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PII: S0944-5013(17)31054-6
DOI: https://doi.org/10.1016/j.micres.2018.04.003
Reference: MICRES 26149

To appear in:

Received date: 25-10-2017
Revised date: 14-3-2018
Accepted date: 6-4-2018

Please cite this article as: Sabaté Daniela C, Brandan Carolina Pérez, Petroselli Gabriela, Erra-Balsells Rosa, Audisio M.Carina.Biocontrol of Sclerotinia sclerotiorum (Lib.) de Bary on common bean by native lipopeptide-producer Bacillus strains.*Microbiological Research* https://doi.org/10.1016/j.micres.2018.04.003

Biocontrol of *Sclerotinia sclerotiorum* (Lib.) de Bary on common bean by native lipopeptide-producer *Bacillus* strains

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Abstract

Bacillus sp. B19, *Bacillus* sp. P12 and *B. amyloliquefaciens* B14 were isolated from soils of Salta province, and PGPR properties on the common bean (*Phaseolus vulgaris* L.) cv. Alubia and antagonistic activity against *Sclerotinia sclerotiorum* were studied.

It was determined that B19 and P12 increased crop germination potential (GP) from the common bean by 14.5 % compared to control seeds; these strains also increased root length (10.4 and 15 %, respectively) and stem length (20.2 and 30 %, respectively) compared to the control; however, as for the B14 strain, no increases in growth parameters were detected. In addition, all the treatments that combined two bacilli: B14+B19, B14+P12 and B19+P12, generated beneficial effects on GP and seedling growth compared to control seeds, but not compared to a single inoculant. B19 and P12 strains synthesized auxins at concentrations of 5.71 and 4.90 mg/mL, respectively, and it was qualitatively determined that they synthesize siderophores. In addition, previous studies have determined that B14 produces auxins in a concentration of 10.10 mg/ml, and qualitatively synthesizes siderophores.

The phytosanitary state of the white bean cv. Alubia control seeds revealed bacterial contamination in 87 % of all the evaluated seeds and different fungi such as *Cladosporium* sp., *Fusarium* sp., and *Rhizopus* sp. Bean seeds treated with B14, B19 or P12 showed no growth of contaminating bacteria or of pathogenic fungi; in fact, bacilli inoculum development was observed in all seeds. Additionally, B19, P12 and B14 strains inhibited *in vitro* the development of 9 native *S. sclerotiorum* strains isolated from the Salta region, with FI ranging between 60 and 100 %. The three *Bacillus* strains synthesized different isoforms of the lipopeptides: surfactin, iturin, and fengycin in the presence of *S. sclerotiorum*, as determined by MALDI-TOF.

In the *in vivo* trials, when common bean seeds were grown in soils contaminated with *S. sclerotiorum*, an incidence of 100% was determined when the seeds were not treated with any *Bacillus*. Seeds treated with the chemical fungicide and sown in *S. sclerotiorum*-infested soil did not produce seed emergence, while the inoculation of the seeds with B14+P12, B14+B19 or B19+P12 reduced the effect of the pathogen by 46, 43 and 25 %, respectively. Disease progression in B14+P12 and B14+B19 treatments was significantly lower than in the remaining treatments, with an AUDPC of 873.75 and 1071, respectively.

Keywords: *Bacillus, Sclerotinia sclerotiorum, Phaseolus vulgaris* L., lipopeptides, biological control.

1. Introduction

Common bean (*Phaseolus vulgaris* L.) production is a key agricultural activity in northwest Argentina (NWA). Crop production is mainly concentrated (70 %) in the province of Salta, with an estimated cultivated area of about 450,000 ha in the last years (De Bernardi, 2016). Sanitary status of seeds is one of the main factors influencing crop production and health and is determined by the presence or absence of crop associated pathogenic microorganisms. The agents causing the most devastating diseases in common bean crops can be transmitted by seeds; therefore, seeds may be a significant means of disease transmission as well as a source of primary pathogen inoculum. White mold, caused by the fungus *Sclerotinia sclerotiorum* Lib. de Bary, is a very detrimental disease affecting the common bean in the province of Salta. The fungus is favored by temperate climates, moderate temperatures and high relative humidity (Mamaní Gonzáles et al., 2015). *S. sclerotiorum* primarily spreads by spores and usually in forms of sclerotia, which may infect stems, leaves and flowers, and even spread to adjacent plants (Zhou and Boland, 1998). Sclerotia of *S. sclerotiorum* can reside in the soil for several years and, under appropriate environmental conditions, germinate to form mycelium, leading to infectious hyphae, or producinge apothecia, which release millions of airborne ascospores (Coley-Smith and Cooke, 1971; Bardin and Huang, 2001). Given its persistence in the soil or in seeds, as well as its tendency to spread, and further, the lack of resistant cultivars, this fungus can cause devastating economic losses in the crops; therefore, its management is of regional importance.

Despite the efforts made by breeding programs, several common bean cultivars used in commercial production are susceptible to white mold. While advances have been made in the development of resistant varieties, selection should also be focused on high-yield varieties (Miklas et al., 2013; Balasubramanian et al., 2014). Other recommended management strategies are crop rotation, wider seeding row spacing and treatment of seeds with chemical products (Vieira et al., 2010; Vizgarra, et al., 2012). As for the last strategy,

intensive use of chemical compounds in crop management has led to insect microbial pathogen resistance to pesticides, and has also caused serious problems for human health and the quality of the environment. Hence, over the last few years, there is a trend in Argentina to apply sustainable agricultural practices to replace, or at least supplement, the use of chemicals, and thus obtain healthy and safe food. This change requires finding non-contaminant and environmentally friendly alternatives.

Different species of the genus *Bacillus* have been widely used both as potential plant growth-promoting rhizobacteria (PGPR) in agriculture, due to their capacity to promote plant growth and as biocontrol agents (Schenck zu Schweinsberg-Mickan and Müller, 2009; Jakab et al., 2011; Pérez-García et al. 2011; Laditi et al., 2012; Stefan et al. 2013). These bacteria have an antagonistic effect against different plant pathogens, which is conferred by their potential to synthesize a wide array of metabolites with antagonistic activity, such as lipopeptides of surfactins, iturins, fengycins, polimixins, 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., 2014a; Thais et. al., 2016; Chandler et. al., 2015; Torres et. al., 2016; Zouari et. al., 2016; Torres et. al., 2017; Sabaté et al., 2017).

