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ORIGINAL ARTICLE

Antifungal activity of *Bacillus* spp. against *Fusarium oxysporum* f. sp. *lycopersici* and *Ascochyta* sp.

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Abstract

The aim of this study is to find bacterial strains with antagonistic effects against *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) and *Ascochyta* sp, which are phytopathogens responsible for fusarium wilt of tomato and ascochyta blight of peas, respectively. One hundred thirty-six bacteria isolated from the rhizosphere of tomatoes were screened. Five strains with the largest inhibition zones were selected. These strains were identified by the phenotypic method, later confirmed by sequencing of 16S rDNA. All strains belonged to the genus *Bacillus* spp. Their inhibition capacity was evaluated by the direct method by doing a dual culture, the inhibition rates ranged from 44.32 ± 0.8 to 61.36 ± 0.2 against *Fol* and 62.04 ± 0.8 to 74.1 ± 0.2% against *Ascochyta* sp. They were then evaluated by the indirect method by evaluating, on one hand, the antifungal effect of the volatile compounds produced by the strains and on the other hand, the antifungal effect of the filtrates. The results showed that volatile compounds inhibited plant pathogens' growth with average inhibition rates of 55% against *Fol* and 17% against *Ascochyta*. For filtrates, the average inhibition rates were 33.01% against *Fol* and 33.74% against *Ascochyta* sp. Finally, the plant growth promoting rhizobacteria (PGPR) effect of *B. halotolerans* RFP57 was evaluated. This involved assessing their ability to stimulate the germination of tomato seeds and the growth of their vegetative organs. The results showed significant improvement in treated seedlings compared to controls. All these results show that the strains selected for this study have the potential for use as a biocontrol agent. However, it is clear that further in-depth studies are needed to confirm their true potentiality.

Keywords: *Ascochyta*, *Bacillus*, biocontrol, *Fusarium*, phytopathogen

Introduction

Plant pests are responsible for an annual loss of 40% of global agricultural production (FAO 2021). Among the most dangerous causative agents are phytopathogenic fungi. They cause various diseases that affect several crops (Almeida *et al.* 2019). In order to control these pests, farmers often rely on chemical pesticides. With time, this use became excessive. Over the past 20 years, the consequences of the use of chemical pesticides have started to appear (Rani *et al.* 2021). These consequences range from the appearance of more and more germs resistant to pesticides, the disruption of the microflora population of soil, to the presence of chemical residues in the food chain and their possible negative effects on consumers' health (Mobin and Usmani 2017). Therefore, finding an alternative to chemical pesticides has become an absolute priority. One alternative is biological control: "the exploitation of living agents (incl. viruses) to combat pestilential organisms (incl. pathogens, pests, and weeds) for diverse purposes to provide human benefits" (Stenberg *et al.* 2021). These living agents include microorganisms such as bacteria and fungi (Bhattacharjee and Dey

2014). Among bacterial agents, the genus of *Bacillus* is one of the most studied and most exploited because of its great potential as a biological control agent. Several biological control products based on this genus are already available commercially (Kumar *et al.* 2021). The genus of *Bacillus* represents a large and diverse group of bacteria having one distinctive point in common: the ability to form an endospore under aerobic conditions when they are in an unfavorable environment (Goldman and Green 2009). In addition, *Bacillus* species have a unique ability to replicate rapidly, are resistant to adverse environmental conditions, and have a very broad biological control capacity (Shafi *et al.* 2017). These bacteria use several mechanisms to inhibit phytopathogens: direct antibiosis, competition by the secretion of spectrum secondary metabolites in the rhizosphere, beneficial actions on the host plant, and stimulation of induced systemic resistance in plants (Fan *et al.* 2018). Most biological control agents (BCA) have a specific action only for a certain type of pathogen. From an environmental point of view this is an advantage, but for growers who need to control several plant pathogens in the same crop, it creates difficulties (Spadaro and Gullino 2005). Therefore, searching for BCA that have action against two or more phytopathogens can be a way to improve the control strategy against pests.

The objective of this study was to find bacterial strains capable of inhibiting two important plant pathogenic fungi and to evaluate their antifungal activity. For this purpose, we isolated bacteria from the rhizosphere and tested their antagonism against *Fusarium oxysporum* f. sp. *lycopersici* and *Ascochyta* sp. The first one is responsible for fusarium wilt of tomatoes. Browning of vascular tissue is strong evidence of this disease (Ignjatov *et al.* 2012). The second one is a pathogen which mainly attacks legumes causing Ascochyta blight. Symptoms of this disease can appear on all aerial parts of a plant where numerous pycnidia develop on necrotic lesions (Pande *et al.* 2005). The antagonistic bacteria were evaluated *in vitro* by direct confrontation and indirect confrontation followed by *in vivo* studies on *Solanum lycopersicum* (tomato) plants.

