & Xing Z. Liu and *Arthrobotrys conoides* Drechsler. Bacterial strains were identified using 16S rDNA, *gyrA* and *rpoB* genes sequence analysis. Bacterial strains identified as *Bacillus* spp. were examined for their ability to synthesize surfactin, iturin, fengycin, kurstakin and bacillomycin using PCR amplification and sequencing of the encoding genes. Additionally, *Bacillus* strains were screened for their antifungal activity against *F. solani*, *D. gephyropaga* and *A. conoides* using the dual culture technique. Lipopeptide from whole cells and from supernatants of *Bacillus* spp. were screened using MALDI-TOF-MS analysis. The majority of the identified bacterial strains belong to the genus *Bacillus* with the predominance of *B. cereus, B. thuringiensis, B. pumilus and B. subtilis*. A total of fifteen *Bacillus* strains demonstrated an antifungal activity against *F. solani*, *D. gephyropaga* and *A. conoides* with the strongest effect found in *B. amyloliquefaciens*. The analysis of lipopeptides showed a high diversity of molecules, including majorly iturin C, bacillomycin D, fengycin A/B and Kurstakin found especially in *B. subtilis* strains. Moreover, MALDI-TOF-MS analysis showed that the responsible antibiotics for the antifungal *Bacillus* strains were associated with the presence of Surfactins/Pumilacidin and Fengycin A/B. Our results demonstrated the wide diversity of lipopeptides among *Bacillus* strains associated with citrus rhizosphere and demonstrated their antifungal ability. Our results extend the importance of *Bacillus* strains as potential candidates for antimicrobial activities due to their ability to synthesize and secrete cyclic lipopeptides.

1. Introduction

Among the predominant bacteria found in the soil *Bacillus* strains inhabit the rhizosphere of various plants and are known for their antimicrobial efficiency (Saxena et al., 2019). The rhizospheric-associated *Bacillus* strains, called PGPR, confer stress tolerance to plants against multiple biotic and abiotic stresses including pathogens (Saxena et al., 2019). However, members of this genus have been implicated as biocontrol agents for their active antimicrobial compounds including species of *B. velezensis*, *B. subtilis*, *B. cereus* and *B. thuringiensis* (Crickmore et al., 2015; Asari et al., 2017; Berini et al., 2018; Fira et al., 2018; Saeid et al., 2018; Borriss et al., 2019). Majority of the antimicrobial metabolites produced by these strains are lipopeptides and oligopeptides (Borriss et al., 2019). The most powerful lipopeptides within *Bacillus* were characterized as surfactin and iturin, including also their contribution to swarming motility and biofilm-forming ability (Zhao and Kuipers 2016) as well as damaging the pathogen cells (Fira et al., 2018). *B. subtilis* and *B. licheniformis* predominantly produce surfactins (Zhao and Kuipers 2016), have demonstrated a strong potential in suppressing many fungal diseases caused by *Colletotrichum* spp., *Alternaria* spp., *Phomopsis*, *Pythium* spp., *Rhizoctonia solani*, *Fusarium* spp. and other fungi (Falardeau et al., 2013; Li et al., 2014; Mora et al., 2015). While

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iturins produced by *B. subtilis* and *B. amyloliquefaciens* (Xu et al., 2020), have been reported as potential antagonists against plant pathogenic fungi like *Penicillium*, *Pestalotia*, *Gloesporidium*, *Colletotrichum*, *Alternaria* and *Botrytis* etc (Falardeau et al., 2013; Ambrico and Trupo, 2017). Fengycin have been also identified as strong antifungal lipopeptides within *B. subtilis* of being capable of damaging the cells of *Fusarium*, *Rhizoctonia* and *Monilinia* etc (Yánez-Mendizábal et al., 2012; Falardeau et al., 2013; Guo et al., 2014). The other implication of these lipopeptides in PGPR *Bacillus* strains have been also reported to be plant growth promoters in crop production (Saxena et al., 2017). Some PGPR *Bacillus* sp. and *B. cereus* strains have shown positive effect on plant growth parameters in both field and greenhouse studies of various crops like wheat (Mukhtar et al., 2017; Raheem et al., 2017), peanut (Goswami et al., 2014), chickpea (Zaheer et al., 2019), tomato (Mehta et al., 2015) and onion (Colo et al., 2014).

In this study, we describe changes in the bacterial community found in the rhizosphere of nematode-infected *Citrus* L. trees and their behavior towards two nematode trapping fungi, *A. conoides* (RGA) and *D. gephyropaga* (CBM) and another fungal isolate, *Fusarium solani,* from the same rhizosphere. A previous study has demonstrated that the interaction between *F. solani* (Mart.) Sacc. and *T. semipenetrans* Cobb, both associated with diseased citrus roots, had significant reduction of citrus plants under greenhouse experiments (Labuschagne et al., 1989). For this purpose, we investigated the antagonistic activity of *Bacillus* strains predominantly found in this rhizosphere against RGA, CBM and *F. solani* fungal isolates and studied their swarming motility and hemolytic activity. In addition, we searched for lipopeptides and putative antagonistic molecules within the antifungal *Bacillus* strains.

