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Decrease in the incidence of charcoal root rot in common bean (*Phaseolus vulgaris* L.) by *Bacillus amyloliquefaciens* B14, a strain with PGPR properties

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ABSTRACT

The objective of this study was to isolate strains of the genus *Bacillus* from different productive soils of the province of Salta, Argentina, which have growth promoting properties in common bean (*Phaseolus vulgaris* L.) and have the ability to inhibit different phytopathogenic fungi, primarily *Macrophomina phaseolina*. Among the 105 strains of *bacilli checked*, *Bacillus* sp. B14 was selected for having the greatest *in vitro* inhibitory effect against *Sclerotium rolfsii*, *Sclerotinia sclerotiurum*, *Rhizoctonia solani*, *Fusarium solani* and *Macrophomina phaseolina*, recording fungal inhibition values that varied between 60 and 80%. In addition, B14 produced auxins in a concentration of 10.10 mg/ml, and qualitatively synthesizes siderophores. Based on 16S rRNA gene sequencing, the strain was characterized as *B. amyloliquefaciens*. Data from greenhouse experiments showed that the black common bean cv. Nag 12 seeds inoculated with B14, had increased germination of 10%, as well as an increase in root length of 2 cm and in shoot length of 6 cm compared with the non-inoculated control seeds. When the phytosanitary state of the B14 inoculated seeds was analyzed, no growth of bacteria, or phytopathogenic fungi and contaminants was observed, while in the non-inoculated seeds, bacteria was found in 46% of seeds, in addition to other phytopathogenic fungi. *B. amyloliquefaciens* B14 reduced the incidence of *M. phaseolina* by 62% in the inoculated black bean cv. Nag 12 seeds. Furthermore, using MALDI-MS it was determined that the bacteria synthesized different lipopeptides in the presence of *M. phaseolina*, such as surfactin, iturin, fengycin, kurstatin and polymyxin, leading us to conclude that they are the main responsible for the antagonistic effect observed and that the nature of lipopeptides synthesized by B14 is influenced by target fungal strain.

1. Introduction

Argentina is the world's leading exporter of the common dry bean (*Phaseolus vulgaris* L.). The cultivation of this legume has been a traditional activity in northwestern Argentina (NOA) since the beginning of the 20th century. The province of Salta is the country's main producer, making up 80 percent of the total production, destined mainly for export (De Bernardi, 2016). In recent years, 532,000 ha have been designated to the planting of this crop. Of the cultivated area, 436,560 ha of beans were harvested, reaching a record production level of 604,817 tonnes (De Bernardi, 2016).

There are diseases caused by microorganisms which affect common bean and result in significant economic loss. Pathogenic fungi cause about 10–20% losses on agricultural crops production (Broughton et al., 2003). Among the most detrimental diseases which affect common bean, the soilborne pathogens are the most dangerous and cause serious damages to the plant. *Sclerotinia sclerotiorum* (Lib de Vary), *Fusarium solani*, *Sclerotium rolfsii* (Sacc), *Rhizoctonia solani* (Khun) and *Macrophomina phaseolina* Tassi (Goid) (Schwartz et al., 2005) are the most frequently isolated pathogens and are the responsible for different types of disease. In the NOA region, especially in the province of Salta, the incidence of the charcoal root rot caused by *M. phaseolina* during

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cultivation has serious negative effects and represents the most important disease in the region, reducing yields and seed quality (Perez-Brandán et al., 2012). *M. phaseolina* is an imperfect fungus which causes dry root rot, stem canker, stalk rot and charcoal rot of over 500 plant species, including common bean (Sinclair and Backman, 1989; Martínez Villarreal et al., 2016). This polyphagous pathogen attacks and infects more than 100 families of monocots and dicots. Charcoal root rot severely damages susceptible cultivars, and epidemics are prevalent in areas with periods of drought and high temperatures such as those registered in northwestern Argentina. Losses in bean yields result from pre-emergence and post-emergence death of infected seedlings, and reduced vigor and premature death of older plants (Abawi and Pastor Corrales, 1990). In heavy infestation, the host plants are destroyed by fungal toxins, such as phaseolinone and vascular obstruction by mycelium (Bhattacharya et al., 1994). Since the pathogen is soil-borne with high saprophytic ability, effective strategies for disease control are not available. To control charcoal rot, resistant bean germplasm and cultivars have been identified and should therefore be used whenever they are available. Clean seed, that is, free of seedborne *M. phaseolina*, should be used. Seed treatment with fungicides is used in controlling charcoal rot during early growth stages (Simonetti et al., 2015). However, in recent years, more sustainable management practices have started to be employed in Argentinian agriculture, leading to the search for non-polluting, environmentally-friendly alternatives, hence minimizing the use of chemical fertilizers and pesticides.