The aim of this work was to evaluate the effect of different native strains of the genus *Bacillus* isolated from soils of Salta province as potential PGPR and biocontrol agents, especially in the incidence of the fungus *S. sclerotiorum* (seed and seedling), on the common bean crop.

2. Materials and Methods

2.1. Bacterial isolation from soil

Rhizosphere soil samples (10 cm depth) were taken from the central-eastern region of the province of Salta, which is the area of the province where most bean crops are cultivated under different production systems (24°52´23.72" S 64°14´54.46" W). Serial dilutions were performed, inoculated in BHI (Brain Heart Infusion, Britania, Argentina) broth and incubated at 37 ºC for 24-48 h. Strains exhibiting visible morphological characteristics of the *Bacillus* strain were preselected and their structure was confirmed via optical

microscopic observation. The selected strains were preserved in BHI broth with 20 % v/v glycerol at -20 ºC.

2.2. In vitro *plant growth-promoting attributes of isolates*

Plant growth-promoting bacteria (PGPB) activities of isolates were determined following standard procedures. The solubilization of inorganic phosphate was measured using the methods described by Goldstein (1986). Auxin and cyanide production were detected using 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).

2.3. Phylogenetic Characterization

DNA was extracted from *Bacillus* spp. B19 and P12 with an active culture after incubation in 5 mL of Brain Heart Infusion broth (BHI, Britania, Argentina) at 37 ºC for 24 h, according to the method of Miller (1972). For the characterization, the strains were genetically characterized by analyzing the 16S rRNA subunit, 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 94ºC for 5 min, followed by 35 cycles at 94 ºC for 1 min, 50 ºC for 2 min and 72 ºC for 2 min, and a final extension at 72 ºC 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 volts 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).

2.4. Effect of Bacillus *cell culture on common bean seed health*

For the following tests, a strain previously used for other studies, *B. amyloliquefaciens* B14 (Sabaté et al., 2017), was incorporated due to the fact that it has beneficial properties on the growth of common black bean cv. Nag 12, and as a biocontrol agent against other pathogens of this crop.

Seeds of the white common bean cv. Alubia were initially sterilized in 70 % alcohol for 30 seconds and then in 1 % sodium hypochlorite solution for 1 min. After this treatment, the seeds were inoculated, submerged for 30 minutes with the 48-h-old cell culture of B19, P12 and B14, at a concentration of 1 x 10^8 cells per mL. Non-inoculated seeds were used as control. Seeds were placed in Petri dishes (9 cm in diameter) containing Potato Dextrose Agar (PDA, Britania); five bean seeds per Petri dish per treatment were placed equidistant from one another and the dishes were then incubated in a heater at 26 ºC for 10 days. After the incubation period, the presence or absence of seed-borne pathogenic microorganisms and other microorganism contaminants in the seeds was assessed. The assays were performed in triplicate.

2.5. Effect of Bacillus *cell culture on common bean growth*

White common bean cv. Alubia seeds were initially sterilized and inoculated with the 48-h-old cell culture of B14, B19 or P12, as mentioned above. The effect of the combination of two of these strains, grown in monoculture, was also tested, as follows: B14+B19, B14+P12, and B19+P12, in 1:1 proportion. Furthermore, the commercial chemical fungicide, Maxim®Evolution Rizobacter (tiabendazol 15 g/L, fludioxonil 2.5 g/L, metalaxil-M 2 g/L), commonly used in the region for this crop, was tested; it was applied to seeds following the manufacturer's instructions. Non-inoculated seeds were used as control. Finally, a total of eight treatments were performed: B14, B19, P12, B14+B19, B14+P12, B19+P12, a commercial fungicide and control (seed non-inoculated). A total of 70 seeds per assay were planted at a depth of 2 cm in plastic trays containing sterile sand used as

substrate (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, 1975).

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 germination potential (GP) effect of each treatment on seeds was analyzed. After 15 days, a total of 45 seedlings were selected at random (15 per repetition) and plant height (shoot and root portion expressed in cm) was determined, also following the protocol of Altamirano et al. (2002). The assays were performed in triplicate.

2.6. Fungal growth inhibition assays

A dual culture technique was used for these trials (Landa et al., 1997). The inhibitory activity of the isolated *Bacillus* spp. B19 and P12 strains and *B. amyloliquefaciens* B14 was evaluated against *Sclerotinia sclerotiurum* (Lib) de Bary native strains isolated from NWA: 15, 27, 33, 39, 48, 58, AN, PV and RF, belonging to the culture collection of Agricultural Microbiology Laboratory of the Estación Experimental Agropecuaria EEA-INTA-Salta, Argentina. The different strains of *Sclerotinia sclerotiurum* were grown on PDA, at an incubation temperature of 28 °C for 7 days.

Five-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 taken from 24-h-old cultures of the isolated bacteria were placed equidistant from one another. After incubation at 28 ºC for 7 days, 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 x 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 mycelial growth without bacterial samples.

The assays were performed in triplicate.

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

Lipopeptide synthesis by B14, B19 and P12 strains was analyzed on PDA medium with the nine previously mentioned strains of *S. sclerotiorum*. The interaction of the three bacilli with each fungus produced an inhibition zone of a large diameter. A portion of 4 mm (sample) was removed from the inhibition zone and kept at -20 ºC. 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). For MALDI-MS experiments dry droplet sample preparation or the sandwich method was used according to Nonami et al. (1997), loading 0.5 µL of matrix solution, analyte solution and matrix solution successively after drying each layer at normal atmosphere and room temperature. Mass spectra were acquired in linear positive ion modes. External mass calibration was made using aqueous solution (1 mg/mL) β-cyclodextrin (MW 1134; [M+Na]=1157.35730 [M+K]= 1173.33010. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively.