2. Material et methods

2.1. Fungal pathogens and culture conditions

Nematophagous fungi (NTF), *A. conoides* (RGA) and *D. gephyropaga* (CBM) were isolated from nematode-infested *Citrus* L. rhizosphere previously identified and characterized by Kallel and Labiadh (2010). RGA and CBM fungal isolates were cultured on CMA (corn meal agar) medium and incubated at 25 °C in the dark (Falbo et al., 2013). Ten-day old CMA plate cultures of RGA and CBM were used for single-conidium isolation and pure subculturing on CMA (Falbo et al., 2013; Xue et al., 2018). Single conidium of each isolate was observed under a binocular microscope and placed on fresh CMA medium. Cultures were then incubated at 25 °C for 10 days and later checked microscopically for germination (Kallel et al., 2008). The CMB fungal isolate was previously characterized as slow-growing-fungi (Kallel and Labiadh, 2010) and therefore was incubated for 14 days.

Fusarium solani isolate was isolated from the same infested citrus rhizosphere, previously identified and characterized as a plant pathogenic isolate by the National Research Institute of Agriculture of Tunisia Collection (INRAT).

2.2. Bacterial strains and culture conditions

Sixty bacterial strains used in this work were previously isolated from of *T. semipenetrans* eggs and from citrus roots (Labiadh et al., 2019). Among these strains, two were associated with RGA and CBM fungal plate cultures (Labiadh et al., 2019). However, the two bacterial strains BRGA and BCBM associated with RGA and CBM isolates were identified as *B. thuringiensis* and *B. subtilis*, respectively (Labiadh et al., 2019). Bacterial strains were maintained on Nutrient agar medium (NA) and incubated at 28 °C for 2 days. BRGA and BCBM bacterial strains previously tested for their antifungal potential (Labiadh et al., 2021) were used as positive control in this study.

2.3. Antifungal screening of the rhizospheric bacteria

All bacterial isolates were screened for their ability to inhibit the mycelial growth of RGA, CBM and *F. solani* fungal isolates. The antifungal activity was performed on CMA plates using the dual culture technique with slight modification (Damasceno et al., 2019). A 10-day old mycelial plug of each fungal isolate (around 3-mm diameter) obtained from the CMA culture was placed 3.0 cm away from the center of a fresh CMA plate. In the opposite side of the fungal mycelial plug, 20 µl of bacterial suspension (10^8 colony forming unit (cfu)/ml) was streaked 3.0 cm away the center of the Petri plate. Control plates were inoculated with 20 µl of sterile distilled water (SDW). Petri plates with 5 replicates for each combination were incubated at 25 °C for 7–14 days. The antagonistic activity of the bacterial strains was evaluated by estimating the percent inhibition of mycelial growth (PI).

Percent inhibition of mycelial growth (PI) was calculated as: PI (%) = [(R-r)/R] × 100; where, R represents the colony radial size (mm) of the fungus in the absence of the bacteria (as a negative control) and r represents the colony radial size (mm) of the fungus proximal to the bacteria (Damasceno et al., 2019).

2.4. Swarming motility

To define the swarming motility in the diverse bacterial strains used for the antifungal assay, bacterial strains were grown in semi-solid Luria–Bertani (LB) medium (10 g tryptone, 5 g yeast extract, 5 g NaCl) supplemented with 0.7% agar and incubated at 37 °C (Kearns and Losick, 2004).

To minimize the swarming of neighboring colonies, a single colony from an overnight bacterial culture was placed using a sterile toothpick in the center of a LB plate and incubated at 37 °C for 18 h (Kearns and Losick, 2004). Following incubation, colonies with diameters reaching 0.3% of the LB agar plates, were scored for swimming and swarming motility (Kearns and Losick, 2004).

2.5. Hemolytic activity of Bacillus strains

Bacillus strains with antifungal activity were selected to study the presence of a hemolytic activity following the protocol suggested by (Hernández-Morales et al., 2018). Bacterial strains were grown on LB agar medium at 28 °C for 2 days. A single colony from the overnight bacterial culture was placed using a sterile toothpick in the center of LB plate containing 5% (v/v) defibrinated horse blood. Petri plates were then incubated at 30 °C and checked after 40 h for the hemolytic phenotype. The ability of these strains to display a production of a biosurfactant was performed through the incubation of bacteria in liquid culture of nutrient broth at 30 °C for 24 h. The incubated test tubes were then checked to observe if there is an emulsification activity in the supernatant (Hernández-Morales et al., 2018).