One alternative is the use of plant growth-promoting rhizobacteria (PGPR), which are beneficial free-living soil bacteria that facilitate plant growth and keep them healthy by controlling pathogens, either directly or indirectly (Kloepper and Schroth, 1978). Different species of the genus *Bacillus*, such as *B. amyloliquefaciens, B. licheniformis, B. pumilus* and *B. subtilis* have been widely used as biocontrol agents and as potential PGPR in agriculture. These bacteria have the ability to produce resistance spores which allow them to resist adverse environmental conditions, a characteristic which makes it possible for them to be used in storage of commercial products (Francis et al., 2010). One of the main beneficial effects that these bacteria have, is their inhibitory activity against pathogenic microorganisms due to their potential to synthesize a wide range of metabolites with antagonistic activity, such as the lipopeptides of the surfactin family, iturins, fengycins, polymyxins, kurstakins, and bacitracins (Hathout et al., 2000; Stein, 2005; Price et al., 2007; Ongena and Jacques, 2008; Banat et al., 2010; Yánez-Mendizábal et al., 2011; Béchet et al., 2012; Cawoy et al., 2014; Thais et al., 2015; Chandler et al., 2015; Torres et al., 2016, 2017; Zouari et al., 2016). These metabolites are environmentally-friendly, have low toxicity and, among other positive effects, have an antagonistic effect on bacteria and fungi (Ongena and Jacques, 2008; Meena and Kanwar, 2015).

Therefore, the objective of this study was to isolate bacteria belonging to the genus *Bacillus* from NOA soils and select those strains that display both growth-promoting properties in the common bean (*Phaseolus vulgaris* L.) and act as a biological control agent against *M. phaseolina*. Moreover, we aimed to determine the chemical nature of the main metabolites involved in the antagonistic activity.

2. Materials and methods

2.1. Bacterial isolation from soil

Soil samples (10 cm depth) were taken from the central-eastern region of the province of Salta, the area of the province where the majority of bean production under different production systems takes place (24 °52′23.72″ S 64 °14′54.46″ W). Each sample (10 g) was dissolved with sterile distilled water and warmed at 80 °C for 10 min to kill vegetative cells and select for endospore forming bacteria. Serial dilutions were performed, inoculated in Brain Heart Infusion (BHI) broth, then incubated at 37 °C for 24–48 h. Strains exhibiting visible morphological characteristics of the *Bacillus* strain were preselected and an optical microscopic observation was performed to confirm the structure. The selected strains were preserved in BHI broth with an added 20% v/v glycerol at −20 °C (normal freezer).

2.2. Fungal growth inhibition assays

For these trials, a dual culture technique was used (Landa et al., 1997). The inhibitory activity of the isolated *Bacillus* spp. strains was evaluated against the following phytopathogenic fungal strains: *Sclerotium rolfsii* (Sacc), *Sclerotinia sclerotiurum* (Lib de Vary), *Rhizoctonia solani* (Khun), *Macrophomina phaseolina* Tassi (Goid) Campichuelo, and *Fusarium solani*. Phytopathogenic fungi were obtained from the culture collection of Agricultural Microbiology Laboratory of the Estación Experimental Agropecuaria EEA-INTA-Salta, Argentina. The different phytopathogens were grown on Potato Dextrose Agar (PDA) (APG, Britania, Argentina), at an incubation temperature of 28 °C for 7 days.

One-day-old fungal discs (4 mm diameter) of each test fungus were placed in the center of 9-cm-diameter Petri dishes containing PDA medium. Then, 10 µl obtained from 24-h-old cultures of the isolated bacteria were placed equidistant from each other. After incubation for 7 days at 28 °C, the mycelial growth diameter of each phytopathogen was measured and the percentage of fungal inhibition (FI) was calculated according to Royse and Ries (1978).

$$FI\ (\%) = RGI \times 100;\ RGI = (C-T)/C$$

where T is the average diameter of mycelial growth in presence of the *Bacillus* sp. strain, C is the average diameter of the mycelial growth without bacterial samples.

The assays were performed in triplicate.

2.3. In vitro plant growth-promoting attributes of isolates

Plant growth-promoting bacteria (PGPB) activities of isolates were determined by following standard procedures. The solubilization of inorganic phosphate was measured using the methods described by Goldstein (1986). Auxin and cianidas production were detected by the method described by de Brito Alvarez et al. (1995). Siderophore production was tested on TSA (Tripteina Soya Agar, Britania) medium supplemented with 8-hydroxyquinoline (de Brito Alvarez et al., 1995).

2.4. Phylogenetic characterization

Bacillus sp. B14 was used in the subsequent studies. DNA was extracted from an active culture after incubation in 5 ml of Brain Heart Infusion broth (BHI, Britania, Argentina) at 37 °C for 24 h, according to the method by Miller (1972). For the genotypic characterization, the strain was genetically characterized by analyzing the subunit 16S of rRNA, and sequencing was performed on both strands by the commercial sequencing services of Macrogen Inc. (Seoul, Korea). 16S was carried out using nucleotide single universal strand primers S-D-Bact-0008-a-S-20 (AGAGTTTGATCCTGGCTCAG) and S-D-Bact-1495-a-A-20 (CTACGGCTACCTTGTTACGA) (Daffonchio et al., 1998). The extracted genomic DNA was amplified in a 25 µl reaction mixture containing: 0.2 µl Taq polymerase, 2.5 µl buffer STR, 0.1 µl primer, 17.5 µl PCR water and 5 µl DNA sample. Amplification consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min, and a final extension at 72 °C for 7 min. Control reaction mixtures lacking template DNA were also included in each experiment. The PCR products were separated in 0.8% agarose gel electrophoresis running at 65 V for 50 min. Gel patterns were visualized by ethidium bromide staining, and photographs taken under UV light. Online search for similarity was carried out at GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov).