2.8. Effect of Bacillus *cell culture on common bean seeds grown in soils contaminated with* S. sclerotiorum

An inoculum of *S. sclerotiorum* was prepared on wheat grains, according to the method proposed by Elsheshtawi et al. (2017). Trays containing 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, 1975) were prepared as substrate; this substrate was artificially infected with the *S. sclerotiorum* RF culture obtained from the wheat (1 % w/w). 70 white bean cv. Alubia seeds were sown per tray and inoculated as mentioned above (item 2.5), using the following treatments: B14, B19, P12, different combinations of two bacteria grown in monoculture: B14+P12, B14+B19 and B19+P12, in 1:1 proportion, and the chemical fungicide (Maxim®Evolution Rizobacter), applied according to the manufacturer's instructions. A total of 8 treatments were performed: B14, B19, P12, B14+P12, B14+B19, B19+P12, commercial fungicide and control (non-inoculated seed). Seeds of each treatment were sown in soils infested with *S. sclerotiorum*

RF. Moreover, non-inoculated seeds were used in infested and non-infested soils, as control. The trays were incubated at 23-26 ºC for 45 days. In these assays, germination energy (GE) at five days from seeding and pathogen incidence were determined during the assay. Pathogen incidence was determined visually as a percentage of plants showing characteristic symptoms.

2.9. Area under the disease progress curve

Visual assessments of white mold incidence were made three times between 5 and 45 days after seeding (same as the previous experiment). A modified version of area under the disease progress curve (AUDPC) (equation 1) (Wilcoxson et al., 1975), was used to evaluate disease incidence over time, as follows:

$$AUDPC = \left(\frac{R_1 + R_2}{2}\right)(t_2 - t_1) + \left(\frac{R_2 + R_3}{2}\right)(t_3 - t_2) \quad (1)$$

where R1 to R3 are incidence ratings corresponding to times t1 to t3.

2.10. Statistical analysis

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

Data obtained from germination potential and energy (GP and GE) and plant height (shoot and root portion, expressed in cm) of each treatment were analyzed to assess the effect of *Bacillus* spp. strains.

B14, B19, P12 and their combinations, and their effect on white common bean seeds growth and incidence in soils contaminated with *S. sclerotiorum* were analyzed using standard ANOVA. In all cases, residuals were tested for normality via the Shapiro-Wilks test. To test for differences between means, an LSD test at a significance level of $P \leq 0.05$ was used.

3. Results

3.1. Isolation and determination of some plant growth promoting attributes

Two strains of *Bacillus* spp., B19 and P12, were isolated from different types of soils, according to their macro and microscopic characteristics. Both B19 and P12 strains synthesized auxins at different concentrations: 5.71 and 4.98 mg/mL, respectively. Both strains grew on TSA medium supplemented with 8-hydroxyquinoline and were therefore considered positive for siderophore production. Neither cyanide synthesis nor inorganic phosphate solubilization was found in any of the strains.

3.2. Phylogenetic characterization of Bacillus *spp. strains*

The 16S rDNA sequence analysis of the selected bacilli showed 98 % DNA sequence identity of the B19 strain to database entries associated with known *Bacillus* sp. strains and 98 % of P12 to known *Bacillus* sp. strains. The 16S rRNA nucleotide sequence data of *Bacillus* spp. B19 and P12 were deposited in the GenBank (accession numbers MF574161 and MF574162, respectively) (http://www.ncbi.nlm.nih.gov).

3.3. Effect of Bacillus *cell culture on common bean seed health*

For these tests *B. amyloliquefaciens* B14, a strain previously used for other studies, was incorporated. This strain was used because in previous studies it had shown beneficial properties in common black bean cv. Nag 12, and to be a biocontrol agent against *Macrophomina phaseolina* (Sabaté et al., 2017).

The phytosanitary state of the white bean cv. Alubia control seeds revealed bacterial contamination in 87 % of all the evaluated seeds. Different fungal contaminants, such as *Cladosporium* sp. (60 %), and phytopathogenic fungi, such as *Fusarium* sp. (26 %) and *Rhizopus* sp. (7 %), were also identified in the non-inoculated seeds (Fig. 1a) based on their macro and microscopic appearance. Bean seeds treated with a single inoculum of B14, B19

or P12, showed no growth of bacteria or of pathogenic fungi; in fact, inoculum development was observed in all seeds (Fig. 1b).

3.4. Effect of Bacillus *cell culture on common bean growth*

The intrinsic germination potential (GP) of white bean cv. Alubia seeds, i.e., without any treatment, was 77.4 %. In addition, seeds inoculated with B19 exhibited a GP of 88.6 %, and an average increase in root length of 10.4 % and in stem length of 20.2 % with respect to control seeds, with significant differences (Table 1). Inoculation with P12 also generated beneficial effects on seedling growth since root length increased by 15 % and stem length by 30 %, approximately, compared to non-inoculated seeds, and a GP of 88.6 % (Table 1). The B14 strain did not have a beneficial effect on white bean cv. Alubia seeds, and a slight reduction of GP and seedling growth parameters were determined with respect to the control (Table 1). Inoculation of seeds with the chemical fungicide exhibited an average increase in root length of 2.6 % and in stem length of 11.8 % compared to control seeds (Table 1) with significant differences. In addition, all the treatments that combined two bacilli generated beneficial effects on GP and seedling growth compared to control seeds (Table 1). So, seeds inoculated with B19+B14, exhibited an increase in GP of about 16.3 %, and significant average increase in root length of 5.2 % and in stem length of 8.1 % compared to the control. Seeds inoculated with B19+P12 exhibited a significant increase in GP of about 21.4 % and an average increase in root length of 4.2 % and in stem length of 18.9 %, with significant differences compared to the control and the singles inoculants. Finally, seeds inoculated with B14+P12 exhibited an increase in GP of about 18.9 % compared to the control, and an average increase in root length of 1.6 % and in stem length of 11.5 % with significant differences compared to control and to the singles inoculants as well (Table 1).

3.5. Fungal growth inhibition assays

The analysis of the individual effect of the three *Bacillus* strains on the different *S. sclerotiorum* strains showed a differential effect on the 9 plant pathogens (Fig. 2). The plant pathogen strains 15 and 27 proved to be the most sensitive as they were inhibited with an FI of 100 % by the three bacilli. The most resistant pathogens were PV and RF, with an FI that ranged between 60 and 80 % showing significant differences compared to the 15 and 27 pathogen strains. Strains 33, 39, 48, 58 and AN were similarly inhibited by the three bacilli, with no significant differences between them. In all these strains the FI ranged between 70 and 90 % (Fig. 2).

