Original article

Bacillus strain selection with plant growth-promoting mechanisms as potential elicitors of systemic resistance to gray mold in pepper plants

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ABSTRACT

Certain soil bacteria produce beneficial effects on the growth and health of plants; hence, their use is steadily increasing. Five strains of *Bacillus* with plant growth-promoting potential were selected in this study, which produced indole-3-acetic acid levels below 50 μg.mL^{-1}. On the other hand, while only strains M8 and M15 dissolved phosphorus, the latter was the only strain that did not produce sidero-phores. Only strains M8 and M16 significantly inhibited the *in vitro* growth of *Botrytis cinerea* and *Fusarium solani* phytopathogens, whose inhibition ranges fluctuated between 60% and 63% for strains M8 and M16 against *B. cinerea* and between 40% and 53% for strains M8 and M16 against *F. solani*. Based on these results, the need to implement resistance induction against gray mold on pepper plants was determined using strains M8 and M16. In this case, strain M16 inhibited the propagation of the necrotic spot by approximately 70%, whereas strain M8 significantly reduced the superoxide dismutase activity in systemic leaves, which substantially increased in plants inoculated with strain M8 and infected with the pathogen. Accordingly, the use of native rhizobacteria may entail biotechnological progress for the integrated management of crops in agriculture industry.

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1. Introduction

Plant growth-promoting rhizobacteria (PGPR) include a small bacterial community that colonizes the rhizosphere, from which they exert positive effects on plant growth and health (Barriuso et al., 2008). PGPR may promote growth directly through indole-3-acetic acid (IAA) secretion, phosphorus dissolution, and sidero-phore production (Olanrewaju et al., 2017) and indirectly through increased tolerance to environmental stress, plague and phytopathogen control, and induced systemic resistance (Abbey et al., 2019; Figuereido et al., 2016; Enebe and Babalola, 2019).

Bacillus species can be found within the PGPR community; such genera are of high biotechnological importance because some strains are potentially antagonistic in the rhizosphere, wherein it has been estimated that virtually 8% of the genome is intended for the production of inhibitory substances such as antibiotics, antimycotics, volatile organic compounds, antimicrobial peptides, and lytic enzymes (Choudhary and Johri, 2009; Kumar et al., 2011), thus allowing for the control of pathogenic microorganism growth. As a result, the isolation and assessment of *Bacillus* species would make it possible to identify the biotechnological potential present in soils (Chowdhury et al., 2015).

PGPR may induce plant resistance to certain pathogens (Kumar et al., 2016; Olanrewaju et al., 2017). Resistance induction is a defense mechanism in plants when they interact with certain factors released by several microorganisms, such as siderophores, homoserine lactones, antimicrobial peptides, and volatile organic compounds or may be conducted by certain parts of cells such as flagella (Jain et al., 2016).

This immune component of plants is an efficient biological control medium against different types of pathogens such as bacteria, fungi, viruses, insects, and nematodes (Kumar et al., 2016). This process begins with the interaction between roots and microorganisms, which leads to a jasmonic acid- and ethylene-dependent response, thereby developing an intracellular signaling cascade in distal tissues (Jain et al., 2016; Verma et al., 2016). Such signaling is completed with the accumulation of the transcription factor NPR-1 (a non-expressor of pathogenesis-related protein 1), which acts as a master regulator of the immune system and ultimately leads to the accumulation of defense proteins in systemic tissues (Jain et al., 2016; Ramšak et al., 2018; Verma et al., 2016), allowing plants to achieve baseline resistance to inhibit the establishment and propagation of phytopathogens (Kumar et al., 2016).

In recent years, the use of PGPR in agriculture industry has improved, thereby promoting sustainable production practices. Therefore, disease control either directly (antagonism) or indirectly (resistance induction) by PGPR is profitable and a sustainable alternative in agriculture industry. This way, the search for alternatives to control gray mold (*Botrytis cinerea* Pers.: Fr) is crucial given that its control involves the use of chemical compounds that negatively affect the environment as well as the health of producers and consumers in traditional practices (Abbey et al., 2019; Gullino, 1992). As a result, this pathogen develops resistance to such chemical compounds (Rupp et al., 2017; Tsitsigiannis et al., 2008).

Pepper (*Capsicum annuum* L.) originates from tropical America and has gained importance as its fruit is used for medicinal and culinary purposes (Singletary, 2011). The pepper crop ranked fifth in Venezuela with regard to vegetable production, with a cultivation of 101,000 tons in 2006 (Gutiérrez, 2008). Over the past decade, pepper production has seen a significant drop; only during 2016–2017, the production decreased by 13%, which can be attributable to the lack of certified seed and absence of fertilizers and agrochemicals in the country (FEDEAGRO, 2018).

Moreover, pepper cultivation is susceptible to attack by *B. cinerea*, with sudden breakage of young leaves being the most common symptom (Tsitsigiannis et al., 2008). Infected leaves show severe symptoms of chlorosis, and the mycelium may infest the stem through the petiole, causing withering in the upper branches (Cheewawiriyakul et al., 2006). Pepper plants grown in greenhouses are highly susceptible to this disease (Jaimez et al., 2010); therefore, comprehensive handling involving the use of biological control as a preventive strategy may prevent its occurrence. Furthermore, it may help reduce the use of toxic fungicides that cause pollution (Gerbore et al, 2014). In Venezuela, the incidence of gray mold has been reported in Mérida, resulting in leaf scorch in Lisianto (Domínguez et al., 2008).

Several studies have focused on the search for *Bacillus* strains capable of inducing resistance to *B. cinerea*. In this sense, different strains of *Bacillus amyloliquefaciens* and *Bacillus subtilis* induced resistance against *B. cinerea* in tomato plants (Cawoy et al., 2014). Two *Bacillus* strains, *Bacillus megaterium* KUDC1728 and *Bacillus pumilus* KUDC17322 significantly increased pepper plant growth and that each strain significantly reduced *Stemphylium lycopersici* incidence in the same crop (Son et al., 2014). Similarly, increased resistance to gray mold in pepper plants inoculated with *B. velezensis* strains 5YN8 and DSN012 has been identified (Jiang et al., 2018). In grapes (*Vitis vinífera* cv. Glera), it has been found that *B. licheniformis* strain GL174 significantly reduced the area of necrotic lesion caused by *B. cinerea* in different forms of foliar application (Nigris et al., 2018). Considering the relevance of pepper cultivation in Venezuela and some regions as well as the high incidence of *B. cinerea* and the problems caused by the use of chemical fungicides, this study focused on the selection of native strains of *Bacillus* spp. isolated from the rhizosphere of a corn plant capable of presenting PGP mechanisms and inducing resistance to gray mold using pepper plants as a reference.