2.5. Effect of Bacillus sp. B14 cell culture on common bean growth

Seeds from the black bean (cv. Nag 12) were used. They were initially sterilized in 95% alcohol for 30 s and then in 1% sodium hypochlorite solution for 1 min. After this treatment, the seeds were inoculated. Prior to inoculation, the seeds were submerged for 30 min with the 48-h-old cell culture of B14 in BHI medium at a concentration of 1×10^8 cells per ml. Non-inoculated seeds were used as control. A total of 70 seeds per assays were planted at a depth of 2 cm in plastic trays containing sterile sand used as substrate.

The trays were placed in a growth chamber with air circulation for germination and temperature control (28 °C ± 2) for 15 days. After 9 days, the potential germination (PG) effect of B14 on seeds was analyzed. After 15 days, plant height (cm) (shoot and root portion) was determined. Fifteen seedlings were selected at random from each repetition (45 total).

The assays were performed in triplicate.

2.6. Effect of Bacillus sp. B14 cell culture on common bean seed health

As previously mentioned, seeds from the black common bean cv. Nag 12 were initially sterilized and inoculated with the 48-h-old cell culture of B14, as mentioned above. Non-inoculated seeds were used as control. Treated and control seeds were placed in Petri dishes (9 cm in diameter) containing PDA; five bean seeds per Petri dish (five treated seeds and five control) were placed equidistant from one another and the dishes were then incubated in a heater at 26 °C for 10 days. After the incubation period, it was assessed the presence or absence of seed-borne pathogenic microorganisms and other microorganism contaminants in seeds. The seed-borne microorganisms were characterized according to their morphological and microscopic characteristics (Barnett et al., 1998).

The assays were repeated five times.

2.7. Effect of Bacillus sp. B14 cell culture on common bean seeds grown in soils contaminated with M. phaseolina

The black bean cv. Nag 12 seeds were disinfected and inoculated with B14 as previously mentioned. Non-inoculated seeds were used as control. In addition, an inoculum of *M. phaseolina* was prepared on rice, according to the method by Castellanos et al. (2011). Trays of sterilized soil (loam soil with 2.91% organic matter, 0.17% total nitrogen, pH 6.9, 32% sand, 44% silt and 24% clay, typic ustorthents according to USDA Soil Taxonomy) were prepared as substrate, which were then artificially infected with the *M. phaseolina* culture obtained from the rice (0.5 g of inoculum per kg of soil). 70 bean seeds were sown per tray, those inoculated as non-inoculated (control), as previously explained. The trays were incubated at 23–26 °C for 21 days. After this period, the incidence of pathogens as a percentage of plants showing root rot symptoms was recorded.

2.8. MALDI-MS analysis of the lipopeptides involved in the antifungal activity

Lipopeptide synthesis by B14 strain was analyzed on the PDA medium with six fungal strains: *M. phaseolina* 02, 03, 06, 27, 32 and Campichuelo after 7 days of incubation at 28 °C. The interaction of B14 with each fungus produced an inhibition zone of a large diameter. A portion of 4 mm (sample) was removed from inhibition zone and kept at −20 °C. Finally, each sample was resuspended in 0.5 ml of water at pH 8 and vigorously shaken for 30 s. The samples were analyzed by MALDI-MS as described by Torres et al. (2016).

Spectra were recorded on a Bruker Ultraflex II TOF/TOF (Bruker Daltonics, Bremen, Germany). As MALDI matrix, 9H-pyrido[3,4b]indole (norharmane, nHo) was used (Sigma-Aldrich, USA).

2.9. Statistical analyses

Data calculation and statistical analyses were performed using Microsoft Office Excel and INFOSTAT software (Di Rienzo et al., 2012) for Windows. Analyses of variance were used (ANOVA) with LSD (least significant difference) to test differences in fungal growth inhibition assays and plant growth-promoting attributes.

Data obtained from potential germination (PG), plant height (cm) (shoot and root portion) of each treatment were evaluated in order to study the effect of *Bacillus* sp.

B14 cell culture common bean growth and health (incidence) were analyzed through standard analyses of variance (ANOVA). In all cases, residuals were tested for normality with the Shapiro-Wilks' test. To test for differences between means, an LSD test at a significance level of $P \leq 0.05$ was used also.

3. Results

3.1. Bacterial isolation from soil and inhibition assays

One hundred and five strains of *Bacillus* spp. were isolated from different types of soils, according to their macro and microscopic characteristics. Out of all isolated strains, seven were found to have the highest and significant antagonistic effect against the test phytopathogens (Table 1). Strains B14, B18, B19, B25 and B30 inhibited all phytopathogens tested. Of all the isolated strains, B14 had the greatest antagonistic effect on *Scl. sclerotiurum, R. solani, F. solani* and *S. rolfsii*, with a %IF of 80, 80, 71 and 80, respectively, showing statistically significant differences respect to the other strains ($p < 0.05$) (Table 1). *M. phaseolina* was more susceptible to bacteria B14, B18 and B25, with close %IF that varied between 60 and 66%. Strain B1 inhibits all pathogens except *S. rolfsii*. The B31 strain inhibited *M. phaseolina* and *S. rolfsii* with a %IF of 12 and 35, respectively, but didn't have antagonistic effect on the rest of the tested pathogenic fungi.

Table 1
Percentage of fungal inhibition (%IF) of bacilli strains agents against some fungal phytopathogens.