Materials and Methods

Isolation of bacteria

In December 2019, nine soil samples were collected from different sites in Oran (Es Senia, Belgaid) western Algeria. These sites are located in a region with a Mediterranean climate with silty and clay soils. These sites were selected because their soils have been cropped with tomatoes for 15 years. The samples were taken from the rhizosphere of tomatoes at a depth of 20 cm. The collected soil samples were put in a paper bag and taken directly to the laboratory for analysis. Each sample was carefully mixed, 1 g was transferred into 9 ml of sterile 0.9% saline solution, and was serially diluted (10^{-3}). An aliquot of 100 µl of the diluted solution was inoculated at the surface of nutrient agar media (NA). The test was carried out in triplicate. The plates were incubated at 25°C and 37°C for 24 to 48 h. After incubation, the colonies having different morphological appearances were selected and streaked onto new plates containing NA media. For each colony, successive subcultures were carried out in order to obtain pure cultures. The isolated bacteria were transferred into slant tubes containing NA media and stored at 4°C.

Origin of phytopathogens

The strain of *Ascochyta* sp. used in this study was isolated from necrosis present on the pea pod. The *Fusarium oxysporum* f. sp. *lycopercisi* (*Fol*) strain was obtained from the collection of the LMA laboratory (Laboratoire Microbiologie Appliquée Es-Senia).

Screening of isolates against *Fol* and *Ascochyta* sp.

Isolated bacterial strains were screened based on antagonistic activity against *Fol* and *Ascochyta* sp. The dual culture method in potato dextrose agar (PDA) described by Oldenburg *et al.* (1996) was followed, with minor modifications. Briefly, the pathogenic disk (5 mm in diameter) was transferred to the center of a Petri dish (90 mm in diameter) containing 15 ml of PDA. The bacterial strains were inoculated to the periphery, 25 mm away from the mycelial disk. Four different strains were spotted on each plate. Plates with no bacterial strains inoculated were set as control. The incubation time was 7 days at 27°C for *Fol* and 10 days at 24°C for *Ascochyta* sp., respectively. An apparent inhibition zone around the colony was considered to be a positive result.

The bacterial strains selected were those with the most apparent inhibition zones against the two phytopathogens in order to evaluate their inhibition rates. For this purpose, the same dual culture method described above was applied, albeit with a slight modification; this time the same strain was spotted to the peripheries. For each isolate, three replicates were used. To determine the inhibition rate, the following formula was used:

$$\text{Inhibtion rates (\%)} = \frac{R1 - R2}{R1} \times 100,$$

where: $R1$ – diameter of the pathogen in control, $R2$ – diameter of the pathogen in treatment.

Physiological and biochemical characterization of the selected antagonist strains

In order to identify the selected antagonist strains, the following steps were applied. Firstly, macroscopic observation was done by studying the characteristics of the colonies (size, color, viscosity, shape). Secondly, microscopic observation (gram staining, shape, grouping, presence of spores) was carried out. Additional tests were performed such as the catalase, respiratory type and motility tests. These different tests enabled us to determine the genus to which the selected isolates belonged. Finally, having determined the genus, we performed various biochemical and physiological tests described in Bergey's Manual of Systematics of Archaea and Bacteria (Logan and Vos 2015) for the genus *Bacillus*.

Molecular identification of the bacterial strains

The extraction of bacterial genomic DNA was performed using the GF-1 nucleic acid extraction kit (Vivantis Technologies Sdn Bhd, Selangor DE, Malaysia) according to the manufacturer's instructions. Extracted DNA was stored at 4°C until required for PCR.

PCR amplification was achieved using the primer set of 16S rRNA gene (27F: 5' – AGAGTTTGATC CTGGCTCAG – 3' and 1492 5' – CTACGGCTAC CTTGTTACGA – 3'). The PCR runs were as follows: initial denaturation at 94°C for 2 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The amplification was repeated in 30 cycles followed by a final extension at 72°C for 7 min. PCR were carried out using a thermocycler (iCycler Bio-Rad, USA).

The PCR products were detected using 1.5% agarose gel electrophoresis (Sigma-Aldrich, USA), purified (Clean-Up kit, Vivantis) and sent to a sequencing agency (Apical scientific Sdn. Bhd.). The generated sequences were analyzed using BLAST which is available on NCBI data base (http://blast.ncbi.nlm.nih.gov).