2. Material and methods

2.1. Isolation and identification of rhizobacteria

Isolates were derived from the rhizosphere of a corn plant collected from a plantation located in La Mucuy Alta (8°37′28.2″N 71°03′19.6″W), Municipality of Santos Marquina, Mérida, Venezuela. The root with attached soil (2.24 g of dry rhizospheric soil) was immersed in a solution of 0.05% Tween 20 for 1 h and constantly stirred at 160 rpm. Serial dilutions were performed from the resulting suspension, the first dilution (in sterile distilled water) was heated at 80 °C for 10 min. The second dilution was performed in LB broth and was maintained at 30 °C under constant stirring (100 rpm) for 2 h. After that, this dilution was heated to 80 °C for 10 min (De Vos et al., 2009). Whereas the two last dilutions were performed in sterile distilled water for subsequent planting in a nitrogen-free medium (20 g.L^{-1} sucrose, 0.1 g.L^{-1} K$_2$HPO$_4$, 0.4 g.L^{-1} KH$_2$PO$_4$, 0.2 g.L^{-1} MgSO$_4$·7H$_2$O, 0.1 g.L^{-1} NaCl, 0.011 g.L^{-1} FeCl$_3$, 0.002 g.L^{-1} Na$_2$MoO$_4$, 0.003 g.L^{-1} MnSO$_4$·4H$_2$O, and 20 g.L^{-1} agar; pH 7.0) modified from Ma and Chen (2008). Thirty isolates were selected from colonies through macroscopic analyses and cultivated at least five times in the same nitrogen-free medium. Finally, the isolates were maintained in a TGL medium at 30 °C (Zeigler, 2013).

2.1.1. Bacterial characterization

To find differences among isolates, Gram and endospores staining (Doetsch, 1981), and biochemical tests (catalase test, urea hydrolysis, nitrate reduction test, citrate use test, Voges–Proskauer test, and methyl red test) (Smibert and Krieg, 1981) were conducted. Isolates that presented characteristics of *Bacillus* (endospores formations and Gram+) were selected to subsequent studies.

2.1.2. Identification of rhizobacteria

Isolated strains were identified using the 16S ribosomal RNA gene analysis. Sequences were amplified using colony polymerase chain reaction (PCR) (Bergkessel and Guthire, 2013). PCR was conducted under standard conditions by the addition of 4 μL bacterial lysis solution using the primers spA 5′-AGA GTT TGA TCC TGG CTC AG-3′ and pcB 5′-TAC CTT GTT ACG ACT T-3′ (Wilson et al., 1990). PCR products were sequenced using Sanger sequencing. Sequence similarity analysis and comparisons were performed using sequences from NCBI's GenBank databases with the BLASTN tool (Altschul, et al., 1997; Benson et al., 2012) and Ribosomal Database Project RDP database (Wang et al., 2007). Relationships analysis was also performed using the MEGA X program (Kumar et al., 2018) and maximum likelihood phylogenetic method with a 2000-replicate bootstrap.

2.1.3. Inoculum preparation

Each inoculum was prepared in sterile saline solution (0.89% NaCl) based on 24-h cultures in a TGL medium. Cells were counted in the Neubauer chamber and inocula were adjusted to a cell density of 1×10^8 cells.mL^{-1}, except for the IAA production test, where the inocula were adjusted to a cell density of 1×10^7 cells.mL^{-1}.

2.2. Determination of plant growth-promoting characteristics

For determining direct mechanisms as growth promoters, isolates were tested for IAA production, siderophore production, and inorganic phosphate solubilization.

2.2.1. IAA production

To determine IAA production by each strain, 0.5 mL inoculum was added to 2.5 mL minimal broth (0.45 g.L^{-1} KH$_2$PO$_4$, 0.10 g.L^{-1} NaCl, 0.40 g.L^{-1} NH$_4$Cl, 0.78 g.L^{-1} KNO$_3$, 0.50 g.L^{-1} MgSO$_4$·7H$_2$O, 0.10 g.L^{-1} CaCl$_2$·2H$_2$O, 0.005 g.L^{-1} FeSO$_4$·7H$_2$O, 0.00156 g.L^{-1} MnSO$_4$·H$_2$O, 0.0014 g.L^{-1} ZnSO$_4$·7H$_2$O, and 10.98 g.L^{-1} D (+)-glucose; pH 6.4) (Reyes et al., 1999) either supplemented or not by 0.5 mg.mL^{-1} L-tryptophan (Glickmann and Dessaux, 1995). An uninoculated control (bacteria-free sterile saline solution) was simultaneously prepared for both treatments, and strain Sp7 of *Azospirillum brasilense* was used as the positive control, which has been reported as an IAA producer with and without tryptophan (Spaepen et al., 2007). Cultures were incubated at 30 °C for 5 days, and constantly stirred at 70 rpm. However, given that a group of bacteria did not grow in minimal broth, they were cultured in MR-VP broth (Smibert and Krieg, 1981).

IAA concentration was established in accordance with the colorimetric reaction with the Salkowski reagent (2:1 ratio; 10 mM FeCl$_3$ and 3.5 M HClO$_4$). Therefore, cultures were centrifuged at 3000 g for 10 min at 20 °C, and 0.1 supernatant was mixed with 2 mL Salkowski reagent. The reaction was conducted in dark for 30 min at room temperature, and absorbance was measured at 530 nm (Bric et al., 1991). IAA concentration was estimated using an absorbance versus IAA standard curve. This trial was performed in triplicate for each isolate.

2.2.2. Siderophore production

Siderophore production was determined using chrome azurol sulfonate (CAS) plates (Schwyn and Neilands, 1987) with King's B medium (Glickmann and Dessaux, 1995) as the base medium with CAS solution (40 mL aqueous solution of 0.182% hexadecyltrimethylammonium bromide in addition to 60 mL of a 0.101% CAS solution in 0.166 mM FeCl$_3$·6H$_2$O and 1.66 mM HCl) (Schwyn and Neilands, 1987). Isolates were planted in this medium and incubated at 30 °C for 7 days. Siderophore production was confirmed based on the presence of a yellowish halo around the growth area.

2.2.3. Phosphorus solubilization

For evaluating their capacity to solubilize phosphorus, strains were planted on minimal medium by replacing KH$_2$PO$_4$ with tricalcium phosphate [0.278 g.L^{-1} Ca$_3$(PO$_4$)$_2$ and 15 g.L^{-1} agar] (Reyes et al., 1999). Plates were incubated at 30 °C for 21 days, and the solubilization index (halo/colony) was estimated (Premono et al., 1996).