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

The MALDI-MS analysis of the lipopeptides synthesized by B14, B19 and P12 individually in solid medium against 9 strains of *S. sclerotiorum* (Table 2, Fig. S1-S18) revealed signals compatible with kurstakin, surfactin, iturin and fengycin homologues. As is shown in Fig. S 1-18 (see supplementary material) observed signals are clearly located in two different m/z regions: those observed in m/z 900-1200 (Fig. S1, S3, S5, S7, S9, S11, S13, S15, S17) and the second group located in the region m/z 1400-1650 (Fig. S2, S4, S6, S8, S10, S12, S14, S16, S18). According to data in the literature (Vater et al., 2002; Yang et al., 2006; Price et al., 2007; Pathak et al., 2012; Torres et al., 2015) in the first region signals can be assigned mainly to kustakins, surfactins and iturins, the second to the m/z region were fengycins.

No significant differences were observed in the production of lipopeptides (number of homologues and relative intensity) by B14 against the 9 plant pathogenic strains used. In all cases relative intensity was higher for surfactins homologues compare with the fengycins ones. Sodiated adduct of kurstakins (m/z= 915, m/z=943, m/z=957 and m/z=971) were detected. Homologues of polymyxin were detected as very low intensity signals (m/z 1145, m/z=1167, m/z=1182, m/z=1202 and m/z=1225). B14 synthetized 7 fengycins homologues against *S sclerotiorum* shown their molecular ions as $[M+H]^+$, $[M+Na]^+$ and/ or $[M+K]^+$ species.

B14 and P12 synthesized 7 isoforms of surfactin and 5 isoforms of iturin in the presence of the different *S. sclerotiorum* strains.

On the other hand, for the bacilli B19, fengycin homologues were more intense than signals assigned as surfactins. Homologues of kurstakins were not detected.

3.7. Effect of Bacillus *cell culture on common bean seeds grown in soils contaminated with* S. sclerotiorum

The *S. sclerotiorum* strain RF was selected for these assays because it was one of the most resistant strains against the bacilli, as indicated by the FI % reported in section 3.5. Untreated common bean seeds (control) sown in soil infested with *S. sclerotiorum* RF exhibited a GE of 7 %. After 15 days of seeding, the disease incidence was 80 % in the control, with infected seedlings showing the typical disease signs such as dark green lesions and watery appearance on the stem, as well as cottony growth of white mold in roots and stems (Fig. 3a, b). The analysis of seeds that did not emerge showed the presence of abundant sclerotia and growth of white mycelium surrounding them (Fig. 3c). After 45 days of the sowing of the seeds without inoculation, the soils infected with the pathogen recorded 100% incidence. Seeds treated with the chemical fungicide and sown in *S. sclerotiorum*-infested soil did not produce seed emergence. The analysis of the non-emerged seeds showed, as in the control, the presence of abundant sclerotia and mycelium growth. Seeds treated with a single inoculant, B14, B19 or P12, showed a lower disease incidence 15 days after seeding: 60, 52 and 50 %, respectively, compared to the control (Table 3). At the end of the trials, an incidence of 100, 76 and 75 %, respectively, was observed with the single inoculant. Seeds inoculated with B19+P12 did not exhibit statistically significant differences from seeds inoculated with a single inoculant of these strains, as the pathogen was present in 50 % 15 days after seeding. After 45 days, the incidence of treatment B19+P12 increased to 75 %, similar to the single inoculant. However, the B14+B19 treatment did show a statistically significant decrease of disease incidence (28.6 % incidence) 15 days after seeding compared to the application of a single inoculant and compared to the control. At the end of the trail, the disease incidence of B14+B19 treatment was 43 % lower than the control. A significantly higher protective effect was detected with the application of B14+P12 (Fig. 3d), which resulted in *S. sclerotiorum* incidence of 12.5 % 15 days after seeding. 45 days after sowing, B14+P12

treatment showed the lowest incidences compared to the rest of the treatments (Table 3). This treatment also yielded the highest GE (34 %) (Table 3). In all treatments, diseased plants initially exhibited watery lesions with white mold growth (Fig. 3a, b). On the other hand, non-emerged seeds showed the presence of sclerotia and pathogen growth in the form of cottony mycelium growth (Fig. 3c).

Disease progress in B14+P12 and B14+B19 treatments was significantly lower than in the remaining treatments, with an AUDPC of 873.75 and 1071, respectively (Table 3).

4. Discussion

In this study, we determined that three *Bacillus* native strains: *Bacillus* spp. B19 and P12 and *B. amyloliquefaciens* B14, isolated from soils of the province of Salta, significantly reduced white mold in common bean (*Phaseolus vulgaris* L.) caused by *S. sclerotiorum* under greenhouse conditions. This plant pathogen is of great concern in the region because it can easily spread in the soil or from infected seeds due to its reproduction mode, causing devastating crop yield losses (Mamaní Gonzáles et al., 2015). The use of pathogen-free seeds is recommended to control this problem; hence, chemicals are frequent applied on seeds (Vieira et al., 2010; Vizgarra, et al., 2012). However, there is currently significant concern about the impact of chemical pesticides on the environment and thus has led to increased interest in strategies for the biocontrol of different phytopathogens. The use of *Bacillus* strains as biocontrol agents to inhibit *S. sclerotiorum* has been previously tested in different crops. Hu et al. (2014) reported a reduction about 10 % of *S. sclerotiorum* incidence in oilseed rape seeds treated with *Bacillus*. Similar results were obtained by Chen et al. (2014) in rapeseed. Fernando et al. (2007) showed that *B. amyloliquefaciens* reduced the incidence of *S. sclerotiorum* on canola petals sprayed at 10 and 30 %. However, few attempts have been made to find bacterial biocontrol agents for controlling diseases that affect the common bean crop in the NWA region (Torres et al., 2016; Torres et al., 2017; Sabaté et al., 2017). In the present study we found that strains B19 and P12 significantly reduced *in vivo* disease incidence (25 % approximately) by the end of the trials. Moreover, the combined application *in vivo* of B14+P12 or B14+B19 was more efficient than a single inoculant, as seen from the fact that disease incidence was reduced by 46 and 43 %,

respectively. During the progression of the disease, the analysis of disease progress curve (AUDPC), confirmed that the combination of B14+P12 was the most effective treatment, followed by B14+B19. The combinations appear to have a synergistic effect. Different authors have shown that the application of more than one biocontrol agent increases the effect of biological control (Guetsky et al., 2001; Jetiyanon and Kloepper, 2002). Sun et al. (2017) determined that the biocontrol effect of an inoculant composed of two *Bacillus* strains (LHS11 + FX2) is more effective than a single inoculant on rapeseed white mold *in vivo*. Correa et al. (2014) also reported that *Bacillus* strains applied in combination were more effective in controlling diseases affecting common bean than when applied as a single inoculant. However, no reports have been found on the effect of native inoculants for the treatment of white mold on common bean from our region.