Evaluation of the inhibitory effect of the filtrates

The antifungal activity of the cell-free filtrate of the antagonistic strains against *Fol* and *Ascochyta* sp. was assessed. The diffusion method described by Mehmood *et al*. (1999) was followed with minor modifications. First of all, it consists of preparing the filtrate. A bacterial culture was prepared in 10 ml of nutrient broth inoculated from a single colony and incubated at 30°C for 48 h. The culture was centrifuged at 12,000 rpm for 20 min and membrane filtered (0.2 µm, Merck Millipore Nylon). Then, 1 ml of this filtrate was added to 15 ml of molten PDA medium, mixed well and allowed to solidify. Afterwards, a 5 mm mycelium disk was placed in the center of the plate. For the negative control, uninoculated NA medium was added to the PDA medium. During the incubation period, 7 days at 27°C for *Fol* and 10 days at 24°C for *Ascochyta* sp., the diameters of the mycelia were measured daily. All treatments were carried out with three replicates.

Evaluation of the antagonistic effects of volatile metabolites

The effects of the volatile compounds released by the antagonist strains on the growth of the two phytopathogens were evaluated. For this purpose, the two Petri dishes method (Zhang *et al*. 2020) was applied. Briefly, it includes using two Petri dishes, one containing PDA medium and one containing NA medium. On the plate containing PDA, a fragment of mycelium from the pathogen tested (5 mm in diameter) was placed in the center. On the plate containing NA, the antagonist strain to be evaluated, sub-cultured from a 24 h culture, was streaked. Afterwards, the bottom parts of the two plates were tightly joined facing each other using parafilm in order to prevent any loss of volatile compounds. Control treatment involved uninoculated NA plates facing the PDA plates containing the fungus. Incubation was at 27°C for 7 days for *Fol* and 24°C for 10 days for *Ascochyta,* respectively. The diameters of the growth of the fungi were measured daily during this incubation period. All treatments were carried out with three replicates.

Tomato seeds germination

Among the advantages of using biological control agents on a crop is their ability to promote plant growth, referred to as plant growth promoting bacteria (PGPR). The ability of our selected antagonist bacteria to stimulate the germination of tomato seeds (*Solanum lycopersicum*) was assessed. In order to perform this assay, the method described by Zouari *et al*. (2020) was applied. The tomato seeds (Super Marmande cultivar) were surface disinfected by dipping them in 70% ethanol for 3 min, followed by 5% sodium hypochlorite for 3 min, and then in sterilized distilled water five times. They were finally washed in water for 2 h. After this the sterilized seeds were dipped in the bacterial suspension adjusted to 10^8 cells · ml^{-1} for 2 h, and placed in a Petri dish containing 15 ml of agar culture medium (2% agar). Seeds dipped in sterilized distilled water were considered as negative controls. For each treatment,

10 plates containing two seeds each were considered. The percentage of germinated seeds during the treatment along with their lengths were determined. The vigor index (*VI*) was calculated according to the formula of Abdul-Baki and Anderson (1973):

$$VI = \text{germination percentage [\%]} \times \times \text{germination lengths [mm]}.$$

Pot culture experiment

The ability of the isolate *B. halotolerans* RFP57 to stimulate the growth of tomato plants as well as its ability to limit the action of the phytopathogen *Fol* were evaluated in a pot culture. The experiment took place from August 17 to September 17, 2021 at Oran Es Senia University site (GPS coordinates: 35.640276, –0.615787).

For this experiment, four groups of treatment were considered: 1 – RFP57 treated; 2 – *Fol* treated; 3 – *Fol* + + RFP57 treated; 4 – control (with no treatment).

For group 1, 10 ml of the inoculum of RFP57 strain (liquid medium/OD620 nm = 0.8) was mixed with 70 g of sterile soil collected from the Es Senia site. For group 2, 10 ml of a 5% diluted suspension of *Fol* mycelial fragments was added to the soil. For group 3, 5 ml each of a mycelial suspension and bacterial inoculum were added to the soil. Finally, for group 4, 10 ml of sterile distilled water was added.

For each group of tests, three seeds per pot were considered with 10 replicates each. The germination rates, the length of the stems as well as the fresh weight of the plants were determined and evaluated after 30 days.

Data analysis

Data from assays *in vitro* and *in vivo* were statistically analyzed using the one-way analysis of variance (ANOVA) and the Tukey HSD multiple--comparison test at the 5% level of significance ($p \leq 0.05$) using STATISTICA software version 10 (www.statsoft.fr).

Results

Isolation of bacteria

At the various sites of the Oran region mentioned above, 136 bacterial strain samples with different morphological appearances were isolated. We found that the soils sampled at the Es-Senia site contained more bacterial diversity and that 37°C was the most favorable temperature for the majority of the bacteria isolated.

Table 1. The inhibition rates of the selected isolates against the phytopathogenic fungi

Strains	Inhibition rates [%]	
	Fol	*Ascochyta* sp.
RFP1	53.4 ± 0.6	71.8 ± 0.3
RFP10	55.67 ± 0.2	72.5 ± 0.3
RFP63	44.32 ± 0.8	63.3 ± 0.6
RFP57	61.36 ± 0.2	74.1 ± 0.2
RFP74	54.54 ± 0.4	62.04 ± 0.8

Data are presented by the means ±SD

In vitro screening of isolates against *Fol* and *Ascochyta* sp.