2.3. In vitro antagonism against phytopathogens

To assess the antagonistic effect of the strains, dual cultures were performed in PDA medium with *Botrytis cinerea* and *Fusarium solani* phytopathogens in individual experiments. Both phytopathogens belong to the strain collection of the Laboratorio de Fitopatología of the Instituto de Investigaciones Agropecuarias of the Universidad de Los Andes. For each isolates, two 10 μL drops (separated by 3.2 cm) of the inoculum were planted on PDA plates. For control, two 10 μL drops of saline solution were planted. After incubation at 30 °C for 24 h, a PDA disc (with the phytopathogen) was placed between both drops, and incubated at 25 °C during 6 days. Experiments were conducted in triplicate, including for control. The growth inhibition percentage of each phytopathogen was estimated using the following equation:

Inhibition (%) = [1 − (diameter of each treatment / control diameter)] × 100 (Yu et al., 2011).

2.4. Induction of resistance against B. cinerea in pepper plants

2.4.1. Plant cultivation and maintenance

Yolo Wonder pepper seeds were disinfected as per the method reported by Blanco et al. (2018); individually planted in an inert and sterile commercial substrate (pH 5.8 and conductivity of 0.127 dS.m^{-1}); and damped with sterile distilled water up to its field capacity. Seedbeds were taken to a growing room with 16-h light, 8-h darkness, and an average temperature of 23 °C ± 1 °C and irrigated every alternate day using sterile distilled water. After 21 days, plants were taken to the greenhouse with an average temperature of 26 °C ± 2 °C, relative humidity of 64.90% ± 0.09%, and brightness of 140.619 ± 103.504 μmol.m^{-2}.s^{-1}. Plants were irrigated on a weekly basis using sterile distilled water and fertilized twice a week with 10 mL sterile Hoagland nutrient solution (Taiz and Zeiger, 2010) at one quarter of its concentration.

2.4.2. Inoculation with rhizobacteria

Strains M8 and M16 were selected for this trial because both had PGP mechanisms and antagonistic activity against both phytopathogens. The M8 inoculum was prepared as stated above, whereas the M16 inoculum was prepared using a culture in MR-VP broth grown for 24 h at 30 °C and constantly stirred at 100 rpm. In this case, cells were washed through centrifugation at 3000 g for 5 min and resuspended in 5 mL of sterile saline solution. This procedure was repeated once, and cells were counted using the last resuspension by adjusting cell density to 1 × 10^8 cells.mL^{-1}. Two groups of 56-day-old plants were inoculated at the root with 10 mL of sterile saline solution (controls) and two groups with 10 mL of the inoculum.

2.4.3. Infection with B. cinerea and determination of the severity index

One culture of *B. cinerea* on PDA was placed under incandescent light bulbs in growing rooms, with a photoperiod of 16-h light/8-h darkness at 23 °C ± 1 °C. After 13 days, conidia were removed from the mycelium with 10 mL sterile distilled water, and the suspension was filtered through four layers of sterile gauze pads. Conidia were counted in the Neubauer chamber and the inoculum was adjusted to a density of 5 × 10^5 conidia.mL^{-1} with a sterile solution of 0.5 mg.mL^{-1} glucose and 0.5 mg.mL^{-1} KH$_2$PO$_4$ (De Meyer et al., 1998).

Infection was conducted 7 days after treating the plants with each isolate. A group of control plants and a group of plants inoculated with the isolate were infected with 20 μL pathogenic inoculum at the center of the firts two leaves. The plants were kept in a wet chamber for 48 h, and the diameter of the expanding lesion was measured from day 3 to 9 after infection (DAI) in five replicates. Diameters were transformed into circular area units; average calculations were made for each individual per day, and the biocontrol efficacy (BE) was estimated on DAI 9 using the following equation:

EB (%) = [diameter of lesion of infected plants − (diameter of lesion of plants treated with rhizobacteria / diameter of lesion of infected plants)] × 100 (Jiang et al., 2018)

2.4.4. Measurement of superoxide dismutase (SOD) activity

A homogenate was prepared as per the report by Dhindsa et al. (1981) by grinding the noninfected leaves in 5 mL cold phosphate buffer (0.05 M, pH 7.0) supplemented with 1% polyvinylpyrrolidone. The homogenate was centrifuged at 3000 g for 50 min at 0 °C; the pellet was discarded, and the supernatant was used as an enzymatic extract by keeping it on ice during the entire process. The inhibition of nitro blue tetrazolium (NBT) photochemical reduction was used to determine the SOD activity (Beauchamp and Fridovich, 1971). Each 3 mL reaction volume contained the following: 0.05 M phosphate buffer (pH 7.8), 13 mM DL-methionine,

75 μM NBT, 0.09 mM EDTA, 0.05 mL extract, and 2 μM riboflavin. Two 20-W actinic lights were shed on the mixtures for 2 min, and absorbance was measured at 560 nm against a blank that comprised the same reaction mixture but under dark conditions. The SOD specific activity is expressed in U.mg^{-1} of protein. One SOD unit equals 50% of NTB photochemical reduction inhibition (Giannopolitis and Ries, 1977). Protein content was determined by Biuret test using bovine serum albumin as the standard (Gornall et al., 1948).

2.4.5. Statistical analysis

Experiments conducted in greenhouses were performed in randomized blocks with triplicates and 5 replicates per repetition. Normally distributed data were analyzed using ANOVA with the LSD test ($p \leq 0.05$) using STATGRAPHICS 5.1, whereas non-normally distributed data distributed were analyzed with Kruskal–Wallis test ($p \leq 0.05$) with INFOSTAT.

3. Theory/calculation

Nie et al. (2017) found that *Arabidopsis* inoculation with *Bacillus cereus* AR256 significantly reduced *Botrytis cinerea* incidence, which was accompanied by PR1 protein accumulation, hydrogen peroxide accumulation, and callose deposition; these parameters were enhanced in plants that were inoculated with both the microorganisms. Moreover, these researchers found that the response elicited by this approach depended on the JA/ET signaling pathways. Conversely, Son et al. (2014) reported that *Bacillus megaterium* KUDC1728 and *Bacillus pumilus* KUDC17322 significantly increased pepper plant growth and that each strain significantly reduced *Stemphylium lycopersici* incidence in the same crop. Furthermore, Jiang et al. (2018) found that the 5YN8 and DSN012 strains of *Bacillus velezensis* efficiently controlled *B. cinerea* growth and that each strain upregulated defense-related genes in pepper plants. These researchers found that a reduction in the incidence of gray mold was accompanied by the accumulation of defense-related enzymes and hydrogen peroxide.