2.6. Detection of nonribosomal peptide synthetases

Genomic DNA of the *Bacillus* strains was previously (Labiadh et al., 2019) and retrieved from the−20 °C storage for PCR amplification.

The NRPSs genes encoding for the lipopeptides surfactin, iturin, fengycin, kurstakin and bacillomycin synthesis were amplified using PCR primers listed in Table 1.

As1–F/Ts2-R primers (Tapi et al., 2010) were used for the detection of *srfAA* gene (419 bp) in the *srfA* operon responsible for surfactin synthesis (KRAAS et al., 2010). Either of the As1–F/Ts2-R or Am1-F/Tm1-R primer pairs (<100% homology) proved the possible detection of Iturin genes with these primers (Tapi et al., 2010). For fengycin detection, Af2-F/Tf1-R primers were used to amplify a partial sequence of the *fenA* gene (443 bp) (Tapi et al., 2010). Am1-F/Tm1-R primers were used for the detection of *mycA* gene responsible for mycosubtilin synthesis (416bp) (Tapi et al., 2010). For bacillomycin D

Table 1
List of primers used for NRPSs encoding for the lipopeptides surfactin, iturin, fengycin, kurstakin and bacillomycin synthesis.

Nonribosomal lipopeptides identified	Gene	Sequences of primers 5'→ 3'	Amplified products size	Annealing temperature "T" (°C)	References
Surfactin	*srfAA*	**As1–F:** CGCGGMTACCGVATYGAGC **Ts2-R**: ATBCCTTTBTWDGAATGTCCGC	419 bp	43 °C	Tapi et al. (2010)
Iturin	*Iturin A*	**Am1-F:** CAKCARGTSAAAATYCGMGG **Tm1-R:** CCDASATCAAARAADTTATC	416	45 °C	Tapi et al. (2010)
Mycosubtilin	*MycA*	**Am1-F:** CAKCARGTSAAAATYCGMGG **Tm1-R:** CCDASATCAAARAADTTATC	416 bp	45 °C	Tapi et al. (2010)
Fengycin	*fenA*	**Af2-F:** GAATAYMTCGGMCGTMTKGA **Tf1-R:** GCTTTWADKGAATSBCCGCC	443 bp	45 °C	Tapi et al. (2010)
Kurstakin	*krsA*	**Aks-F:**TCHACWGGRAATCCAAAGGG Tks-R:CCACCDKTCAAAKAARKWATC	1125 bp	44.4 °C	Abderrahmani et al. (2011)
Bacillomycin	*bmyA*	**Abl1-F:**GATSAWCARGTGAAAATYCG **Tbl1-R:** ATCGAATSKCCGCCRARATCRAA		45 °C	Moyne et al. (2004)

detection, primer pair of Abl1-F/Tbl1-R were used to amplify the *bmy*A gene (428 bp) (Moyne et al., 2004). The primers designed for the detection of Surfactin, fengycin, iturin and bacillomycin gene families were specifically based on *B. subtilis* strains which are characterized by the production of these lipopeptides (Moyne et al., 2004; Tapi et al., 2010). For kurstakin lipopetide detection, primers were designed based on NRPS encoding genes from *B. thuringiensis* (Abderrahmani et al., 2011). However, for kurstakin detection, Aks-F/Tks-R primers were used to amplify the *krsA* gene with an expected size of 1125 bp (Abderrahmani et al., 2011).

The amounts of genomic DNA template in each PCR mixture (10–30 ng µl−1) as well as PCR mixtures were performed as described by Tapi et al. (2010) and Abderrahmani et al. (2011). The PCR mixture was performed in a volume of 25 µl consisting of 1.5 µl of each primer (10 µM), 12.5 µl (1.2 µM) of Master Mix (Thermo Fisher Scientific), 9.5 µl of H_2O, and 1.5 µl of DNA template. PCR amplifications of NRPSs encoding genes were determined based on the annealing temperature of each pair of primers (Table 1). The PCR thermal cycling conditions was programmed following the protocol described by Tapi et al. (2010): an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30s, annealing at step from 43 to 45 °C (Table 1) for 30s and extension at 72 °C for 45s, with a final extension at 72°c for 5 min. PCR amplifications were performed in a Mastercycler® Personal (Eppendorf, Hambourg, Germany). Five microliters of the PCR amplified products were visualized by electrophoresis in 1.5% (w/v) agarose gel stained with ethidium bromide and checked with an Ultraviolet Illuminator. The presence of the NRPSs genes were scored positive when a band of the appropriate size was detected (Tapi et al., 2010; Abderrahmani et al., 2011).