Strains	Origin	Phytopathogens				
		Scl. sclerotiurum	*R. solani*	*Mp. phaseolina*	*F. solani*	*S. rolfsii*
B1		64 b*	50 b	50 b	25 d	NI
B14		80 a	80 a	60 a	71 a	80 a
B18		63 b	57 b	66 a	50 b	77 a
B19		70 b	57 b	55 b	43 c	54 b
B25		57 c	51 b	60 a	43 c	52 b
B30		47 c	51 b	58 ab	33 cd	57 b
B31		NI	NI	12 c	NI	35 c

NI: Did not inhibit.
Different letters indicate values that are significantly different ($p \leq 0.05$).
* Percentage of fungal inhibition (IF).

Table 2
Main metabolites produced by the 7 preselected bacillus strains with potential of PGPRs.

Strains	Metabolites			
	Auxins (mg/ml)	Siderophores*	Cyanides*	Solubilization of inorganic phosphates*
B1	+[a]	+	−[b]	−
B14	10.10 a	+	−	−
B18	5.08 c	+	−	−
B19	5.71 c	+	−	−
B25	3.35 c	+	−	−
B30	7.39 b	+	−	−
B31	+	+	−	−

Different letters indicate values that are significantly different ($P \leq 0.05$).
* Qualitative determination.
[a] Produce, qualitative determination.
[b] Not produce.

3.2. Determination of plant growth promoting characteristics

The seven *Bacillus* spp. strains synthesized auxins in different concentrations (Table 2). B14 and B30 were the two that produced the greatest and significant concentrations: 10.10 and 7.39 mg/ml, respectively ($p < 0.05$). B1 and B31 also synthesized the metabolite but their concentrations were not determined. It was also observed that all strains grew on TSA medium supplemented with 8-hydroxyquinoline, and were therefore considered as positive for siderophore production. However, neither cyanide synthesis nor inorganic phosphate solubilization were found in any of the strains with the trials used.

Strain B14 was selected as the best isolate for showing maximum and appreciable growth inhibition against all the tested pathogens and by the synthesis of metabolites with PGPB characteristics. The selected isolate was further screened for other biocontrol properties.

3.3. Phylogenetic characterization of Bacillus sp. strain

The 16S rDNA sequence analysis of the selected bacilli was determined, and it was observed that the B14 strain exhibited the 99% DNA sequence identity to database entries associated with known *B. amyloliquefaciens* strains. The 16S rRNA nucleotide sequence data of *B. amyloliquefaciens* B14 has been deposited in the GenBank (accession numbers KY659315) (http://www.ncbi.nlm.nih.gov).

3.4. Effect of B. amyloliquefaciens B14 cell culture on common bean growth

The germination percentage (GP) of black bean cv. Nag 12 seeds, i.e., without any treatment, was 85%. When the seeds were inoculated with B14 there was a 10% increase in GP, reaching 95% GP compared with the non-inoculated control seeds, showing statistically significant differences (Table 3).

Untreated controls had an average root length of 14.12 ± 0.07 cm, and stem length of 31.08 ± 0.45 cm. In the seedlings inoculated with B14, it was recorded a significant increase in the length of both, the root and shoot, measuring 16.75 ± 0.78 cm and 37 ± 2 cm, respectively (Table 3). This result shows that inoculating the seeds with B14 increased significantly ($p < 0.05$) the root length by approximately 2 cm and the area by almost 6 cm.

Table 3
Effect of *B. amyloliquefaciens* B14 cell culture on common bean growth.

	Black common bean cv Nag12 seeds		
	GP (%)	Root length (cm)	Stem length (cm)
Control seeds	85 b	14.12 ± 0.07 b	31.08 ± 0.45 b
Inoculated seeds whit B14	95 a	16.75 ± 0.78 a	37.00 ± 2.00 a

Different letters indicate values that are significantly different ($P \leq 0.05$).

3.5. Effect of B. amyloliquefaciens B14 cell culture on common bean seed health

The phytosanitary state of the black bean cv. Nag 12 control seeds revealed a bacterial contamination in 46% of the total evaluated seeds. Different fungal contaminants such as *Cladosporium* sp. (26%), and phytopathogenic fungi such as *Fusarium* sp. (20%), and *Alternaria* sp. (7%) were also identified on the total analyzed seeds (Fig. 1a) based on their macro and microscopic appearance. When the black bean seeds were treated with B14, neither growth of pathogenic bacteria and fungi, nor contaminants were detected; indeed, it was observed the inoculum development in all seeds (Fig. 1b).

3.6. Effect of B. amyloliquefaciens B14 cell culture on common bean seeds grown in soils contaminated with M. phaseolina

When the soils were infected with *M. phaseolina* and the black bean seeds were not inoculated with B14, only 65 ± 9% of the plants emerged. Throughout the assay the initial symptoms of charcoal root rot were detected on emerged seedlings such as dark and irregular lesions of different sizes on the cotyledons and on the stem tissues of the bean. Infected cotyledons always remained attached to the stems. Symptoms, observed also included bright and systemic chlorosis on young leaves above the site of infection (Fig. 2a). After two weeks, the pathogen had 100% of incidence in the control, showing highly significant differences between treatments ($p < 0.05$), (Fig. 2b). When the seeds were treated with *B. amyloliquefaciens* B14, 94 ± 8% of the plants emerged. Of those a 38 ± 3% incidence of the pathogen was recorded after two weeks of treatment, which means that inoculation of B14 on black bean seeds reduced significantly the incidence of *M. phaseolina* by 62% compared to the non-treated seeds showing highly significant differences between them (Fig. 2c). The results remained the same up to the end of the 21-day assay.