Using the dual culture method, the bacteria having antagonistic activities against *Fol* and *Ascochyta* sp. were selected from the isolates. Of the 136 strains tested, 17 showed inhibition of the growth of *Fol* after 7 days of incubation. The five that showed the greatest zone of inhibition were selected for further study.

Tested against *Ascochyta* sp., these five strains also showed a large zone of inhibition. The inhibition rates of the isolates against the two phytopathogens *Fol* and *Ascochyta* sp..were determined (Tab. 1). The bacterial strains had a higher capacity to inhibit the growth of *Ascochyta* sp. than Fol. Three strains had an inhibition rate of more than 70% of which RFP57 had the largest with 74.1 ± 0.2%. RFP74 and RFP63 had the lowest rates with 62.04 ± 0.8 and 63.3 ± 0.6, respectively. Against *Fol*, the inhibition rates varied from 44.32 to 61.36%, the lowest belonged to RFP63 while the highest belonged to RFP57. The ANOVA test ($p < 0.05$) demonstrated the significant effect of these strains against the growth of both pathogens.

Physiological and biochemical characterization of the selected strains

After performing a Gram stain, the slides were viewed using a light microscope at 1000× magnification. The characteristics observed were Gram-type, shape, cell arrangement, and the presence or absence of endospores. All the selected strains are spore formers, Gram positive, and rod shape. Some biochemical and physiological tests were carried out and the results are presented in Table 2.

Molecular identification of the isolates

The 16S rDNA of the five selected strains were partially sequenced and the obtained sequences were analyzed and aligned with the reference sequences at a percentage between 99 and 100% using BLAST. The sequences of 16S rDNA of these strains were submitted to NCBI

Table 2. Physiological and biochemical characterization of the selected antagonistic strains

Tests	RFP1 (*Bacillus halotolerans*)	RFP10 (*Bacillus halotolerans*)	RFP63 (*Bacillus cereus*)	RFP74 (*Bacillus halotolerans*)	RFP57 (*Bacillus halotolerans*)
Gram	+	+	+	+	+
Spore presence	+	+	+	+	+
Catalase	+	+	+	+	+
Anaerobic growth	+	+	+	+	+
Mannitol	+	+	–	–	+
Starch hydrolysis	+	+	+	+	+
Gelatin liquefaction	+	+	+	–	+
Casein hydrolysis	+	+	+	+	+
Citrate utilization	–	–	+	+	–
Nitrate reduction	+	+	+	+	+
Growth at pH 6	+	+	+	+	+
Growth at pH 8	+	+	+	+	+
Voges-Proskauer	–	–	+	+	–
Growth in NaCl 7%	+	+	–	+	+
Growth at 50°C	+	+	+	+	+
Growth at 65°C	–	–	–	–	–

Table 3. Molecular identification of the selected strains

Strains	Identification	% Identity	Accession number
RFP74	*Bacillus halotolerans*	99.73	OM439612
RFP57	*Bacillus halotolerans*	99.65	OM439613
RFP10	*Bacillus halotolerans*	100.00	OM439614
RFP1	*Bacillus halotolerans*	99.74	OM439615
RFP63	*Bacillus cereus*	99.72	OM439616

(http://blast.ncbi.nlm.nih.gov) and the accession numbers were obtained (Tab. 3). Four of the strains belonged to *B. halotolerans* while RFP63 belonged to *B. cereus*.

Evaluation of the inhibitory effects of the filtrates

The growth of *Fol* in the presence of the filtrates excreted from the different antagonist strains was evaluated. All filtrates from five strains significantly reduced the growth of the pathogen ($p < 0.05$) compared to the control. The average inhibition rate for all the strains was 33.01%. RFP63 had the highest inhibition rate with 44.5% after 7 days of incubation (Fig. 1A).

Against *Ascochyta* sp., the filtrates of the antagonist strains had an average inhibition rate of 33.74% compared to the control. RFP63 strain had the highest inhibition rate with 44.89% after 10 days of incubation (Fig. 1B). Overall, all the tested filtrates significantly ($p < 0.05$) slowed down the fungal growth.

Assessment of the inhibitory effects of volatile compounds

All of the tested bacterial strains produced some volatile compounds with antifungal activity that significantly ($p < 0.05$) slowed down the growth of *Fol*. Strain RFP57 had the highest inhibition rate with 67% compared to the control while RFP74 was the least effective with 34%. The average inhibition rate for all the strains was 55% (Fig. 2A).