2.7. MALDI-TOF MS application for the detection of antifungal antibiotics of Bacillus species

Among the *Bacillus* strains showing the presence of multiple biosynthetic genes of antifungal lipopeptides surfactin, iturin A, bacillomycin D, and fengycin, whole cell and supernatant extract of these strains were subjected to MALDI-TOF-MS analysis for the detection of the antifungal antibiotics. Preparation of bacterial whole cell and surface extracts was performed according to the methodologies described by Vater et al. (2003) and Athukorala et al. (2009).

B. subtilis (BCBM) and *B. thuringiensis* (BRGA) strains (Labiadh et al., 2019) were used as the reference control for surfactin and iturin C production (Athukorala et al., 2009). Bacterial strains were grown on LB agar medium at 28 °C for 2 days and supernatants were obtained by inoculating the bacterial culture into 100 ml-flasks containing 10 ml of Landy broth (Sun et al., 2019). The bacterial suspensions were placed on a rotary incubator at 28 °C with shaking at 160 r min^{-1} for 36 h. To remove the bacterial cells and impurities, the cultures were centrifuged at 10,000 r min^{-1} for 15 min and the supernatant was collected. The supernatant formed was filtrated (0.2 µm) and stored at 4 °C for further analysis. Bacterial pellet was collected by centrifugation at 10,000 r min−1 for 20 min and used for MALDI-TOF-MS analysis. Matrix solution was prepared in 500 mL of 70% acetonitrile with 0.1% trifluoroacetic acid. Then, an aliquot of 10 µl of the surface extract was spotted onto the target of the mass spectrometer with an equal volume of matrix solution (Athukorala et al., 2009). Samples of whole cell were air-dried and subjected to MALDI-TOF-MS analysis (Athukorala et al., 2009). NaCl and KCl were added to the matrice to generate Na+ and K + -doped spectra to aid in the identification of the molecular ion peaks.

2.8. Statistical analysis

Data were analyzed using SPSS software version 16.0. Average of fungal growth rate was compared using analysis of variance ($P < 0.05$) and means were separated using Student-Newman-Keuls test. All data were tested for the stability of the variance before analysis by Levene test.

3. Results

3.1. Antifungal screening of the rhizospheric bacteria

Among the tested bacterial strains, 16 *Bacillus* strains showed strong antifungal activity with predominance of *B. subtilis* and *B. pumilus* strains (Table 2; Fig. 1). From the *B. subtilis* group, strains B17, X32, BCBM, B11 and X5 were all effective against *F. solani*, *A. conoides* and *D. gephyropaga* (Table 2). However, strain X10 in this group did not show any inhibition against *F. solani* and *D. gephyropaga* isolates (Table 2). Most of the strains of the *B. subtilis* showed a strong antifungal activity against the NTF isolates with high percent of inhibition ranging between 40 and 60% (Table 2). Four strains of the *B. pumilus* were mostly effective against *A. conoides*, and one strain X33 was only effective against *F. solani* (Table 2). Within the *B. cereus* group, the two strains B7 and X3 inhibited only the mycelial growth of the NTF isolates. Within the *B. thuringiensis* group, strains B18 inhibited the growth of the two NTF isolates, whereas strain BRGA inhibited only the mycelial growth of *F. solani* (Table 2). *B. amyloliquefaciens* strain X28 showed a strong antifungal activity against all fungal isolates and showed the highest percent of inhibition compared to the other *Bacillus* strains (Table 2). *B. amyloliquefaciens* strain X28 inhibited more than 40, 60 and 70% of the mycelial growth of *F. solani*, *D. gephyropaga* and *A. conoides*, respectively. Similarly, *B. mojavensis* strain B12 was effective against all the tested fungal isolates and showed a strong antifungal potential compared to the other *Bacilli*. This strain inhibited more than 64 and 65% of the mycelial growth of *D. gephyropaga* and *A. conoides*, respectively. Overall, *D. gephyropaga* was the most sensitive isolate towards all tested *Bacillus* strains followed by *A. conoides*.

In contrast, *F. solani* was moderately resistant to the application of

Table 2
Percentage of inhibition growth of *Bacillus* spp. against *Fusarium solani*, *Dactylellina gephyropaga* et *Arthrobotrys conoides*.

Bacteria strain			Antifungal activity		Arthrobotrys conoides
			Fusarium solani	Dactylellina gephyropaga	
Bacillus subtilis group	*B. subtilis* subsp. *Subtilis*	B17	38.82 b	40.7 d	50.58 bc
	B. subtilis	X32	34.58 bc	38.11 de	46.58 c
		BCBM	24.47 d	26.11 f	37.88 d
		B11	32.23 c	64.7b	64.11a
		X5	33.88 bc	61.88 b	56.47 b
		X10	–	–	26.35 ef
	B. amyloliquefaciens	X28	46.54 a	71.29 a	63.05a
	B. mojavensis	B12	29.17 c	64.94 b	65.41a
	B. pumilus	X15	–	–	25.88 ef
		X4	–	–	23.76 f
		X33	24.23 bc	–	–
		X24	–	–	28.47 ef
Bacillus cereus group	*B. cereus*	B7	–	45.88 c	34.58 de
		X3	–	–	19.29 f
	B. thuringiensis	B18	–	34.2 e	32.94 de
		BRGA	12.23 e	–	–

The means in same column followed by the same letters (a, b, c … …) are not significantly different according to the test of Student, Newman and Keuls with the probability P = 0.05.