The use of *Bacillus* strains as potential elicitors of resistance against gray mold has dramatically increased in recent years. Therefore, the isolation, characterization, and evaluation of the isolated strains are important steps in this field. The application of characterized and isolated bacteria as plant growth-promoting bacteria (PGPB) in the roots of pepper plants would allow addressing questions related to the capacity to induce resistance against *B. cinerea*. This can be achieved via the inoculation at the root level of several selected strains followed by infection of the leaves with the pathogen. Subsequently, several physiological parameters can be studied to assess whether the inoculation with the strain and one round of infection with the pathogen alter the physiological state of the plants.

4. Results

4.1. Strain isolation, selection, and characterization

In total, 30 colonies were isolated; however, based on *Bacillus* characteristics (endospores formation and Gram+), only 5 were selected. These isolates were morphologically characterized by biochemical tests, and molecular identification was conducted using *16S ribosomal* RNA gene analysis (Table 1).

Three isolates (M3, M15, M16) reduced nitrates to nitrites and two (M3 and M16) hydrolyzed urea. Two isolates (M8 and M16) showed positive results Voges–Proskauer test. Positive results were obtained for Gram staining and endospore production for all isolates. Based on the identification using *16S* sequences, all isolates were found to belong to *Bacillus*, with a similarity between 99% and 100% in relation to the species reported in the GenBank and RDP databases. Phylogenetic analysis confirmed the identity of these species when grouped in the same clades (Fig. 1).

4.2. In vitro scrutiny in plant growth promotion

During the selection of a bacterial strain capable of acting as PGPR, the direct mechanisms that may promote plant growth are evaluated. In this sense, the plant growth-promoting characteristics presented by the maize rhizosphere strains were observed (Table 2). At this point, it is important to note that all strains selected during isolation grew in medium not supplemented with nitrogen. Therefore, the biological fixation of nitrogen may be one of the plant growth-promoting mechanisms of these strains.

Only strain M10 produced IAA ($p \leq 0.05$) in minimal broth supplemented with tryptophan and had a higher concentration than the positive control (Sp7), whereas strain M16 did not significantly differ from the negative control (without inoculum). However, this strain was also significantly similar to the positive control (Sp7) but had a lower concentration. In minimal broth without tryptophan, no strain produced IAA. Regarding MR-VP broth, only strain M15 was found to be an IAA producer ($p \leq 0.05$) regardless of the presence or absence of tryptophan.

All strains, except strain M15, produced siderophores, but only strains M8 and M15 dissolved tricalcium phosphate. Accordingly, strain M8 had a greater efficiency of phosphorus solubilization than strain M15 given that the halo/colony diameter ratio was significantly higher ($p \leq 0.05$) than that estimated for strain M15 at 14 days of culture.

4.3. In vitro antagonism against phytopathogens

The antagonistic potential that a strain can have in the rhizosphere can be estimated *in vitro* by dual cultures with other microorganisms. In this study, 5 strains were assessed against phytopathogens *B. cinerea* and *F. solani*. Only strains M8 and M16 significantly inhibited ($p \leq 0.05$) the growth of both phytopathogens (Table 3, Fig. 2), and strain M16 had a significantly greater inhibition percentage ($p \leq 0.05$) against *B. cinerea* than strain M8.

In the co-culture of *F. solani* and strain M16, the area of inhibition and thickening of colony edges were observed (Fig. 2C), whereas in the co-culture with strain M8 and *F. solani*, it not was observed that last behavior (Fig. 2B).

4.4. Induction of resistance against gray mold

Based on the PGPR properties displayed by strains M8 and M16 (Table 2) and their antagonistic potential (Table 3, Fig. 2), these isolates were selected for resistance induction against the necrotrophic phytopathogen *B. cinerea* in pepper plants. Progression of necrotic lesions in pepper leaves infected with *B. cinerea* for DAI 9 can be seen (Fig. 3). Necrotic lesion area did not display significant differences ($p > 0.05$) between plants inoculated with strain M8 and control plants (Fig. 3A).

On the other hand, necrotic lesion area showed significant differences ($p \leq 0.05$) between control plants and plants inoculated with strain M16 at DAI 9. Similarly, the development of necrotic lesions in plants inoculated with strain M16 did not vary significantly between DAI 3 and DAI 9, showing that with this treatment, necrotic lesions did not spread to leaves. This differs from what occurred in control plants wherein there was significant difference ($p \leq 0.05$) in terms of necrotic lesion area at the beginning and end of the experiment, thus showing a tendency of necrotic lesions to continue spreading (Fig. 3B).

Table 1
Morphological and biochemical characteristics of species isolated from maize rhizosphere. Species were identified with 16S ribosomal RNA gene analysis.

Isolate	Nitrate reduction	Urease	Citrate use	Voges–Proskauer	Methyl red	Catalase	Related species	Similarity (%)
M3	+	+	+	−	−	+	*Bacillus megaterium*	99
M8	−	−	+	+	+	+	*B. pumilus*	99
M10	−	−	−	−	+	+	*B. clausii*	99
M15	+	−	−	−	+	+	*B. megaterium*	100
M16	+	+	+	+	+	+	*B. licheniformis*	100