Against *Ascochyta* sp., all of the tested strains emitted volatile compounds that significantly ($p < 0.05$) slowed down the growth of the pathogen except strain RFP57 whose inhibition rate was only 7%. The average inhibition rate for all the strains was 17%. RFP1 had the highest rate with 24.8% compared to the control after 10 days of incubation (Fig. 2B).

The volatile compounds produced by the tested strains showed higher antifungal activity against *Fol* than *Ascochyta* sp.

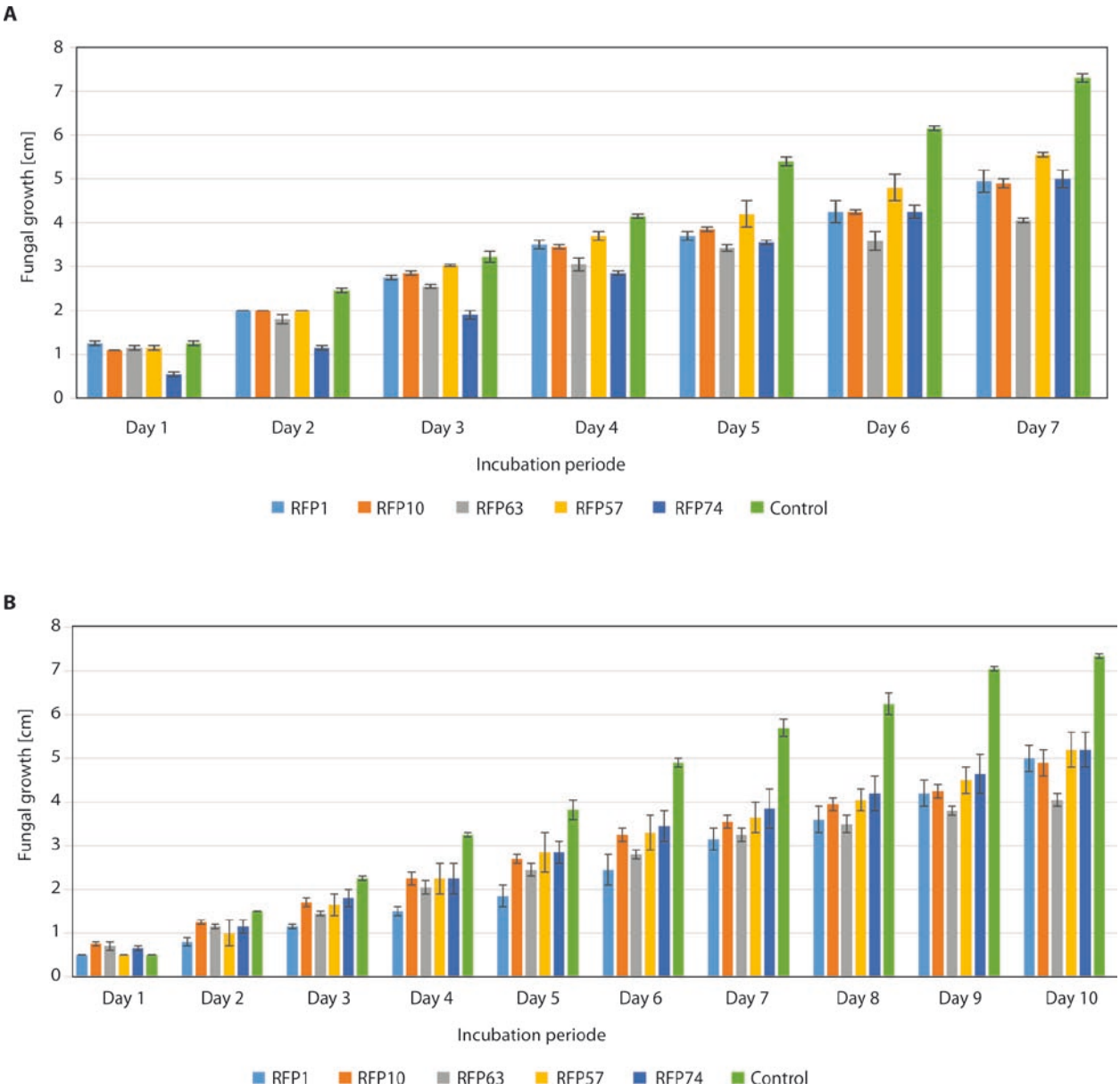

Fig. 1. Effect of the bacterial filtrates on the growth of the phytopathogenic fungi: A – the effect on the growth of *Fusarium oxysporum* f. sp. *lycopersici;* B – the effect on the growth of *Ascochyta* sp.
Values in the column bar are the means ±SE

The ability of strains to stimulate the germination of tomato seeds

The ability of the selected bacterial strains to stimulate the germination of tomato seeds was investigated and the vigor index was calculated (Fig. 3). Statistical analysis using Tukey's test showed that among the strains tested, RFP57 was the only one that showed a significant ability to stimulate seed germination compared to the control (VI = 12.315,7). It was then chosen for the study on the ability of antagonist bacteria to stimulate the growth of tomato plants.