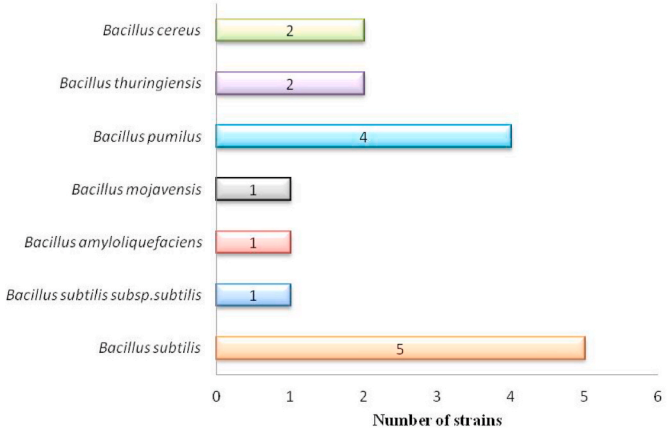

Fig. 1. Number of *Bacillus* strains showing antifungal activity.

antagonistic *Bacillus* strains.

3.2. Swarming motility

To investigate whether *Bacillus* strains are capable of swarming motility, Petri plates containing LB medium supplemented with 0.7% agar were inoculated at a central location. Swarming motility was determined by measuring the colony diameter of the swarming zone after 18 h of incubation at 37 °C. *B. subtilis* X10, *B. pumilus* X24 and *B. amyloliquefaciens* X28 colonized the Petri plates indicating a mechanism of surface motility with colony diameters over 45 mm (Table 3). *B. pumilus* X4 and *B. cereus* B7 strains were not capable of spreading from the site of inoculation and their growth was restricted to a tight central colony (Table 3). The colony diameters of the others *Bacilli* ranged from 13 to 43 mm (Table 3) where the colonies were restricted to the center of the plate.

To determine the swarming phenotypes of the antifungal strains of *Bacillus*, the plates were photographed after 18 h incubation at 37 °C (Fig. 2). Swarming phenotypes included dendritic, snowflake and Ginkoleaf circle-like growth (Fig. 2) with different colony diameters of the swarming zone. *B. subtilis* X10, *B. pumilus* X24 and *B. amyloliquefaciens* X28 were the most invasive strains (Fig. 2). *B. subtilis* X10 and *B. pumilus*

Table 3
Summary of the properties of the antifungal *Bacilli*.

Bacterial strain			Antifungal activity [a]			spreading[b]	hemolytic test	
			F. solani	*D. gephyropaga*	*A. conoides*		Complete lysis	Partial lysis
Bacillus subtilis group	*B. subtilis* subsp. *subtilis*	B17	+	+	+	++	–	–
	B. subtilis	X32	+	+	+	++	+ (3 mm)	–
		BCBM	+	–	+	+	+ (2 mm)	–
		B11	+	+	+	+++	+ (2 mm)	–
		X5	+	+	+	++	+ (4 mm)	–
		X10	–	–	+	++++	–	–
	B. amyloliquefaciens	X28	+	+	+	++++	+ (2 mm)	–
	B. mojavensis	B12	+	+	+	++	–	–
	B. pumilus	X15	–	–	+	–	–	–
		X4	–	–	+	++	+ (1 mm)	–
		X33	+	–	–	+	–	+ (2 mm)
		X24	–	–	+	++++	–	–
Bacillus cereus group	*B. cereus*	B7	–	+	+	–	+ (27 mm)	–
		X3	–	–	+	++	+ (1 mm)	–
	B. thuringiensis	B18	–	–	+	++	+ (2 mm)	–
		BRGA	+	+	+	+	–	+ (3 mm)

[a] Inhibtion activity, (−) no inhibtion, (+) inhibition.
[b] Diameter of the colony after 48 h of growth (−)<15 mm, (+) 15–25 mm, (++) 25–35 mm, (+++) 35–45 mm, (++++) >45 MALDI-Tof-MS analysis: ++: …, +++: …..