In common bean production areas in the NWA, sclerotia are usually present in the soils, which guarantee the presence of the pathogen in future crop seasons. This problem causes considerable crop yield losses and requires the use of fungicides to reduce the high disease levels affecting the crop (Vizgarra et al., 2012). McCreary et al. (2016) showed that fungicides still play a major role in the control of white mold in dry bean, and Vieira et al. (2010) found that fluazinam combined with reduced plant density offered great disease suppression. In this study, however, we determined that the chemical fungicide frequently used in our study region to prevent fungal diseases did not have a protective effect against *S. sclerotiorum*; moreover, bean seeds showed abundant presence of sclerotia and exhibited white mold growth. In the *S. sclerotiorum*-contaminated soils, the application of B14+P12 or B14+B19 on seeds can be considered as an alternative for biocontrol of white mold in order to reduce the incidence of the disease in the crop. This possibility should be given serious consideration due to the fact that the common bean is one of the most widely grown crops in the region and because white mold is both one of the most harmful diseases affecting this crop and to date there are no cultivars resistant to the disease.

In this study, we also found that the three strains significantly inhibited pathogen growth *in vitro*, with FI values ranging between 60 and 100 %. Numerous studies have demonstrated that the antifungal effect of *Bacillus* strains against phytopathogenic fungi is due to lipopeptides synthesis (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; Kumar et al., 2016; Torres et al., 2017; Sabaté

et al., 2017). Elkahoui et al. (2014) determined that *S. sclerotiorum* inhibition by *Bacillus* sp. BCLRB2 is due to the synthesis of bacillomycin and iturin. In this study, using the MALDI-TOF MS method, we demonstrated the ability of B14, B19 and P12 to synthesize kurstakins, surfactins, iturins, polimyxin and fengycins against *S. sclerotiorum* when the strains are in contact with the fungus. However, no correlation was found between the different types of synthesized lipopeptides and their isoforms and the FI % found (Data not shown). This result may be due to the synergistic effect of metabolites among them. The lipopeptides iturins and fengycins are known for their antifungal activity (Ongena and Jacques, 2008; Falardeau et al., 2013), which suggests that these metabolites are the main factors responsible for the antagonistic activity against the phytopathogen used in this study. Surfactin primarily exhibits antibacterial activity but it also has a synergistic effect on iturin (Thimon et al., 1992) or fengycin (Koumoutsi et al., 2004); this may be the phenomenon observed with the B14 + P12 or B14 + B19 when applied on common bean seeds, with its antagonistic effect increasing in the presence of *S. sclerotiorum*. Furthermore, Sabaté et al. (2017) determined that *B. amyloliquefaciens* B14 synthesized surfactins, iturins, fengycins and, moreover, co-produced kurstakins and polymyxins when grown in the presence of *M. phaseolina.* Thus, from these results we can infer that the nature of lipopeptides synthesized by *Bacillus* influences the target species, which is consistent with results reported by Cawoy et al. (2014). Biocontrol efficacy of *Bacillus* on *S. sclerotiorum* was more efficient *in vitro* than *in vivo*, possibly due to the complexity of the soil microenvironment. We determined *in vitro* the synthesis of lipopeptides, and that they are responsible for the antagonistic effect; however, the antifungal substances that strains produce in the soil still remains to be determined in a future study.

Seed health is one of the main factors influencing final seed quality of a crop and is determined by the presence or absence of pathogens. Pathogenic microorganisms associated with seeds generate diseased seedlings that may not survive in the field. In our study, we identified *Cladosporium* spp., *Rhizopus* spp. and *Fusarium* spp. in white bean cv. Alubia seeds; these results are consistent with those obtained by Persa et al. (2016) and Sabaté et al. (2017). In the present study, we detected *Fusarium* spp., which presented an incidence of 26 % in the *in vitro* trials. We demonstrated that seeds inoculated with B14, B19 or P12 suppressed all fungi identified in the control seeds. This result is extremely

important, mainly due to the presence of seed-borne *Fusarium* spp. in seeds, given that the fungi could be transmitted to the seedlings and cause significant economic losses (Persa et al., 2016). Similarly, although *S. sclerotiorum* did not grow in the studied seeds, it is imperative to recognize that because the fungus is a soil pathogen, the importance of control is not only to have a seed free of other pathogens, but also to control its incidence in the field.

In this study, shoot length increased 7.7 and 11.4 cm, and root length, 2.0 and 2.9 cm in B19 and P12, respectively, as a single inoculant. These increases were more effective than in the combined application and than in the fungicidal-seed treated. Both strains, B19 and P12, synthesize IAA (5.71 and 4.90 mg/mL, respectively). IAA production by microbes promoted root growth by directly stimulating plant cell elongation or cell division (Patten and Glick, 1996; Babalola, 2010). This metabolite would be responsible for the increase in growth of common bean caused by these strains. These results suggest that these strains are more effective than the fungicide seed treatment and could be used as potential agents promoting growth in the common bean, thus minimizing the use of chemicals. Moreover, these strains would synthesize siderophores, which act specifically as chelates that can 'sequester' iron and solubilize it, therefore supplying the plant with soluble iron and ultimately promoting its growth while also limiting the growth of phytopathogenic fungi and bacteria (Babalola, 2010).