3.7. MALDI-MS analysis of the lipopeptides involved in the antifungal activity

After seven days of incubation, the dual culture method was used to determine the *in vitro* antagonistic activity of B14 against 6 *M. phaseolina* strains (02, 03, 06, 27, 32 and Campichuelo). The six strains of *M. phaseolina* were significantly inhibited by B14, with inhibition halos of similar size. Strains 02 and 06 were inhibited with an IF that varied between 45 and 50%, strains 03, 27 and 32 with an IF of 50%, and the Campichuelo strain with an IF of around 60% (Fig. 3).

The MALDI-MS analysis of the lipopeptides synthesized by B14 in solid medium against six strains of *M. phaseolina* showed several signals as listed in Table 4 and shown in Figs. S1–S5 included as Supplementary material.

The signals observed were identified as kurstatin, surfactin, iturin, polymyxin and fengycin homologues (Table 4). Some homologues were detected simultaneously as protonated $[M+H]^+$ and/or sodiated $[M+Na]^+$ and/or potassiated $[M+K]+$ adduct. B14 synthesized surfactin, iturin, fengycin and kurstatin isoforms, in addition to polymyxin D1 in the presence of strain 02. B14 did not produce the $C_{41}H_{68}N_{11}O_{12}$ kurstatin isoforms, nor $C_{42}H_{70}N_{11}O_{12}$ in the presence of the strains 03, 06 and 07. The isoform of kurstatin $C_{41}H_{68}N_{11}O_{12}$, was only detected in the presence of Campichuelo strain. B14 did not synthesize kurstatin in the presence of the strain 32. B14 synthesized 2 isoforms of iturin, $C_{49}H_{76}N_{11}O_{15}$ and $C_{52}H_{82}N_{11}O_{15}$ and polymyxin D1 $C_{50}H_{93}N_{15}O_{15}$ when it was in the presence of all strains. Additionally, in the presence of all strains of *Macrophomina*, B14 synthesized 5 isoforms of surfactin, however, B14 did not synthesize the isoform $C_{54}H_{94}N_7O_{13}$ in contact with the strain 27. The synthesis of 5 isoforms of fengycin in the presence of all phytopathogenic strains was also determined, with the

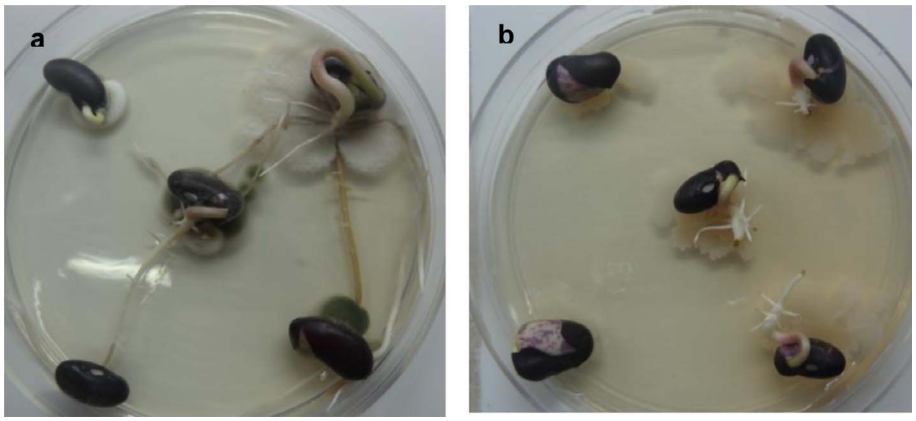

Fig. 1. Seeds of black bean cv. Nag 12 arranged at random on agar PDA. a) Presence of contaminating fungi on black bean seeds un-inoculated. b) Presence and development of *Bacillus* B14 inoculated in black bean seeds.

exception of strain 03, where the isoforms $C_{70}H_{106}N_{12}O_{20}$ and $C_{75}H_{116}N_{12}O_{20}$ were not detected.

The *m/z* of the lipopeptide homologues detected and the relative intensity of the signals, for the samples 03, 06, 27, 32 and Campichuelo were quite similar (Figs. S2–S6; similar fingerprint's sample); for sample 02 the signal pattern showed different relative intensity ratio although the *m/z* values are the same (Fig. S1). The signals corresponding to the surfactin homologues were the most intense and those assigned to kurstakins the less intense (i.e., Figs. S2–S6, samples 03, 06, 27, 32 and Campichuelo, higher intensity at *m/z* = 1060; Fig. S1, sample 02, higher intensity (signals at *m/z* = 1075 and 1060).

4. Discussion

In this study, *Bacillus amyloliquefaciens* B14 was isolated from soils belonging to the common bean producing regions province of Salta and selected for its properties as potential PGPB and PGPR. This strain synthesizes the phytohormone indole-3-acetic acid (IAA), which influences cell elongation, root growth, plant growth promotion and tissue differentiation (Patten and Glick, 1996; Babalola, 2010), and this is a very important metabolite used for plant growth promotion. One essential nutrient in plant nutrition is iron, however it has very low solubility in natural environments. B14 would synthesize siderophores, which act specifically as chelates that can 'sequester' iron and solubilize it, then supplying the plant with soluble iron, thus promoting its growth while also limiting the growth of phytopathogenic fungi and bacteria (Babalola, 2010).