Evaluation of the PGPR effect of agent RFP57

The ability of strain RFP57 to stimulate tomato growth as well as its ability to reduce the symptoms of *Fol* on the crop was evaluated. The results obtained are set out in Table 4.

Compared to the control, seeds planted in soil inoculated with strain RFP57 had a higher germination rate. Moreover, the elongation of their shoots and their fresh weight were superior to the other groups. Their shoot elongations were significantly ($p < 0.05$) greater than those of the plants grown in soil inoculated with the phytopathogen *Fol* only.

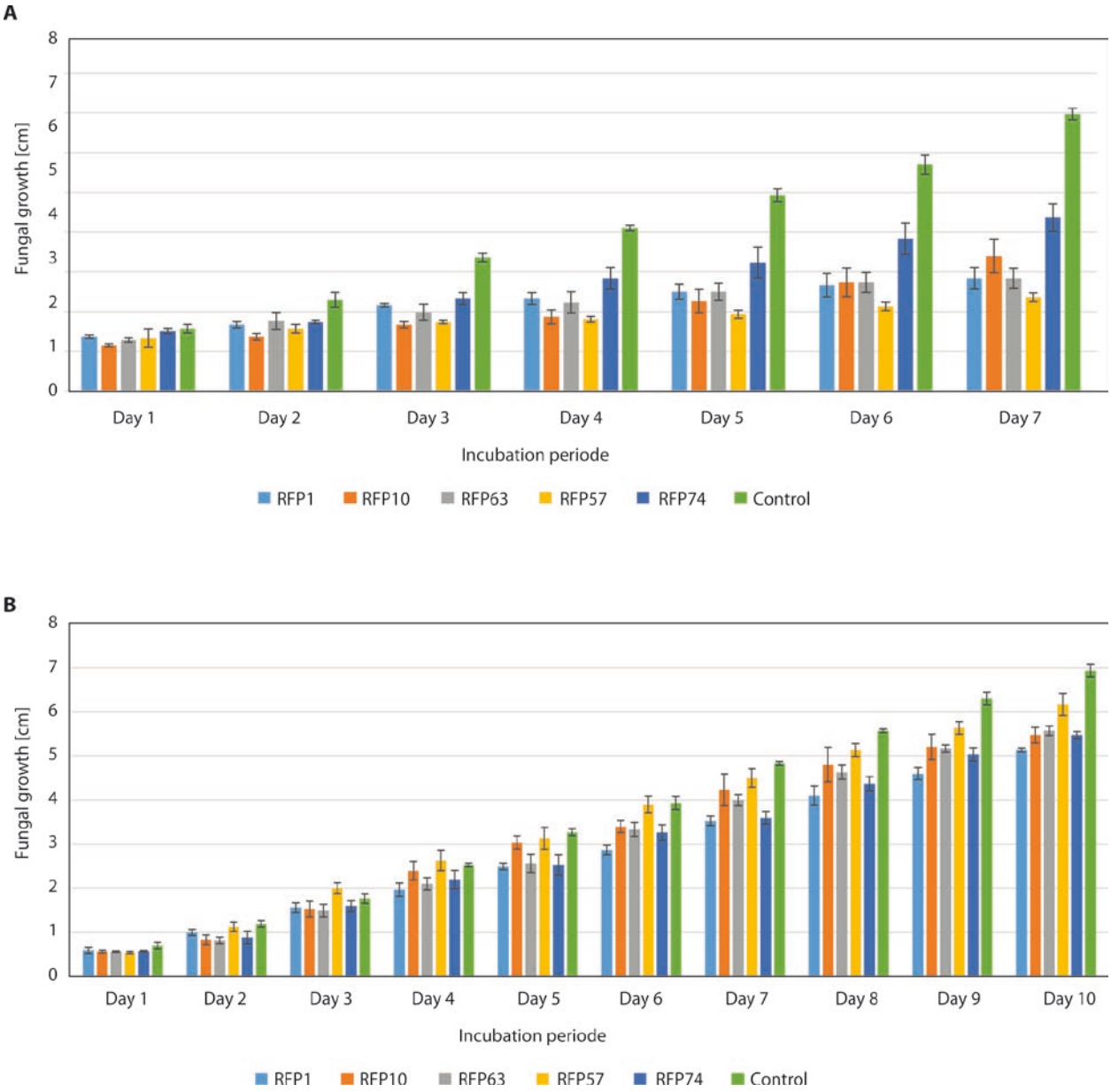

Fig. 2. Effects of the volatile compounds produced by the selected antagonist strains on the growth of the phytopathogenic fungi: A – the effect on the growth of *Fusarium oxysporum* f. sp. *lycopersici*; B – the effect on the growth of *Ascochyta* sp.
Values in the column bar are the means ±SE