Symbols (+) indicate a positive test result.
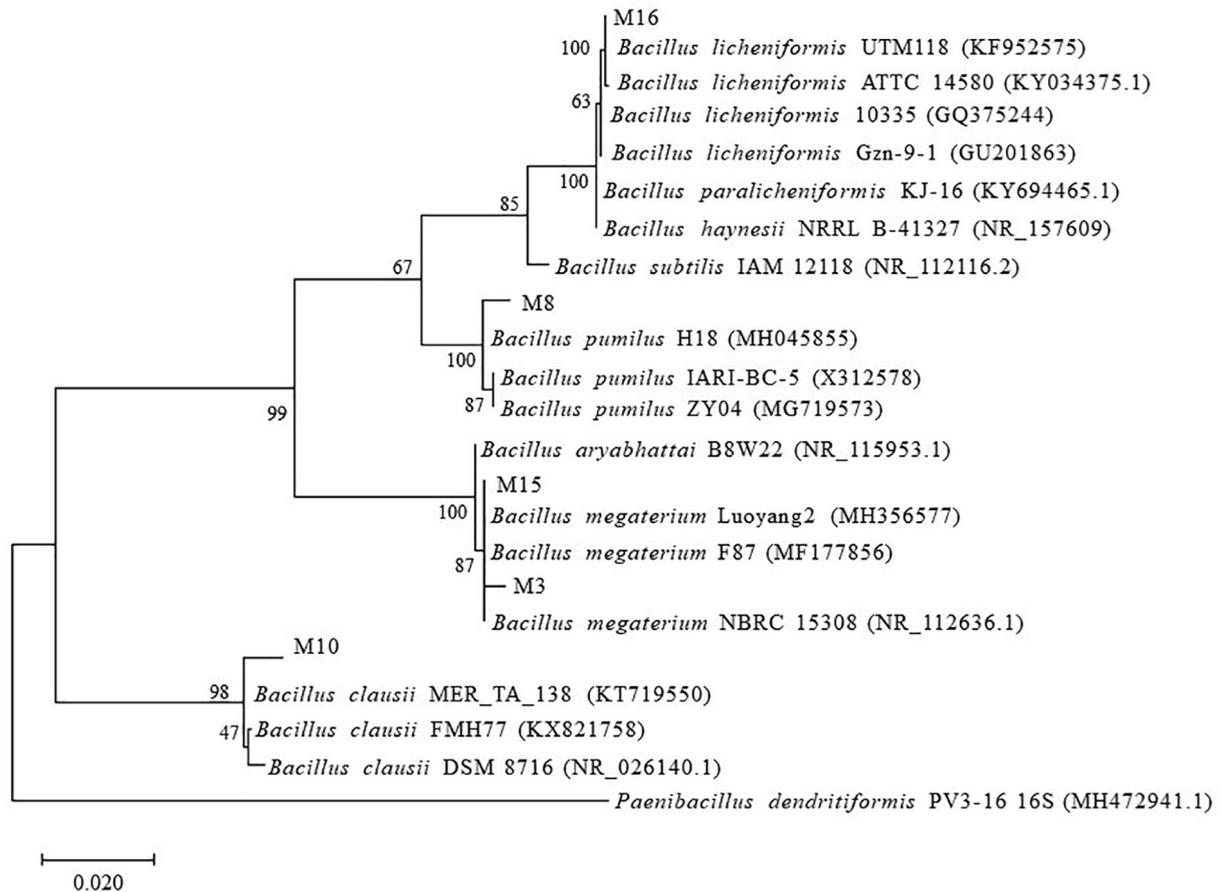
Fig. 1. Phylogenetic tree of isolates from maize rhizosphere. The bootstrap contained 2000 replicates and phylogenetic relations established following the Hasegawa-Kishino-Yano model.

Table 2
In vitro plant growth-promoting mechanisms for the 5 selected *Bacillus* strains.

	Indole-3-acetic acid production (μg mL^{-1})					Ca$_3$(PO)$_2$ solubilization index
	Minimal medium			MR-VP broth		
Strains	(+Trp)	(−Trp)	(+Trp)	(−Trp)	Siderophores	Halo/Colony
M3	13.85 ± 1.33 [BC]	0.00 ± 0.00[b]	ND	ND	+	−
M8	ND	ND	5.56 ± 1.96[b]	1.71 ± 1.48 [bc]	+	1.21 ± 0.06[a]
M10	43.59 ± 9.25 [A]	0.00 ± 0.00[b]	ND	ND	+	−
M15	ND	ND	18.80 ± 1.96 [a]	3.42 ± 1.48[b]	−	1.02 ± 0.04[b]
M16	17.09 ± 1.48 [ABC]	3.42 ± 1.48[b]	ND	ND	+	−
Control (−)	0.86 ± 1.48[C]	0.00 ± 0.00[b]	2.14 ± 0.74[b]	0.00 ± 0.00[c]	ND	ND
Sp7 (+)	23.08 ± 0.00 [AB]	9.66 ± 5.77 [a]	21.80 ± 5.59 [a]	13.68 ± 1.48 [a]	ND	ND

Data are presented as the average of three replicates ± standard deviation. The symbol (+) indicates positive test result.
ND: not determined, uppercase letters: Kruskal–Wallis test, lowercase letters, LSD test. Different letters within the same column indicate significant difference ($p \leq 0.05$).

Table 3
Percentage of inhibition of phytopathogen growth for the 5 selected *Bacillus* strains.

Treatments	Percentage of inhibition (%)	
	Botrytis cinerea	*Fusarium solani*
Control	0.00 ± 0.00^c	0.00 ± 0.00^C
M3	0.00 ± 0.00^c	6.67 ± 6.67^{BC}
M8	60.77 ± 1.15^b	40.00 ± 0.00^{AB}
M10	0.00 ± 0.00^c	22.22 ± 7.70^{ABC}
M15	0.00 ± 0.00^c	6.67 ± 11.55^{BC}
M16	62.69 ± 2.67^a	53.33 ± 0.00^A

Values are shown as average ± standard deviation. Uppercase letters: Kruskal–Wallis test, lowercase letters, LSD test. Different letters within the same column indicate significant difference ($p \leq 0.05$).

Further, the BE percentage at DAI 9 significantly differed ($p \leq 0.05$) between strains M8 and M16 (Fig. 4). Strain M8 displayed a BE percentage of approximately 40%, whereas strain M16 biologically controlled *B. cinerea* at approximately 70%.

Fig. 5 shows the necrotic lesion caused by infection with *B. cinerea* in the first two leaves of pepper plants. In this figure, the necrotic lesion is more intense at DAI 2 in control plants than in plants inoculated with strain M8 or M16. A similar behavior was observed at DAI 9 when the lesion was greater in control plants.

In this case, the necrotic lesion is less evident in plants inoculated with strain M16 than in those inoculated with strain M8, consistent with the results shown in Figs. 3 and 4.

Fig. 6 shows the SOD activity of systemic plant leaves in all treatments. Inoculation with strain M8 had significantly ($p \leq 0.05$) lower SOD activity than the other treatments (Fig. 6A). Additionally, the SOD activity was 214% higher ($p \leq 0.05$) in plants inoculated with strain M8 and infected with the pathogen than in plants inoculated with strain M8 only. On the other hand, the SOD activity did not differ significantly ($p > 0.05$) for treatments with strain M16 (Fig. 6B).