When the black bean cv. Nag 12 (*Phaseolus vulgaris* L.) seeds were inoculated with *B. amyloliquefaciens* B14, the shoot and root length

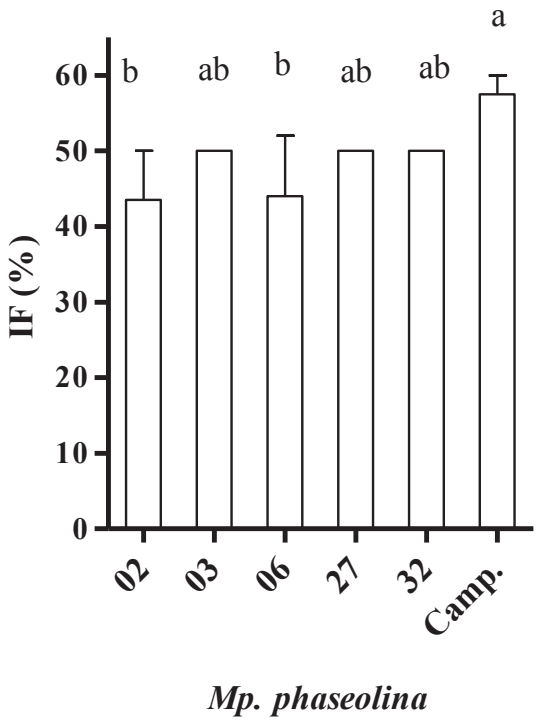

Fig. 3. Percentage of fungal inhibition (%FI) of *Bacillus* B14 against different strains of *M. phaseolina* (identified as 02, 03, 06, 27, 32 and Campichuelo).

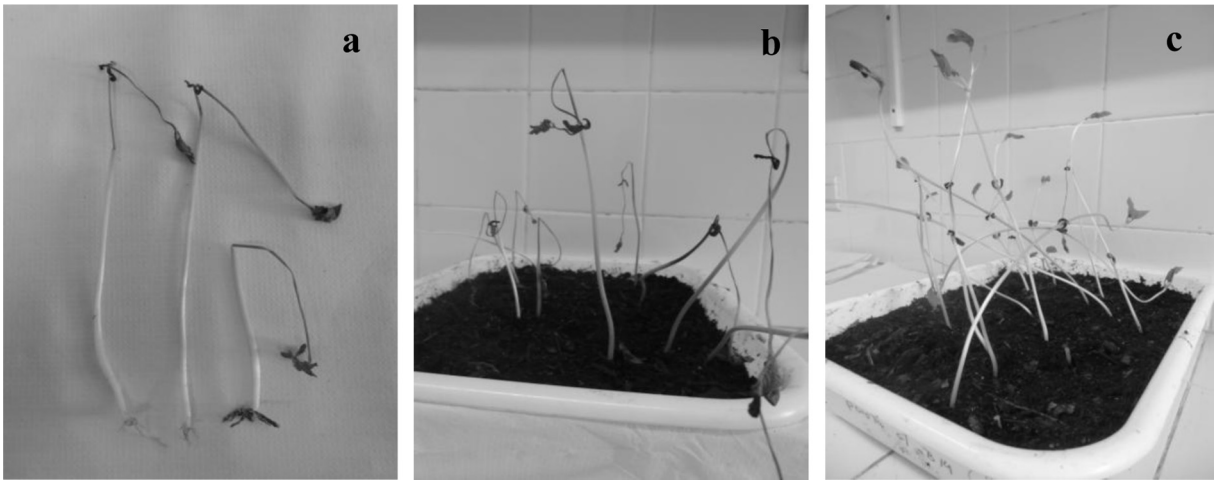

Fig. 2. Symptoms and lesions caused by *M. phaseolina* in black bean cv. Nag 12 seedlings in soils infected with the pathogen. a) and b) Black bean seedlings un-inoculated showing symptoms of strangulation in the stem and root. c) Healthy black bean seedlings inoculated with *Bacillus* B14.

Table 4
Lipopeptides synthesized by *B. amyloliquefaciens* B14 against *M. phaseolina* characterized by MALDI-MS.