The preliminary results obtained in this study suggest that the isolated bacteria *Bacillus* spp. B19 and P12 and *B. amyloliquefaciens* B14, in combination, have the potential to control white mold in the common bean used in our region as well as possessing potential PGPR properties.

5. Conclusion

The treatments B14+P12 or B14+B19 were the most effective combinations in reducing the incidence of *S. sclerotiorum*. Furthermore, B19 and P12 had beneficial properties on the growth of common bean cv. Alubia. Therefore, these strains or combined strains could be a potential growth promoter in commercial bean cultivation in the NWA region.

Acknowledgements

The authors would like to thank CIUNSa (C-2453; A-1974), CONICET (PIP 0072CO) and National Science and Technology Promotion Agency (ANPCyT) of Argentina for the financial support (PICT-2013-0634 and PICT 2012-0888) and the collaboration of the INTA PE-Suelos 1134043, directed by Dra. Silvina Vargas Gil. MC Audisio, DC Sabaté, R Erra-Basells and G Petroselli are Research Members of CONICET. The Ultraflex II (Bruker) TOF/TOF mass spectrometer was supported by a grant from ANPCYT, PME2003 No.125, CEQUIBIEM, DQB, FCEN, UBA.

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Figure captions

Fig. 1. Seeds of white bean cv. Alubia arranged at random on agar PDA.

a) Presence of contaminating fungi on non-inoculated white bean seeds.

b) Presence and development of *Bacillus* B19 inoculated in white bean seeds.

Fig. 2. Percentage of fungal inhibition (%FI) of *Bacillus* B14, B19 and P12 against different strains of *S. sclerotiorum* (identified as 15, 27, 33, 39, 48, 58, AN, PV and RF).

Fig. 3. Symptoms and lesions caused by *S. sclerotiorum* in white bean cv. Alubia seedlings in soils infected with the pathogen.

a) and b) Inoculated white bean seedlings showing symptoms in the stem.

c) Sclerotia of and seeds infected with *S. sclerotiorum*.

d) Comparison of seedlings inoculated with *Bacillus* B14+P12 and single inoculant (B14 or P12).

Fig. 1.

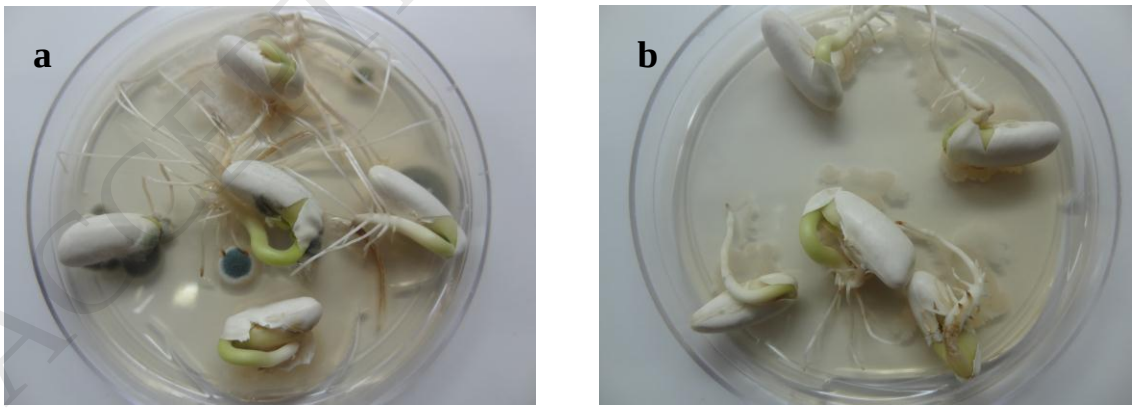

Fig. 2.

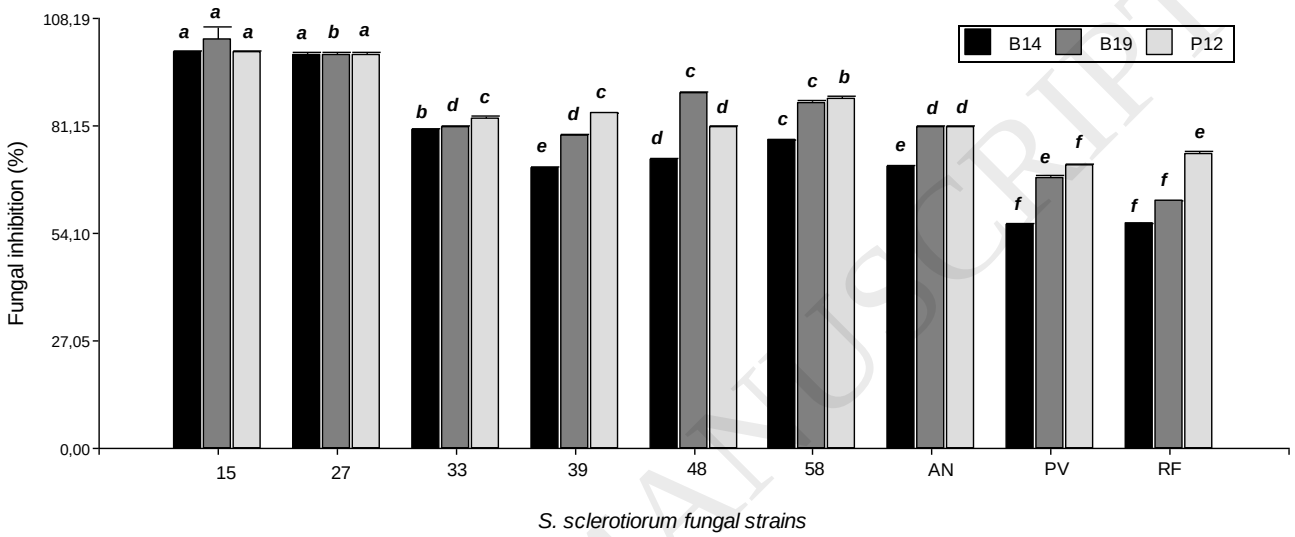

Fig. 3.