5. Discussion

In agriculture, the use of beneficial bacteria has increased in the past few years. Therefore, the isolation, identification, and evaluation of these bacteria are important steps to suggest multifaceted inoculants to producers. In this study, *Bacillus* species isolated from the rhizosphere of a corn plant showed broad characteristics as promising PGPR. Furthermore, it is important to know the microbial interactions of the soil to improve fertilization, particularly when they are influenced by different phosphate sources (Gumiere et al., 2019).
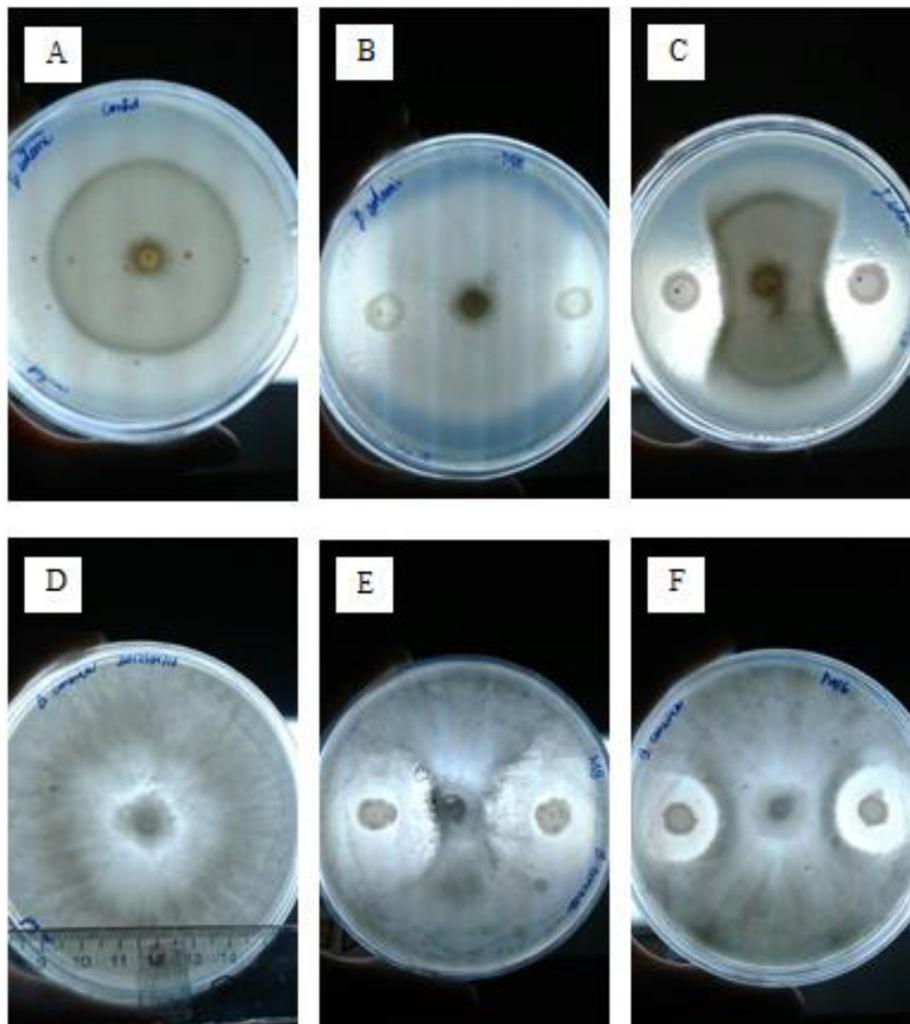
Fig. 2. Growth inhibition of two phytopathogens (A and D) *Fusarium solani* and *B. cinerea* in PDA, respectively. (B) Growth inhibition halos of *F. solani* compared with those of strain M8. (C) Growth inhibition halos of *F. solani* compared with those of strain M16. (E) Growth inhibition halos of *B. cinerea* compared with those of strain M8. (F) Growth inhibition halos of *B. cinerea* compared with those of strain M16.
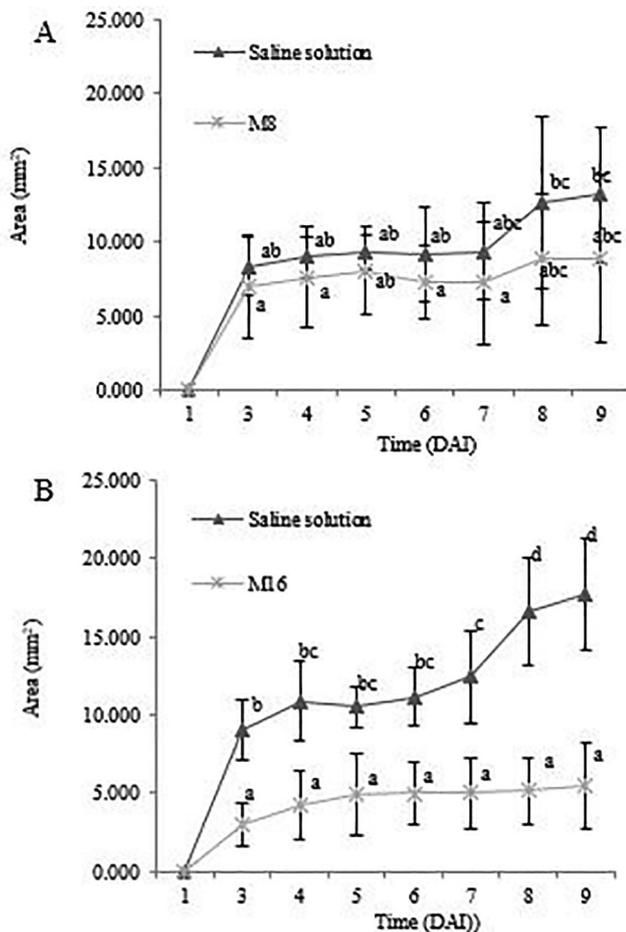
Fig. 3. Progression of necrotic lesion area in infected pepper plant leaves inoculated with strains M8 (A) and M16 (B). Dots indicate the average of 5 repetitions and bars indicate standard deviations. Different letters indicate significant difference ($p \leq 0.05$) as per the LSD test. DAI = days after infection.
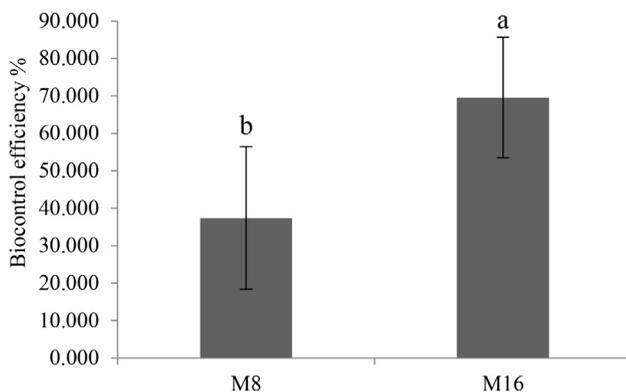
Fig. 4. Percentage of biocontrol efficacy against gray mold in pepper plants inoculated with *Bacillus* strains M8 and M16.