Lipopeptide	Chemical formula	Calculated	Macrophomina phaseolina[a]					
			02	03	06	27	32	Campichuelo
Kurstatin	$[C_{41}H_{68}N_{11}O_{12}K]^+$	945.47	945.15	nd[b]	nd	nd	nd	945.26
Kurstatin	$[C_{42}H_{70}N_{11}O_{12}K]^+$	959.48	961.35	nd	nd	nd	nd	nd
Kurstatin	$[C_{43}H_{72}N_{11}O_{12}K]^+$	973.50	972.23	973.34	973.01	973.44	nd	972.60
Surfactin	$[C_{48}H_{82}N_7O_{13}Na]^+$	987.59	988.40	987.28	987.37	987.64	986.92	987.38
Surfactin	$[C_{49}H_{84}N_7O_{13}Na]^+$	1001.60	1001.54	1001.20	1001.43	1000.62	1001.02	1000.95
Surfactin	$[C_{50}H_{86}N_7O_{13}Na]^+$	1015.62	1016.44	1015.42	1015.20	1015.63	1016.19	1015.65
Surfactin	$[C_{51}H_{88}N_7O_{13}Na]^+$	1029.63	1031.38	1031.11	1031.27	1031.37	1031.32	1031.50
Surfactin	$[C_{52}H_{90}N_7O_{13}Na]^+$	1043.65	1045.34	1045.34	1045.28	1045.47	1045.09	1045.25
Surfactin	$[C_{52}H_{90}N_7O_{13}K]^+$	1059.62	1060.34	1059.53	1059.46	1059.86	1059.70	1059.75
Not assigned			1067.35	nd	nd	nd	nd	nd
Surfactin	$[C_{53}H_{92}N_7O_{13}K]^+$	1073.64	1075.62	1075.38	1075.38	1075.70	1075.65	1075.87
Iturin	$[C_{49}H_{76}N_{11}O_{15}Na]^+$	1081.54	1081.68	1082.866	1082	1082.615	1082	1081.508
Surfactin	$[C_{54}H_{94}N_7O_{13}K]^+$	1087.65	1089.20	1089.61	1089.23	nd	1089.29	1089.40
Iturin	$[C_{49}H_{76}N_{11}O_{15}K]^+$	1097.51	1097.46	1097.48	1097.46	1098.64	1097.46	nd
Iturin	$[C_{52}H_{82}N_{11}O_{15}H]^+$	1101.61	1103.36	1103.68	1103.48	1104.03	1104.07	1103.94
Not assigned			1113.59	1113.32	1113.52	nd	1113.63	1103.94
Not assigned			1120.07	1119.54	1119.93	1119.84	1119.90	1119.97
Not assigned			1136.17	1135.40	1135.41	1136.01	1135.67	1136.30
Not assigned			nd	1158.21	1159.20	1159.68	1159.07	1159.82
Polymyxin	$[C_{50}H_{93}N_{15}O_{15}K]^+$	1182.66	1183.515	1182.02	1181.70	1182.78	1182.15	1182.43
Not assigned			1198.77	1199.35	1198.44	nd	nd	nd
Fengycin	$[C_{70}H_{106}N_{12}O_{20}Na]^+$	1457.75	1458.95	nd	nd	nd	nd	nd
Not assigned			nd	nd	1465.59	1465.72	nd	1465.02
Fengycin	$[C_{70}H_{106}N_{12}O_{20}K]^+$	1473.33	1472.94	nd	1472.24	1472.95	1472.67	1472.80
Fengycin	$[C_{73}H_{112}N_{12}O_{20}H]^+$	1477.82	nd	nd	1478.65	1477.96	1478.51	1478.34
Fengycin	$[C_{72}H_{110}N_{12}O_{20}Na]^+$	1485.78	1486.57	1486.47	1486.48	1486.48	1486.82	1486.82
Not assigned			nd	nd	nd	1494.104	nd	nd
Fengycin	$[C_{72}H_{110}N_{12}O_{20}K]^+$	1501.75	1501.54	1501.37	1501.66	1501.52	1501.21	1501.36
Fengycin	$[C_{73}H_{112}N_{12}O_{20}K]^+$	1515.77	1515.43	1515.95	1516.65	1515.48	1516.32	1516.56
Fengycin	$[C_{74}H_{114}N_{12}O_{20}K]^+$	1529.79	1530.93	1530.53	1530.37	1530.97	1530.79	1530.97
Fengycin	$[C_{75}H_{116}N_{12}O_{20}K]^+$	1543.81	1544.58	nd	1544.911	1544.99	1545.15	1545.24
Not assigned			1562.25	nd	nd	nd	nd	nd
Not assigned			1575.18	nd	nd	nd	nd	nd

[a] *m/z* values obtained for each experiment are listed. For details see Materials and Methods, sec.8.
[b] nd: no detected.

increased in 19.0 and 14.5 percent, respectively, while also recording an increase in the seed germination potential. Perhaps the stimulation of the growth on common bean inoculated with B14 could be related to the IAA produced by the bacterium. In previous studies, our research team determined the ability of *B. amyloliquefaciens* PGPBacCA1 to increase root length when used in inoculation experiments. Torres et al. (2017) did not observe increases in shoot length and in potential germination; however, they demonstrated that PGPBacCA1 had an antagonistic effect against different plant phytopathogens mainly by the production of several metabolites. Kumar et al. (2012) determined metabolite production with PGPB properties and antagonistic effect of *Bacillus* strains used in bean cultivation. In later studies, Kumar et al. (2016) proved that *Bacillus* strains increased crop growth showing in addition an antifungal effect against plant pathogens. Martins et al. (2013) also concluded that *Bacillus* strains increased bean crop growth in Brazil. However, to our knowledge, this would be the first report of a native isolated *Bacillus* strain from the Salta province that synthesizes metabolites involved in plant growth promotion and improve commercial bean crop germination and seedling growth in our region.

Parsa et al. (2016) were the first to document the seed-borne fungal diversity in bean crops out of 11 common bean cultivars from Latin America, and they identified 42 different taxons of pathogenic and saprophytic fungi. In our study we identified *Cladosporium* spp., *Fusarium* spp. and *Alternaria* spp. in black bean cv. Nag 12 seeds, which are consistent with those identified by Parsa et al. (2016). In addition and as we expected, we found the common contaminating fungus *Rhizopus* spp. In the present study, a low count of seed-transmitted pathogens of common bean were identified, the only exception was *Fusarium* spp. which presented an incidence of 20% in the *in vitro* trials. The prevalence of *Fusarium* spp. compared with other seed-transmitted phytopathogens was also found in other studies of the common bean pathosystem (El-Samawaty et al., 2014; Parsa et al., 2016). In this work we proved that when seeds were inoculated with *B. amyloliquefaciens* B14, all fungi identified in the control seeds disappeared. This result is extremely important, mainly due to the presence of *Fusarium* spp. seed borne fungal in black bean seeds, given that the fungi could be transmitted to the seedlings, damaging the crop and causing significant economic losses (Parsa et al., 2016).