Table 1. Effect of *Bacillus* cell culture on common bean growth

Treatments	White bean cv Alubia seeds		
	GP (%)	Root length (cm)	Stem length (cm)
Control seeds	77.4^{c}	19.3±3.7^{bc}	38.2±3.6^{d}
Seeds inoculated with B19	88.6^{b}	21.3±2.0^{a}	45.9±3.5^{b}
Seeds inoculated with P12	88.6^{b}	22.2±1.8^{a}	49.6±3.0^{a}
Seeds inoculated with B14	70^{d}	18.0±2.0^{c}	34.3±2.5^{d}
Seeds inoculated with fungicide	90^{ab}	19.8±2.0^{ab}	42.7±2.7^{bc}
Seeds inoculated with B19 + B14	90^{ab}	20.28±1.0^{a}	41.3±2.0^{c}
Seeds inoculated with B19 + P12	94^{a}	20.1±1.0^{a}	45.4±1.5^{b}
Seeds inoculated with B14 + P12	92^{a}	19.6±1.30^{bc}	42.6±2.6^{bc}

Table 2. Lipopeptides synthesized by *Bacillus* B14, B19 and P12 against *S. sclerotiorum* characterized by MALDI-MS

Lipopeptide	Chemical formula	Calculated	*Bacillus* strain	*S. sclerotiorum*								
				15	**27**	**33**	**39**	**48**	**58**	**AN**	**PV**	**RF**
kurstakin	$[C_{40}H_{66}N_7O_{11}Na]^+$	915.48	B14	+	-	-	+	-	-	-	-	+
			B19	-	-	-	-	-	-	-	-	+
			P12	-	-	-	-	-	-	-	-	-
kurstakin	$[C_{42}H_{70}N_7O_{11}Na]^+$	943.51	B14	+	-	+	-	-	-	+	+	-
			B19	-	-	-	-	-	-	-	-	-
			P12	-	-	+	-	-	-	-	-	-
kurstakin	$[C_{43}H_{72}N_7O_{11}Na]^+$	957.52	B14	+	-	-	-	+	-	-	-	+
			B19	-	-	-	-	-	-	-	-	-
			P12	-	-	+	-	-	-	-	-	-
kurstakin	$[C_{44}H_{74}N_7O_{11}Na]^+$	971.54	B14	+	+	+	+	+	-	+	+	+
			B19	-	-	-	-	-	-	-	-	-
			P12	+	-	+	-	-	-	+	+	-
surfactin	$[C_{48}H_{82}N_7O_{13}Na]^+$	987.59	B14	+	+	-	+	+	-	-	-	-
			B19	-	-	-	-	-	-	-	-	-
			P12	-	-	-	-	+	+	+	+	-
surfactin	$[C_{49}H_{84}N_7O_{13}Na]^+$	1001.60	B14	+	+	+	+	+	+	+	+	+
			B19	-	-	-	-	-	-	-	-	-
			P12	+	+	+	+	+	+	+	+	+
surfactin	$[C_{50}H_{86}N_7O_{13}Na]^+$	1015.62	B14	+	+	+	+	+	+	+	+	+
			B19	+	-	-	-	-	-	+	+	+
			P12	+	+	+	+	+	+	+	+	+
surfactin	$[C_{51}H_{88}N_7O_{13}Na]^+$	1029.63	B14	+	+	+	+	-	+	+	-	-
			B19	+	-	-	+	+	-	+	+	+
			P12	+	+	+	+	+	+	+	+	+
surfactin	$[C_{53}H_{92}N_7O_{13}H]+$	1035.68	B14	+	+	+	+	+	+	+	+	+
			B19	-	+	-	+	-	+	+	+	+
			P12	+	-	+	+	+	+	-	+	+
surfactin	$[C_{52}H_{90}N_7O_{13}Na]^+$	1043.65	B14	+	+	+	+	+	+	+	+	+
			B19	+	+	+	+	+	+	+	+	+
			P12	+	+	+	+	+	+	+	+	+
iturin	$[C_{47}H_{71}N_{11}O_{15}Na]^+$	1052.50	B14	+	+	+	+	+	+	+	+	+

			B19	-	-	-	-	-	+	+	-	+
			P12	+	+	+	-	+	+	-	-	+
surfactin	$[C_{53}H_{92}N_7O_{13}Na]^+$	1057.66	B14	+	+	+	+	+	+	+	+	+
			B19	+	+	+	+	+	+	+	+	+
			P12	+	+	+	+	+	+	+	+	+
iturin	$[C_{48}H_{73}N_{11}O_{15}Na]^+$	1066.52	B14	-	-	-	-	+	-	-	+	-
			B19	+	-	-	-	-	-	-	+	-
			P12	-	-	-	-	+	-	-	-	+
surfactin	$[C_{53}H_{92}N_7O_{13}K]^+$	1073.64	B14	+	+	+	+	+	+	+	+	+
			B19	+	+	+	+	+	+	+	+	+
			P12	+	+	+	+	+	+	+	+	+
iturin	$[C_{49}H_{75}N_{11}O_{15}Na]^+$	1080.53	B14	+	+	+	+	+	+	+	+	-
			B19	+	-	-	+	+	+	+	+	+
			P12	-	-	+	+	+	+	+	+	+
surfactin	$[C_{54}H_{94}N_7O_{13}K]^+$	1087.65	B14	+	+	+	+	+	+	+	+	-
			B19	+	-	-	+	+	-	-	+	-
			P12	+	+	+	+	+	+	+	+	+
iturin	$[C_{49}H_{75}N_{11}O_{15}K]^+$	1096.51	B14	+	+	+	+	+	+	+	+	-
			B19	+	-	-	+	+	+	+	+	+
			P12	+	+	+	+	+	+	+	+	+
iturin	$[C_{52}H_{81}N_{11}O_{15}H]^+$	1100.60	B14	+	+	+	+	+	+	+	+	+
			B19	+	+	-	+	+	+	+	+	+
			P12	+	+	+	+	+	+	+	+	+
iturin	$[C_{53}H_{83}N_{11}O_{15}H]^+$	1114.61	B14	+	+	+	+	+	+	+	+	+
			B19	-	-	+	+	+	-	+	+	+
			P12	+	-	+	-	+	+	-	+	-
Polimyxin D1	$[C_{50}H_{93}N_{15}O_{15}H]^+$	1144.70	B14	-	-	-	+	-	+	-	-	-
			B19	-	-	+	+	-	-	-	-	-
			P12	-	-	-	-	-	-	+	-	-
Polimyxin D1	$[C_{50}H_{93}N_{15}O_{15}Na]^+$	1166.69	B14	+	+	+	+	+	+	+	+	-
			B19	-	-	-	-	-	-	-	-	-
			P12	+	+	-	-	-	-	-	+	-
Polimyxin D1	$[C_{50}H_{93}N_{15}O_{15}K]^+$	1182.66	B14	+	+	+	+	+	+	+	+	+
			B19	-	-	+	+	+	+	+	+	-
			P12	+	+	+	-	+	-	+	+	-
Polimyxin B1	$[C_{56}H_{98}N_{16}O_{13}H]^+$	1202.76	B14	-	+	-	+	+	-	+	-	-
			B19	-	-	-	-	-	-	-	+	-

