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Biological activity of the lipopeptide-producing *Bacillus amyloliquefaciens* PGPBacCA1 on common bean *Phaseolus vulgaris* L. pathogens

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HIGHLIGHTS

- *B. amyloliquefaciens* PGPBacCA1 inhibited endophytic and soil-borne fungi of common beans.
- Surfactins, iturins and fengycins were responsible for the fungi antagonism.
- Lipopeptides lethally injured key structures of *Sclerotinia* and *Fusarium* strains.
- Bacilli cells did not affect the potential germination of either type of bean.
- Common bean seeds cv. Alubia and cv. Nag 12 were protected by PGPBacCA1.

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ABSTRACT

Bacillus amyloliquefaciens PGPBacCA1 was studied regarding its aptitude to protect common bean seeds from their intrinsic pathogens. Also, the inhibition of different environmental phytopathogenic fungi was tested. Two cultivars of *Phaseolus vulgaris* L. were evaluated: cv. Nag (black bean) and cv. Alubia (white bean). *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. constituted the natural fungal biota of both seeds, whereas white bean and black bean also exhibited *Cladosporium* spp. and *Rhizopus* spp., respectively. *B. amyloliquefaciens* PGPBacCA1 prevented the development of the endophytic fungi of black bean, while only *Cladosporium* spp. survived in the white variety. Growth chamber assays were carried out and bacilli cells were applied on seeds without affecting neither the vigor nor the germination potential of either type of bean. In addition, *B. amyloliquefaciens* PGPBacCA1, by dual cultures, was able to inhibit the development of the following phytopathogenic fungi: *Sclerotium rolfsii* (35%), *Sclerotinia sclerotiorum* (76.5%), *Rhizoctonia solani* (73%), *Fusarium solani* (56.5%), and *Penicillium* spp. (71.5%). The UV-MALDI TOF MS analysis showed that *B. amyloliquefaciens* PGPBacCA1 co-produces different homologues of the lipopeptides surfactin, iturin and fengycin in the presence of *S. sclerotiorum* and *F. solani*. These compounds were identified as the main responsible for the antagonistic effect. SEM analysis confirmed the antifungal effects of the lipopeptides, which also caused damage to chlamydospores and sclerotia of *Fusarium* and *Sclerotinia*, respectively. *B. amyloliquefaciens* PGPBacCA1 can thus be applied to these bean seeds varieties as a potential bioprotection agent.

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1. Introduction

The cultivation of *Phaseolus vulgaris* L., known as common bean, has been a traditional activity in the northwestern region of Argentina since the early 20th century. This crop is of great economic importance in the region and its final destination is the export market, Argentina being the leading exporter of white bean variety. Its production is concentrated mainly in the provinces of Salta, Tucumán and Jujuy, where the most widely cultivated varieties are white bean and black Nag Bean type 12 (García Medina, 2002). In the crop cycle of 2013–2014, 242.920 Tn were exported (90% of total production) which represented around USD 278,272,000.

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Different bacterial and fungal pathogens affect the growth of beans, causing significant economic losses. That situation has led to the indiscriminate use of chemical pesticides, which also have a strong environmental impact because they generate resistance in pathogenic microorganisms and contaminate the environment. Therefore, there is a growing interest in searching for alternatives to control crop diseases. The use of beneficial microorganisms, such as plant growth-promoting rhizobacteria (PGPR), is considered one of the most promising tools for applying safer, more rational and eco-friendly crop management practices (Droby et al., 2009). PGPR have also several advantages because only low concentrations of them are required to be effective; they grow easily and can be used for phytopathogenic control (Choudhary and Johri, 2009; Francis et al., 2010; Gray and Smith, 2005).