In recent years, the genus *Bacillus* has held considerable importance in agriculture due to its ability to synthesize a large variety of metabolites with antifungal effects against plant pathogens (Singh et al., 2008; Kumar et al., 2012; Liu et al., 2014; El Arbi et al., 2016; Torres et al., 2016; Kumar et al., 2016). In this work it was shown the *in vitro* antifungal effect of B14 on the development of *S. sclerotiurum, R. solani, F. solani*, *S. rolfsii* and *M. phaseolina*. Numerous studies have demonstrated that the antifungal effect of *Bacillus* strains against phytopathogenic fungi is due to lipopeptide synthesis (Singh et al. 2008; Kumar et al., 2012; Cawoy et al., 2014; Li et al., 2014; Liu et al., 2014; El Arbi et al., 2016; Torres et al., 2016, 2017; Kumar et al., 2016). In our previous studies, it was evaluated, using the MALDI-TOF MS method, the synthesis of surfactin, iturin and fengycin by *B. amyloliquefaciens* PGPBacCA1 when up against *M. phaseolina* (Torres et al., 2016), the same against *F. solani* and *S. sclerotiorum* (Torres et al., 2017). In this study it was demonstrated the ability of *Bacillus* B14 to synthesize surfactins, iturins, fengycins and, moreover, to co-produce kurstakins and polymyxins when grew in the presence of *M. phaseolina.* This reveals the variety of metabolites produced by *B. amyloliquefaciens* B14, which could be responsible for its antagonistic effect against the

different phytopathogenic fungi tested in this work. Thus, from these results we can infer that in addition to influencing the target species in the nature of lipopeptides synthesized by *Bacillus* (Cawoy et al., 2014), we can also suggest that the type of lipopeptides produced depends on the producing strain. Price et al., (2007), determined the coproduction of surfactins, iturins, fengycins, kurstakins and polymyxins by different *Bacillus* strains, but when grown in monocultures in the absence of pathogenic fungi. Similar results were reported by El Arbi et al. (2016). As far as we know, there are not scientific articles mentioning the co-production of these five lipopeptides by *Bacillus* strains in the presence of *M. phaseolina*.

The lipopeptides iturins, fengycins and kurstakins are known for their antifungal activity (Hathout et al., 2000; Ongena and Jacques, 2008; Béchet et al., 2012; Falardeau et al., 2013), which suggests that these metabolites are the main responsible for the antagonistic activity against the phytopathogens used in this study. Surfactin primarily has antibacterial action but it also has a synergistic effect on iturin (Thimon et al., 1992) or fengycin (Koumoutsi et al., 2004); and perhaps this situation was the one here observed with B14, which synthesized all these three compounds in the presence of *M. phaseolina* mycelium. On the other hand, polymyxins are the oldest-known class of lipopeptides isolated from *Bacillus* species and display strong activity against Gram-negative bacteria (Cochrane and Vederas, 2016). However, Adams et al. (2016) demonstrated the synergistic effect of polymyxin B on antifungal lipopeptide caspofungin in *Candida* fungal species that were resistant to antifungal lipopeptide. Perhaps, in our work the same effect could occur, and then the polymyxin could exert synergism on the lipopeptides iturins, fengycins and kurstakins synthesized by B14.

There are few studies regarding the effect of *Bacillus* strains as a biocontrol agent on *M. phaseolina in vivo* in common bean cultivation. Corrêa et al. (2014) studied the effect of *B. cereus* strains as biocontrol agents in soils infected with *M. phaseolina* on the common bean from Brazil. They determined, in two independent trials, that *B. cereus* DFs093 reduced the severity and progress of the pathogen on the crop, in 28 and 14%, compared to control, according to the area under disease progress curve (AUDPC). In this study, it was determinate that the inoculation of *B. amyloliquefaciens* B14 on black bean seeds reduced the incidence of *M. phaseolina* by 62% compared with the non-inoculated seeds as well as promoting its growth. This issue is very important to consider since the bean crop is one of the most exploited in the region and the incidence of the charcoal root rot causing by *M. phaseolina* represents the most important disease in the region, commonly reducing bean yield and quality, which causes significant economic losses (Perez-Brandán et al., 2012). However, there is not sustainable management practices employed in northwestern Argentina (NOA) on common bean crop, so it would be very important to develop a proper bio-inoculant to combat this disease. The preliminary results obtained in this work, suggest that the isolated bacteria *B. amyloliquefaciens* B14 have the potential to control charcoal rot root in the common bean (*Phaseolus vulgaris* L.) used in our region in addition to possessing potential PGPR properties.

From all our results, we conclude that *B. amyloliquefaciens* B14 would be a potential growth promoter in commercial bean cultivation in the NOA region, acting in addition as a biocontrol agent against different phytopathogenic fungi.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2017.06.008.

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