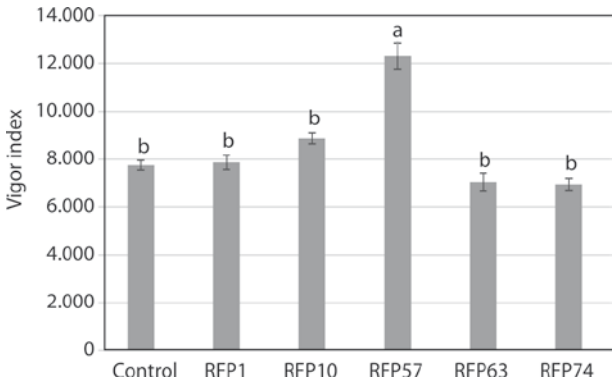

Fig. 3. The vigor index for the selected strains.
Values in the column bar are the means ±SE. Same letters indicate no significant difference ($p \leq 0.05$)

For the groups that were cultivated in soils inoculated with *Fol* + RFP57, a slight increase in their total fresh weight was observed.

Discussion

The bacteria used in this biocontrol study were isolated from the same site where the *in vivo* testing took place. This is important since according to Weller (1988), biological control agents are much more effective when they are applied in the same environment in which they were isolated. As the objective of this study was to select any strains capable of inhibiting both

Table 4. The plant growth promoting rhizobacteria (PGPR) effect of *Bacillus halotolerans* RFP57 on *Solanum lycopersicum* growth in presence of *Fusarium oxysporum* f. sp. *lycopersici*

Treatment	Germination [%]	Shoot elongation [mm]	Total fresh weight [g]
Control	72.5	873 ± 294 ab	27.42
RFP57	85.0	988 ± 237 a	41.91
Fusarium oxysporum f. sp. *lycopersici*	55.0	800 ± 25 b	16.90
RFP57 + *Fusarium oxysporum* f. sp. *lycopersici*	67.5	912 ± 258 ab	34.76

phytopathogens *Fol* and *Ascochyta* sp., a heat treatment was not performed during the isolation step since it would exclusively select the sporulating bacteria. If all the selected antagonist strains, belong to the single genus *Bacillus*, this simply shows their predominance in the soil samples studied and that their antagonistic capacity is higher than other soil microorganisms. *Bacillus* have long been used as a biological control agent on different crops. Their ubiquity and ability to adapt to different types of environment make them an ideal candidate. In our experiment, the strains were able to grow at different temperatures, pH and even at high salt concentrations. *Bacillus halotolerans* have already been isolated from an extreme environment and are known for their ability to withstand harsh environments (Wu *et al*. 2021). The study by Yao *et al*. (2012) asserted that *B. subtilis* had the ability to grow between pH 5.7–8.0 and at temperatures 30–50°C and under 10% NaCl concentration. Inhibition of *F. oxysporum* by *Bacillus* species has already been reported several times (Zhao *et al*. 2014; Jangir *et al*. 2018; Ramírez-Cariño *et al*. 2020; Xu *et al*. 2020). *Bacillus halotolerans* are considered as a warden against *Fusarium* infection in plants (Slama *et al*. 2019). In our study, the highest inhibition rate was obtained by strain RFP57 (61.36 ± 0.2). A similar study reported an inhibition rate of 79.37–87.5% (Jangir *et al*. 2018). High inhibition rates can be attributed to a better formula containing a higher cell density or the presence of more antifungal secondary metabolites (Zhu *et al*. 2020). Among our isolated bacterial strains, those that were antagonists against *Fol* also showed antagonistic activity against *Ascochyta* sp. The RFP57 strain was the most active with an inhibition rate of 74.1 ± 0.2%. Overall, *Ascochyta* was much more susceptible to our bacterial agents than *Fol*. Similar results were also reported by Diabankana *et al*. (2021) where the *B. mojavensis* PS17 strain demonstrated strong inhibition against several phytopathogens including *Fusarium* sp. and *A. pisi*. Other studies have also reported the ability of *Bacillus* spp. to inhibit multiple phytopathogens (Wang *et al*. 2003; Liu *et al*. 2016).