On comparing the morphological and biochemical characteristics of the isolates with those of isolates reported in Bergey's Manual of Systematic Bacteriology (Logan and De Vos, 2009), it was found that the 5 selected isolates belong to *Bacillus*. This was confirmed with molecular and phylogenetic analysis using the GenBank database, which it was found that all isolates belong to *Bacillus* and that there is a high similarity of strains M3 and M15 with *Bacillus megaterium*, M8 with *B. pumilus*, M10 with *Bacillus clausii*, and M16 with *Bacillus licheniformis*.

Bacillus species have many biotechnological applications, particularly in agriculture industry (Tiwari et al., 2019). Several *Bacillus* species have shown plant growth-promoting mechanisms (Akinrinlola et al., 2018; Castellano-Hinojosa et al., 2018). The mechanisms employed by PGPR to promote growth vary, with IAA production being one of the most used mechanisms by bacteria (80% bacteria living in the rhizosphere are IAA producers) (Podile and Kishore, 2006). In this study it was quantified the amount of IAA produced by strains M3, M10, and M16 in minimal broth supplemented or not with tryptophan, and in MR-VP broth for strains M8 and M15. IAA production by strain M10 in minimal broth with tryptophan represents the first report on IAA production by *B. clausii*. Given that the analysis of IAA production was performed at 5 days of culture and that strain M16 did not differ from both control groups, it is suggested that M16 produced IAA during a certain growth phase considering that IAA production or degradation depends on the stage of growth (Duca et al., 2014). Additionally, *B. licheniformis* strains have been proven to be IAA producers (Baldan et al., 2015). On the other hand, only strain M15 produced IAA significantly in MR-VP broth with or without tryptophan, producing six times more IAA in broth with tryptophan than without tryptophan. This suggests that strain M15 uses alternative routes to produce IAA. Based on these results, the selected strains produced IAA at a concentration of 50 μg.mL^{-1}, consistent with the results reported by Kavamura et al. (2013).

Siderophore production was proven in most strains, with strain M15 showing negative results. Siderophores are organic compounds with a low molecular weight secreted by some bacteria to sequester the rhizosphere's ferric ion (Aguado-Santacruz et al., 2012). Siderophore–iron complex formation increases the possibility of iron uptake by the roots of certain plants, thus causing increased plant growth. Several studies have proven siderophore production by strains belonging to *Bacillus* (Kesaulya et al., 2018; Son et al., 2014; Yu et al., 2011). In this study, *B. clausii* strain M10 is reported as a siderophore producer, which may indicate its use as a possible PGPR because this species is usually promoted only as a probiotic (Lippolis et al., 2013). Likewise, siderophore production may be indicative of a strain's potential to induce resistance as being part of the factors that trigger this defense mechanism (Saikia et al., 2005; Yu et al., 2011).

On the other hand, the tricalcium phosphate solubilization was only positive for strains M8 and M15, with strain M8 being more efficient in dissolving phosphorus than strain M15. However, *in vitro* phosphate solubilization depends on the type of phosphate and medium characteristics (Wang et al., 2012). Phosphorus is the second most important element for living organisms and one of the most abundant elements found in soil. However, it has low availability due to the reactions it undergoes with iron, aluminum, and calcium, thereby producing insoluble phosphates (Khan et al., 2014). Therefore, it is crucial to isolate and identify microorganisms capable of dissolving this mineral. The application of *Bacillus* species is one of the methods employed for phosphorus solubilization in agriculture (Tiwari et al., 2019). Accordingly, *Bacillus* species such as *B. megaterium*, *B. cereus*, and *B. pumilus* have proven to have phosphorus-dissolving capacity (Kavamura et al., 2013; Son et al., 2014; Timmusk et al., 2011).

Additionally, among the 5 selected strains, the antagonistic effect of *B. pumilus* strain M8 and *B. licheniformis* strain M16 against the phytopathogens *B. cinerea* and *F. solani* was proven. Other studies have proven the antagonism of *Bacillus* strains against *F. solani* (Yu et al., 2011). In this study, the growth inhibition percentage of *F. Solani* was 40% and 54% for strains M8 and M16, respectively. At this point, it is important to note that the behavior that this pathogen showed with M16 (thickening of colony edges) without direct contact, may be due to a substance secreted by this strain. Conversely, with strain M8, the behavior
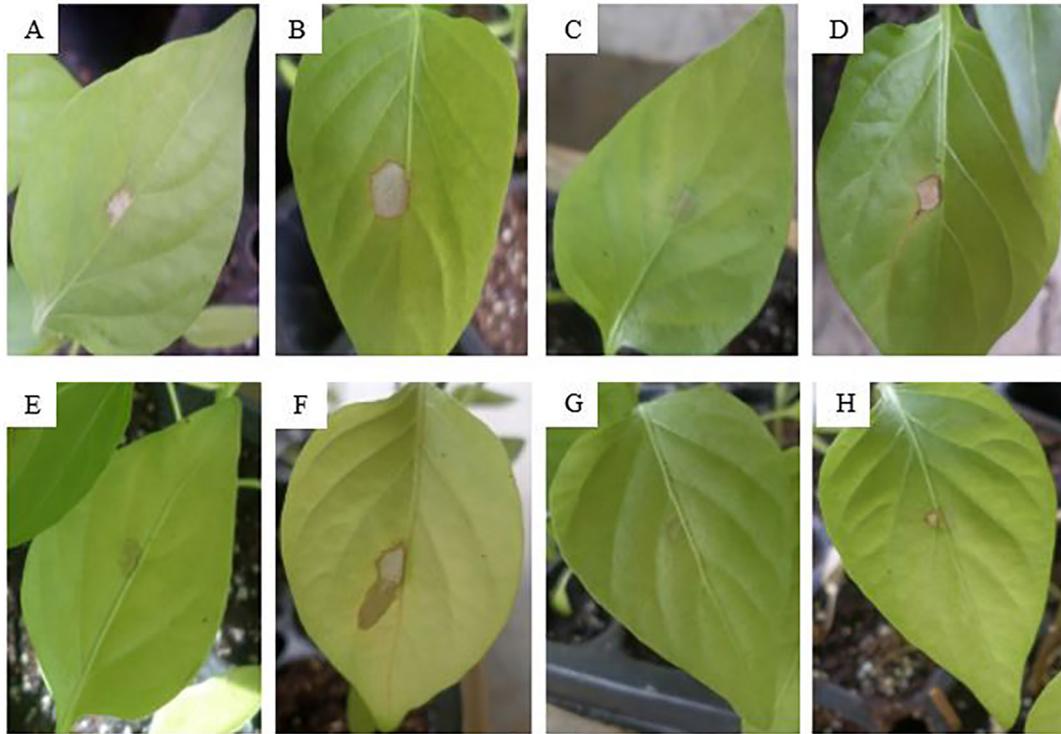
Fig. 5. Necrotic lesion caused by infection with *Bacillus cinerea* in the first two pepper plant leaves. (A and E) Control plants at DAI 2. (B and F) Control plants at DAI 9. (C and G) Plants at DAI 2 inoculated with strains M8 and M16, respectively. (D and H) Plants at DAI 9 inoculated with strains M8 and M16, respectively. DAI = days after infection.
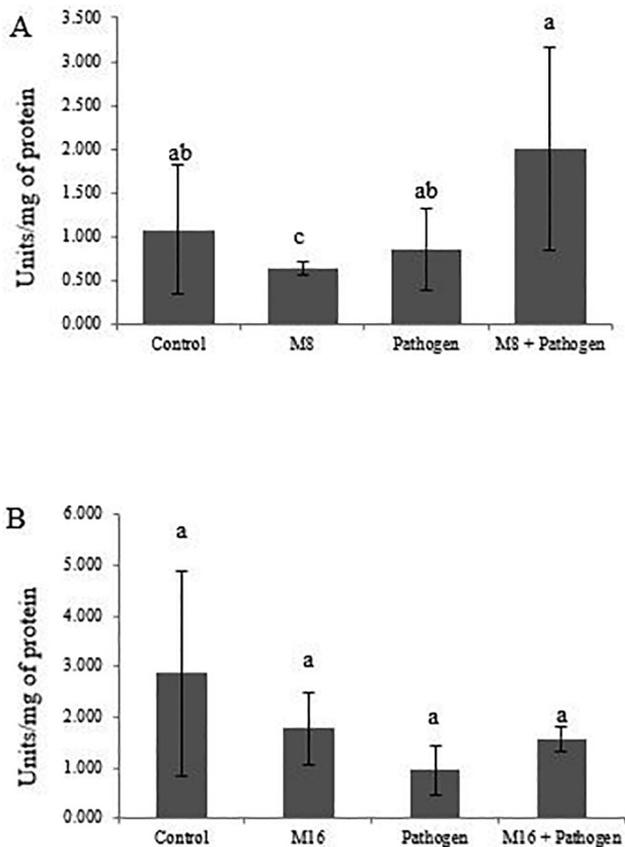
Fig. 6. Superoxide dismutase (SOD) activity in pepper plants. (A) SOD activity in pepper plants inoculated with strain M8. (B) SOD activity in pepper plants inoculated with strain M16.