Members of the genus *Bacillus* are widely used in agriculture due to their potential properties as PGPR, mainly as biocontrol agents (Babalola, 2010; Berg, 2009; Ludueña et al., 2012; Lugtenberg and Kamilova, 2009; Pérez-García et al., 2011). Due to their versatility as bioactive-compound producers, different *Bacillus* species are being studied for several potential applications. For example, many bacilli strains produce a variety of antifungal cyclic lipopeptides (CLPs), including members of the surfactin, iturin, and fengycin families, and it has been suggested that these CLPs are mainly responsible for plant-disease reduction following treatment with *Bacillus* strains (Torres et al., 2016). In particular, phytopathogenic fungi are one of the major threats for crops and plant production. Hence, the control of fungal diseases by bacilli would represent a relevant opportunity for agricultural biotechnology.

In a previous study, two *Bacillus* strains, *B. amyloliquefaciens* PGPBacCA1 and *B. subtilis* subsp. *subtilis* PGPMori7 showed a significant ability to inhibit *Macrophomina phaseolina* (Tassi) Goid, the causal agent of charcoal root rot in common bean and soybean, by at least two different mechanisms: lipopeptide synthesis and competition between microorganisms. In particular, *B. amyloliquefaciens* PGPBacCA1 exerted a fungicidal effect by producing lethal damage on its reproductive structures (Torres et al., 2016). Thus, the aim of this study was to evaluate the ability of *B. amyloliquefaciens* PGPBacCA1 to protect seeds from their natural seedborne pathogens as well as to inhibit *Fusarium solani* and *Sclerotinia sclerotiorum* Sacc, relevant soilborne phytopathogenic fungi. In addition, the chemical nature of metabolites responsible for antifungal activity and the changes in the morphology of theses fungi due to antifungal effect were evaluated.

2. Materials and methods

2.1. Microorganisms and culture conditions

Bacillus amyloliquefaciens PGPBacCA1 (GenBank access code JX120520) isolated from an air sample (Torres et al., 2016), was studied in the Laboratory of Applied Bacteriology of the Research Institute for Chemical Industry (INIQUI). This strain was cultured in Mueller Hinton broth (MH, Britania, Argentina) at 37 °C during 24 h, without agitation.

Strains of *Sclerotium rolfsii* (Sac 2 and Sac 3), *Sclerotinia sclerotiorum* (Acceso Norte and Puesto Viejo), *Rhizoctonia solani* (15), *Fusarium solani* (1 and 2) and *Penicillium* spp. were obtained from a stock culture of the Laboratory of Soil Microbiology (National Institute of Agricultural Technology INTA-Salta- Cerrillos, Argentina), and grown on potato-dextrose-agar (PDA, Britania, Argentina) at 28 °C for 7 days. All the fungal strains were isolated from roots of soybean and common bean from the Salta province following the protocol of Pérez Brandán (2009).

2.2. Cell suspension of PGPBacCA1 for the biological assays

To obtain the cell suspension (CS), 5 mL of Mueller Hinton broth was inoculated with a pure culture of the bacterial strain in a percentage of 1% (v/v) and incubated at 37 °C for 24 h. After this period, concentrations of about 1×10^7 cells per mL were obtained. The number of cells was determined by the counts of serial dilutions in 0.1% (w/v) meat peptone on MH agar plates.

2.3. Impact of cell culture of PGPBacCA1 on white and black common beans

The natural microbiota of white common bean cv. Alubia and black common bean cv. Nag 12 seeds was analyzed, and the impact of bacilli cells was also evaluated. Seeds were initially sterilized in 95% alcohol for 30 s and then in 1% sodium hypochlorite solution for 1 min. After this treatment, seeds were inoculated with the CS of PGPBacCA1 at a concentration of 1×10^7 cells per mL and kept in contact with it for 1 h. Non-inoculated seeds were used as control. Then, treated and control seeds were placed in Petri dishes (9 cm in diameter) containing PDA; five bean seeds per Petri dish (five seed treated and five seed control) were placed equidistant from one another and the dishes were then incubated in an heater at 26 °C for 10 days. Each treatment was done in quintuplicate. After the incubation period, the presence or absence of seedborne pathogenic microorganisms and others microorganism's contaminants in seeds was determined.