This ability of *Bacillus* strains to inhibit phytopathogenic fungi is mainly based on their production of a wide variety of secondary metabolites with antimicrobial properties (Keswani *et al*. 2020). Among these metabolites are cyclic lipopeptides like iturin, fengycin and bacillomycin, which have an inhibitory effect against several types of phytopathogens (Lee *et al*. 2008). These lipopeptides are excreted in the extracellular environment and are therefore present in the culture supernatant (Velho *et al*. 2011). For this reason, in our study, we tested the effect of the cell-free filtrate of each strain against the plant pathogens *Fol* and *Ascochyta*. The results (Fig. 1) showed that the filtrates had different effects against the two phytopathogens. They significantly ($p < 0.05$) slowed down the growth of *Fol* and *Ascochyta* sp. after 7 days and 10 days of incubation, respectively. Their average inhibition rates were similar, 33.74 and 33.01%, respectively. Toral *et al*. (2018) also presented similar results where the cell-free filtrate of *Bacillus* sp. inhibited the growth of the pathogen *Botrytis cinerea* by more than 60%. These results could suggest that antifungal lipopeptides were present in the cell-free filtrate of these bacterial strains and played a role in slowing down the growth of plant pathogens especially because *Bacillus* sp. are known to produce these lipopeptides. Cossus *et al*. (2021) have successfully extracted lipopeptides from culture media containing *B. subtilis* PTB185, an antagonistic bacterium against plant pathogens. Moreover, the research of Caulier *et al*. (2018), suggests that *Bacillus* strains which have remarkable antagonistic activities also have genes related to known antimicrobial peptides.

Besides the production of secondary metabolites, another mechanism is also known to contribute to the antagonistic capacity of *Bacillus*: the production of volatile compounds (Raza *et al*. 2016; Gao *et al*. 2017; Massawe *et al*. 2018; Zhang *et al*. 2020). Isolated, these compounds can indeed be potential antifungal agents against several phytopathogens (Zhang *et al*. 2020). For this study, in order to demonstrate the antifungal capacity of these volatile compounds, we applied the two Petri dish method instead of using a two compartments Petri dish (Ting *et al*. 2011). The latter, as indicated by Khan *et al*. (2018), is difficult to apply because the bacteria do not remain confined in their half compartment. Our results show that the volatile compounds released by the tested strains were more effective against *Fol* than against *Ascochyta* sp. RFP57

did not significantly inhibit the growth of *Ascochyta* sp. It should be noted, however, that, according to the results reported by Khan *et al*. (2018) some fungi may not be inhibited using the two Petri dish method unless pure volatile compounds extracted from the strain were used. This could explain the poor inhibition rate observed during this assay. On the other hand, previous studies have successfully demonstrated the effect of volatile compounds released by *Bacillus* sp. on plant pathogens (He *et al.* 2020; Zhang *et al*. 2020; De la Cruz-López *et al.* 2022; Ramírez *et al*. 2022). These volatile compounds produced by *Bacillus* could be dimethylsulfoxide, 1-butanol and 3-hydroxy-2-butanone (or acetoin) (Lim *et al*. 2017).

Bacillus are also known as plant growth promoting rhizobacteria (PGPR) because they can positively influence plant growth through the synthesis or excretion of phytohormones like auxin or cytokinin (Santoyo *et al*. 2012). These hormones play an important role in seed germination and plant growth. We tested this ability to stimulate seed germination in tomato plants (*Solanum lycopersicum*). The RFP57 strain significantly stimulated the germination of the tomato seeds compared to the control. The ability of *Bacillus* spp. to stimulate germination of plants has been reported (Masmoudi *et al*. 2019; Zouari *et al*. 2020). The mechanism behind this ability as described by Knežević *et al.* (2021) could be the production of phytohormones like indole-3-acetic acid, or siderophores. The latter are small molecules that can bind iron with high affinity and specificity, making it available for the plants which are sometimes capable of taking up a ferric complex of siderophores and using these as a source of iron unlike phytopathogenic fungi (Yu *et al*. 2011). Valencia-Cantero *et al*. (2007) attributed the ability of *B. megaterium* strain to stimulate plant growth by its ability to produce siderophore molecules. Production of siderophores in *B. halotolerans* has been reported (Jiménez-Gómez *et al*. 2020; Sarwar *et al*. 2020). Therefore, we can assume that these mechanisms have been used by our strain *B. halotolerans* RFP57 to stimulate the germination and promote the growth of the tomato plant.

Furthermore, the presence of RFP57 in soil contaminated by *Fol* reduced the effect of the pathogen on the growth of vegetative organs. This shows the ability of this strain (RFP57) to inhibit the growth of *Fol in vivo*.

The results of this study show that some strains of *Bacillus* spp. can be used as biocontrol agents against different phytopathogens. In general, our study has shown the ability of five strains of *Bacillus* spp. to inhibit the growth of *Fol* and *Ascochyta* sp. either by direct or indirect mechanisms. In addition, they showed a high ability to stimulate germination and plant growth. These results confirm why they are excellent PGPRs. With these characteristics, these strains can be considered as potential biocontrol agents. However, further studies must be carried out such as the detection of genes that code for secondary metabolites, the demonstration of siderophore production and a large-scale *in vivo* test in order to elucidate the mechanisms used by the strains to control the plant pathogens and thus enhance their potential use in pest management.

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