was different because there was a reduction in colony strength, which may indicate the production of a volatile component.

On the other hand, the growth inhibition percentage of *B. cinerea* was 60% and 63% for strains M8 and M16, respectively, higher than that reported in other studies (Kim et al., 2013; Xu et al., 2016). Recent studies have proven the controlling effect *Bacillus* strains can have on *B. cinerea* by the production of lipopeptides (Nigris et al., 2018), secondary metabolites, or volatile organic compounds (Jiang et al., 2018). Therefore, strains M8 and M16 may present many of these mechanisms to inhibit the growth of *F. solani* and *B. cinerea*, such as the secretion of siderophores, antimicrobial peptides, volatile organic compounds, and lytic enzymes (Chowdhury et al., 2015; Grady et al., 2016). This study proved the production of siderophores by these isolates, which may represent a mechanism of competition for ferric ion. Additionally, strain M16 demonstrated chitinase production in a parallel experiment conducted by other researchers in the Laboratorio de Fitobiotecnología, Departamento de Biología, Facultad de Ciencias, Universidad de Los Andes (unpublished data).

Based on the plant growth-promoting characteristics and antagonistic activity of strains M8 and M16, these strains were selected for the resistance induction test against gray mold in pepper plants. According to the results, strain M8 did not significantly inhibit the spread of necrotic lesions in pepper leaves infected with *B. cinerea* (Fig. 3A), inconsistent with the results of strain M16, which significantly inhibited the growth of necrotic lesions during the 9-day experiment (Fig. 3B). The hypersensitive response (HR) at DAI 3 it was reduced by 66% in plants previously inoculated with strain M16. This reduction is important given that it has already been proven that *B. cinerea* infection is favored in *Arabidopsis* based on HR (Govrin and Levine, 2000); therefore, the use of strain M16 as an inoculant may reduce long-term damages produced by this pathogen. Therefore, strain M16 reduced necrotic lesion spread at DAI 9 by 71% (Fig. 4). Compared with these results, other studies have showed induction of resistance against *B. cinerea* in

Arabidopsis thaliana by *B. cereus* strain AR156 (Nie et al., 2017). In turn, in grapes (*Vitis vinífera* cv. Glera), it has been found that *B. licheniformis* strain GL174 significantly reduced the area of necrotic lesion caused by *B. cinerea* in different forms of foliar application (Nigris et al., 2018). In peppers, the induction of resistance against *B. cinerea* by *B. velezensis* strains 5YN8 and DSN012 has been proven, with BE of 50% at DAI 30 (Jiang et al., 2018). In comparison with last study, M16 produced a BE was 69.59% at DAI 9 in pepper plants.

Meanwhile, SOD activity did not vary in the experiment using M16 (Fig. 6B). While in the M8 experiment, SOD activity was lower in pepper plants inoculated with M8 only, and increased significantly when infected with *B. cinerea* (Fig. 6A). In another study, SOD activity varied between 0.7 to 1.4 U.mg^{-1}, being higher in pepper plants inoculated with rhizobacteria and infected with *B. cinerea* (Jiang et al., 2018). Similar results have been found in cucumber plants inoculated with *B. megaterium* L8 and infected with *Pythium aphanidermatum* (Liang et al., 2011).

6. Conclusions

Rhizobacteria belonging to *Bacillus* was isolated that displayed mechanisms that may increase plant growth. Strains M8 and M16 significantly inhibited the growth of *F. solani* and *B. cinerea*, representing an alternative to control these phytopathogens. This fact leads to the evaluation of these strains against phytopathogens being of economic importance. Lastly, strains M8 and M16, when inoculated separately at the root level in pepper plants, promoted a physiological response that led to the inhibition of the spreading of the necrotrophic pathogen *B. cinerea*; therefore, in addition to controlling this pathogen directly, these strains also induced resistance against it in pepper plants. Accordingly, it would be possible to produce a biocontroller for on-site evaluation against these phytopathogens in pepper. These finds represent a potential of bacteria for global agribusiness and agricultural microbiology. It is recommended to test plant growth promotion *in vivo* for this crop and other crops by strains M8 and M16 both in greenhouses and on site and to determine the molecular and biochemical mechanisms that may induce resistance.

Declarations of interest

None.

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