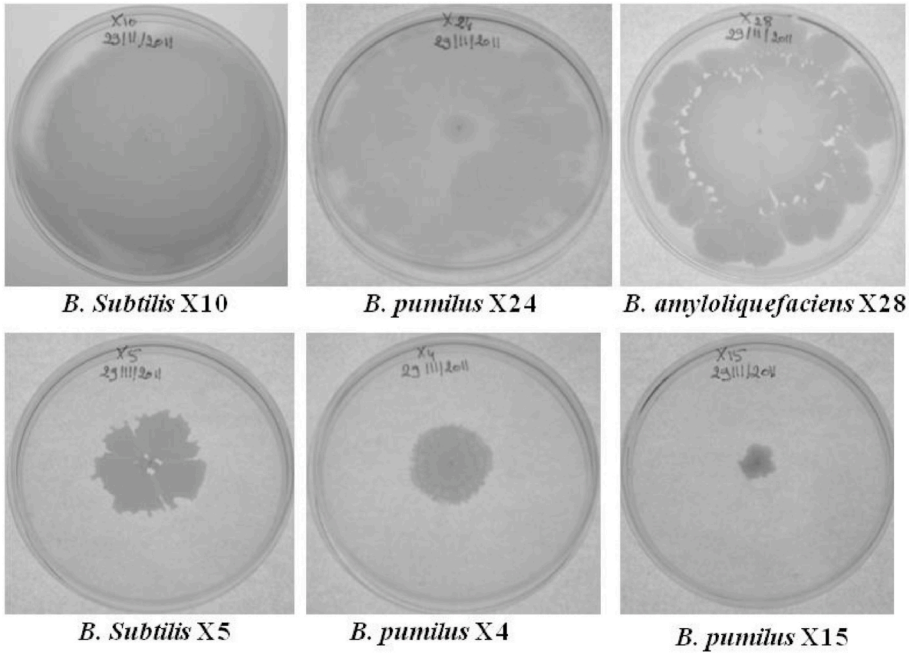

Fig. 2. Swarming patterns of antifungal strains of *Bacillus* inoculated on LB medium supplemented with 0.7% agar.

X24 colonized the agar plate following successive waves of growing, beginning with the formation of successive rounds of translucent finger-formed expansion which then fuse together (Fig. 2). *B. amyloliquefaciens* X28 started at three or four points from the central colony with a Ginko-leaf form.

3.3. Hemolytic activity of Bacillus spp

Bacillus strains with antifungal activity were selected to study the presence of a hemolytic activity on agar blood medium after 40 h at 30 °C. *Bacillus* strains exhibited clear rings of lysed erythrocytes around each colony. Nine strains from sixteen showed hemolytic zone, producing a light halo of 1 mm–27 mm indicating a complete lysis of erythrocytes (Table 3). *B. pumilus* X33 and *B. thuringiensis* BRGA showed partial lyses of red blood cells with the formation of green ring around the colony.

3.4. Detection of nonribosomal peptide synthetases

Primers specific for surfactin, iturin C, fengycin, kurstakin and bacillomycin D were used to amplify biosynthetic genes from these antifungal strains of *Bacillus* using PCR amplification.

The majority of *Bacillus* strains harbored surfactin biosynthetic gene (Tables 3 and 4).

B. subtilis subsp. *Subtilis* (B17), *B. subtilis* (X32), *B. subtilis* (BCBM) and *B. subtilis* (X10) strains were positive for surfactin, whereas *B. subtilis* (B11) and *B. subtilis* (X5) showed the presence of the iturin C biosynthetic gene (Tables 3 and 4). The Bacillomycin D gene was detected in *B. amyloliquefaciens* (X28). Surfactins/Pumilacidin and Fengycin A/B biosynthetic genes were detected in *B. mojavensis* (B12) (Tables 3 and 4). *B. pumilus* strains X15, X4, and X33 showed the presence of the Surfactins/Pumilacidin biosynthetic genes (Tables 3 and 4). Kurstakin biosynthetic gene was detected in *B. pumilus* (X24), *B. cereus* (B7) and *B. thuringiensis* (B18).