			P12	-	-	-	-	-	-	-	-	-
Polimyxin B1	$[C_{56}H_{98}N_{16}O_{13}Na]^+$	1225.74	B14	-	+	-	+	-	+	-	-	-
			B19	-	-	-	+	-	-	-	-	-
			P12	-	-	+	+	+	-	-	+	-
fengycin	$[C_{73}H_{112}N_{12}O_{20}H]^+$	1477.82	B14	+	+	+	+	+	-	+	+	+
			B19	+	+	+	+	+	+	-	-	+
			P12	-	-	+	+	+	-	-	-	-
fengycin	$[C_{72}H_{110}N_{12}O_{20}Na]^+$	1485.78	B14	+	+	+	+	+	+	+	+	+
			B19	+	+	+	+	+	+	+	+	+
			P12	+	+	+	+	+	+	+	+	+
fengycin	$[C_{74}H_{114}N_{12}O_{20}H]^+$	1491.83	B14	+	+	+	+	+	+	+	-	+
			B19	+	+	+	+	+	+	+	-	+
			P12	-	-	+	+	+	+	-	+	-
fengycin	$[C_{73}H_{112}N_{12}O_{20}Na]^+$	1499.80	B14	+	+	+	+	+	+	+	+	+
			B19	+	+	+	+	+	+	+	+	+
			P12	+	+	+	+	+	+	+	+	+
fengycin	$[C_{75}H_{116}N_{12}O_{20}H]^+$	1505.85	B14	+	+	+	+	+	+	-	+	-
			B19	+	+	+	+	+	+	+	+	+
			P12	-	+	-	-	+	+	-	-	-
fengycin	$[C_{74}H_{114}N_{12}O_{20}Na]^+$	1513.82	B14	+	+	-	+	+	+	+	+	+
			B19	+	+	+	+	-	+	+	+	+
			P12	+	+	+	+	+	+	+	+	+
fengycin	$[C_{73}H_{112}N_{12}O_{20}K]^+$	1515.77	B14	+	+	+	+	+	+	+	+	+
			B19	-	+	+	+	-	-	+	+	+
			P12	+	+	+	+	+	+	+	+	+
fengycin	$[C_{74}H_{114}N_{12}O_{20}K]^+$	1529.79	B14	+	+	+	+	+	+	+	+	+
			B19	+	+	+	+	+	+	+	+	+
			P12	+	+	+	+	+	+	+	+	+
fengycin	$[C_{75}H_{116}N_{12}O_{20}K]^+$	1543.80	B14	+	+	+	+	+	+	+	+	+
			B19	+	+	+	+	+	+	+	+	+
			P12	+	+	+	+	+	+	+	+	+
fengycin	$[C_{76}H_{118}N_{12}O_{20}K]^+$	1557.82	B14	+	+	+	-	+	-	+	+	-
			B19	+	-	+	-	-	-	+	-	-
			P12	-	-	-	-	-	-	-	-	-
fengycin	$[C_{80}H_{127}N_{12}O_{20}H]^+$	1575.93	B14	+	+	+	-	+	-	-	+	-
			B19	+	-	+	+	+	+	+	+	+
			P12	-	-	-	-	-	-	-	-	-

fengycin	$[C_{81}H_{129}N_{12}O_{20}H]^+$	1590.95	B14	-	+	+	-	-	-	-	-	-
			B19	+	-	+	-	+	-	-	-	-
			P12	-	-	-	-	-	-	-	-	-

Table 3. Effect of different *Bacillus* treatments on the white mold of common bean caused by *S. sclerotiorum*

	GE (%)	After 15 days			After 30 days			After 45 days			AUDPC
		LP	HP%	I%	LP	HP%	I%	LP	HP%	I%	
Control	7.20^{c}	5^{c}	20^{d}	80^{b}	1^{c}	20^{d}	80^{b}	0^{d}	0^{c}	100^{a}	2550^{a}
Comercial Fungicide	0^{d}	0^{d}	0^{e}	100^{a}	0^{c}	0^{e}	100^{a}	0^{d}	0^{c}	100^{a}	3000^{a}
B14	14.3^{b}	10^{b}	40^{c}	60^{c}	0^{c}	0^{e}	100^{a}	0^{d}	0^{c}	100^{a}	2700^{a}
B19	30.0^{a}	21^{a}	48^{b}	52^{c}	6^{b}	28.5^{c}	71.5^{b}	5^{b}	23.8^{b}	76.2^{b}	2034^{b}
P12	5.70^{c}	4^{c}	50^{b}	50^{c}	2^{c}	50^{b}	50^{c}	1^{c}	25^{b}	75^{b}	1687.5^{c}
B14+P12	34.3^{a}	24^{a}	87.5^{a}	12.5^{e}	18^{a}	75^{a}	25^{d}	11^{a}	46^{a}	54^{c}	873.75^{d}
B14+B19	10.0^{b}	7^{bc}	71.4^{a}	28.6^{d}	5^{b}	71.4^{a}	28.6^{d}	3^{b}	43^{a}	57^{c}	1071^{cd}
B19+P12	5.70^{c}	4^{c}	50^{b}	50^{c}	1^{c}	25^{c}	75^{c}	1^{c}	25^{b}	75^{b}	2062.5^{b}

GE=germinative energy
LP=number of living plants
HP%= Healthy plants percentage
I%= incidence pathogen percentage