3.5. MALDI-TOF MS application for the detection of antifungal antibiotics of Bacillus species

Among the antifungal *Bacillus* strains showing the presence of the lipopeptedic biosynthetic genes, production of particular antibiotics was confirmed through MALDI-TOF-MS of bacterial whole cell and supernatant extract. Mass spectra obtained from *Bacillus* strains showed very clear peak clusters with particular mass peaks and the corresponding antibiotics listed in Table 4. The analysis of lipopeptides showed a high diversity of molecules, especially for *B. subtilis* group. In the spectrum of *B. subtilis*, peaks corresponding to Surfactins/Pumilacidin and iturin C were detected in more or less similar intensities. However, a clear peak clusters representing iturin C, bacillomycin D, fengycin A or B and Kurstakin were detected separately by different strains of *B. subtilis* group. In the spectra of *B. mojavensis* (B12), peaks representing Fengycin A and B were prominent, whereas peaks representing Surfactins/Pumilacidin in this strain were less important. The group of peaks at m/z = 1513.8, 1515.8, 1527.8, 1529.8, and 1543.8 and 1599.8 detected in the mass spectra of *B. mojavensis* (B12) could be attributed to sodium and potassium additions of C14–C19 fengycin A and B (Table 4). Whereas, the peaks at m/z = 1030.6, 1046.6, 1044.6, 1060.6, 1058.6, 1074.6 could be attributed to the protonated forms of C15–C17 Surfactins/Pumilacidin (Table 4). Mass spectra obtained from strains *B. subtilis* subsp *subtilis* B17, *B. subtilis* X32, *B. subtilis* X10, *B. pumilus* X15, *B. pumilus* X4, *B. pumilus* X33, *B. cereus* X3 and *B. thuringiensis* (BRGA) showed only peaks corresponding to surfactin/pumilacidin. The group of peaks at *m/z* = 892, 914, 900,916,928 and 944 detected in the mass spectra of strains *B. pumilus* X24, *B. cereus* B7 and in more intensity for *B. thuringiensis* B18, are characteristic of C12, C11 and C13 kurstakin. In agreement with the results indicated above, each strain produces *in vitro* at least one lipopeptide. Only *B. subtilis* BCBM and *B. mojavensis* B12 showed the capacity to secrete at once two lipopepetides, surfactin/iturin C and surfactin/fengycine, respectively (Table 4).

4. Discussion

Most of our *Bacillus* strains have shown considerable antifungal activity against *F. solani*, *A. conoides* and *D. gephyropaga* fungal isolates with *A. conoides* being the most sensitive one. *A. conoides* and *D. gephyropaga* have been reported to be the most efficient nematode trapping fungi of *T. semipenetrans* (Kallel and Labiadh, 2010), whereas the interaction between *F. solani* and *T. semipentrans* has increased the disease on citrus roots (Labuschagne et al., 1989). Antifungal strains of

Table 4
Peak masses corresponding to particular antibiotics produced by the antifungal Bacilli strain detected by MALDI-TOF-MS analysis.

Strain	Peak mass (*m/z*)	Antibiotics
B. subtilis subsp. *Subtilis* (B17)	**1058.6, 1074.6** [H, Na,K]$^+$, **1100.6, 1116.6** [H,Na,K]$^+$	Surfactins/ Pumilacidin
B. subtilis (X32)	**1046.6** [K]+ ,**1044.6, 1060.6** [H, Na, K]$^+$ **1058.6, 1074.6** [H, Na, K]$^+$	Surfactins/ Pumilacidin
B. subtilis (BCBM)	**1058.6, 1074.6** [Na,K]$^+$ **1078, 1094** (C15) **1106,1122**(C17) **1120,1136**(C18) **1134,1150** (C19)	Surfactins/ Pumilacidin iturine C
B. subtilis (B11)	**1078, 1094** (C15) **1106,1122** (C17)	Iturin C
B. subtilis (X5)	**1106,1122**(C17) **1120,1136** (C18)	Iturin C
B. subtilis (X10)	**1058.6, 1074.6** [Na,K]$^+$	Surfactins/ Pumilacidin
B. amyloliquefaciens (X28)	**1053,1069** (C14) **1067,1083** (C15) **1081,1097** (C16) **1111** (C17)	Bacillomycin D
B. mojavensis (B12)	**1030.6, 1046.6** (C15) **1044.6, 1060.6** (C16) **1058.6, 1074.6** (C17) **1599.8** K^+ FengA (C16) = ou FengB (C14) **1513.8** forme K^+ de FengA (C17) = ou FengB (C14) = **1527.8** forme K^+ de FengA C18 = ou FengB C15 = **1515.8** forme K^+ de FengA (C17) ou FengB (C14) **1529.8** forme K^+ de FengA (C18) ou FengB (C15) **1543.8** forme K^+ de FengA (C19) ou FengB (C16)	Surfactins/ Pumilacidin, Fengycin (A or B)
B. pumilus (X15)	**1058.6** Na^+, **1072.6, 1088.6** [Na, K]$^+$, **1086.6** Na^+	Surfactins/ Pumilacidin
B. pumilus (X4)	**1058.6** Na^+, **1072.6, 1088.6** [Na, K]$^+$**1086.6** Na^+	Surfactins/ Pumilacidin
B. pumilus (X33)	**1058.6** Na^+ **1072.6, 1088.6** [Na, K]$^+$ **1086.6** Na^+	Surfactins/ Pumilacidin
B. pumilus (X24)	892,914(C12) 900,916(C11) 928, 944(C13)	Kurstakin
B. cereus (B7)	892,914(C12) 900,916(C11) 928, 944(C13)	Kurstakin
B.cereus (X3)	994, 1036.8, 1050.8 [Na,K]$^+$	Surfactins/ Pumilacidin
B. thuringiensis (B18)	892,914(C12) 900,916(C11) 928, 944(C13)	Kurstakin
B. thuringiensis (BRGA)	**1002, 1018** [Na,K]$^+$, **1032.6 6** [K]+ **1030.6, 1046.6** [H,Na,K]$^+$ **1058.6, 1074.6** [Na,K]$^+$	Surfactins/ Pumilacidin

B. subtilis X10, *B. pumilus* X24 and *B. amyloliquefaciens* X28 exhibited vigorous swarming motility with the formation of distinct wrinkled-colony architecture. Several of our *Bacillus* spp. strains were able to produce highly surface-active compounds following the formation of clear rings of lysed erythrocytes around each colony. The majority of our *Bacillus* strains harbored surfactin and iturin biosynthetic gene with *B. subtilis* BCBM and *B. mojavensis* B12 simultaneously secreting both genes-related peptides.

Many *Bacillus* species are known for their biocontrol capacity to plant pathogens through lipopeptide antibiotic production (Saxena et al., 2019). Our results support the findings in which surfactin and iturin are among the most common lipopeptide antibiotics found in a wide variety of *Bacillus* sp., including *B. subtilis* as the model strain. Additionally, bacillomycin and fengycin antibiotics have been associated with the inhibition of plant pathogenic fungi through the destruction of the structure and permeability of cell membrane (Deleu et al., 2005; Velho et al., 2011). In our study, Iturin C, bacillomycine D and fengycine have been implicated in the antifungal activity against *F. solani*, whereas Kurstakin inhibited the growth of *D. gephyropaga* and *A. conoides* isolates. kurstakin was detected in *B. pumilus* X3, the only strain in the *pumilis* group showing antifungal potential againt *F. solani*. According to Abderrahmani et al. (2011), Kurstakin was only involved in the biosynthesis of the siderophore bacillibactin implicated in iron transport of *B. subtilis*. However, the potential properties of kurstakins, produced by *B. thuringiensis* displaying antifungal activity have been recognized in 2012 by Béchet et al. (2012).

Surfactin, residing in the extracellular surfactants of *B. subtilis* has been reported to have an essential role in sliding motility of the bacteria (Hao et al., 2011). However, Leclère et al. (2006) demonstrated that the overproduction of mycosubtilin, a lipopeptide of the iturin family, has been also implicated in spreading activity of the bacteria. In our study, iturin C, bacillomycine D and fengycine were directly implicated in the spreading activity of our *Bacillus* strains, thus facilitating their invasive growth. The implication of the kurstakin biosurfactant in growth phenotypes is yet unknown (Abderrahmani et al., 2011). In our study, *B. cereus* B7, known for producing Kurstakin, has a weak spreading activity with a colony diameter lower than 15 mm. Moreover, *B. thuringiensis* B18, with a high content of kurstakin, has a medium spreading activity. Whereas, *B. pumilus* X24, with a medium production of kurstakin was the most invasive strain on culture media. However, previous results suggested that kurstakin secreted from *B. thuringiensis* strains with the most efficient biosurfactant activity, has a high affinity for cell membranes (Béchet et al., 2012).

The bioactive lipopeptide kurstakin was detected in our *B. pumilus* strain, although it has been reported to be only present in *B. thuringiensis* and *B. cereus* (Abderrahmani et al., 2011; Béchet et al., 2012). Three years after discovery of kurstakin, homologous series of three ions at m/z 892, 906, and 920 similar to those of kurstakins were detected by Madonna et al. (2003) in *B. subtilis* (ATCC6051). However, these results were not confirmed through genetic analysis. Although the production of some antibiotics can be produced by a particular strain, their synergistic activity may be occasional under a given set of conditions (Koumoutsi, 2006). This may have caused the differences observed in peak intensities of iturin C, bacillomycin D, fengycin A or B and Kurstakin which have been separately detected by different strains of *B. subtilis*. However, *B. mojavensis* strain B12 is a good example of this where it was found to produce 4 of the aforementioned antibiotics. The MALDI-TOF analysis of *B. mojavensis* B12 showed high intensities for fengycin A and B and low for Surfactins/Pumilacidin. Additionally, surfactin has never been detected in *B. thuringiensis*, (Abderrahmani et al., 2011), while our strains *B. thuringiensis* (BRGA) and *B. cereus* X3 harbor this lipopeptide.

5. Conclusion and perspectives

Our results highlight the potential production of lipopeptides by different strains of *Bacillus* obtained from citrus rhizosphere and highlight their antifungal potential. Additionally, the results of this study along with previous studies emphasize the need of new research focus on biosurfactant lipopeptides, which can be used as biopesticides.

Declaration of competing interest

Informed consent was obtained from all individual participants included in the study. The authors report no conflicts of interest. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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