2.4. Greenhouse experiment

Seeds of white common bean cv. Alubia and black common bean cv. Nag 12 were disinfected as explained above. A total of 70 seeds of each bean variety were inoculated with CS of PGPBacCA1 at a concentration of 1×10^7 cells per mL. Then, these treated seeds and 70 non-inoculated seeds (control) were sowed at a depth of 2 cm in plastic trays containing sterile fertile-soil (loam soil with 2.91% of organic matter, 0.17% total nitrogen, pH 6.9, 32% sand, 44% silt and 24% clay, typic ustorthents according to USDA Soil Taxonomy) as substrate.

The trays were placed in a growth chamber with air circulation for germination and temperature control (28 °C ± 2) for 15 days, according to Pérez Brandán (2009). The vigor, or germination, potential effect of PGPBacCA1 on seeds of each bean variety was analyzed considering the following parameters: i) percentage of emerged seedlings (emergence); ii) plant height (cm), and iii) dry matter of seedlings (% dry matter), following Pérez Brandán et al., 2005. Theses parameters were evaluated as indicators of plant growth promotion (GP) in seeds of white bean cv. Alubia and black bean cv. Nag 12, after treatment with the strain PGPBacCA1.

2.5. Study of fungal phytopathogens inhibition by dual culture assays

The inhibitory activity of the CS of the PGPBacCA1 was evaluated by the dual culture technique, against the following phytopathogenic fungi: *Penicillium* spp., *Fusarium solani* 1 and 2, *Sclerotium rolfsii* Sac 2 and Sac3, *Sclerotinia sclerotiorum* Acceso Norte and Puesto Viejo, and *Rhizoctonia solani* N° 15. The trials were performed according to Torres et al. (2016). Briefly, 25 μL of the CS was inoculated in a well-made in 9 cm-diameter Petri dishes containing DPA medium. In the center of each dish were placed plugs of 4 mm, taken from the leading edge of a 7 day-old culture of each fungal strain. Plates without bacterial samples were used as controls. After 7 days of incubation at 28 °C, the mycelial growth diameter of each phytopathogen was measured and the

percentage of fungal inhibition (FI) was calculated according to Landa et al. (1997):

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

Where RGI is radial growth inhibition, **T** is the average diameter of the mycelial growth in the presence of the CS, and **C** is the average diameter of the mycelial growth in the absence of CS.

2.6. UV-MALDI TOF analysis to identify the lipopeptides involved in the antifungal activities

Lipopeptide synthesis by *B. amyloliquefaciens* PGPBacCA1 was analyzed on the PDA medium with two fungal strains: *F. solani* 2 and *S. sclerotiorum* Puesto Viejo, after 7 days of incubation at 28 °C. The interaction of PGPBacCA1 with each fungus produced an inhibition zone of a large diameter; a sample was extracted from the area surrounding the bacterial colony (ZBC) and another one from the area surrounding the fungus mycelium (ZFM). A portion of 4 mm (sample) was removed and extracted from each zone and resuspended in 0.5 mL of acetonitrile. Finally, each sample was vigorously shaken for 30 s and kept at −18 °C. The samples were analyzed by UV - MALDI TOF as described by Torres et al. (2016).

2.7. Scanning electron microscopy (SEM) evaluation of control and treated fungal

For the SEM analyses, different portions of the fungal mycelia were taken from the Petri dishes where either *F. solani* 2 and *S. sclerotiorum* Puesto Viejo had been faced against *B. amyloliquefaciens* PGPBacCA1 cells in independent trials. As control of normal growth, mycelium fractions were recovered from Petri dishes where the fungi had grown without bacterial cells. Then, the samples were studied according to Torres et al. (2016). SEM analyses were carried out with a Joel JMS 6480 LV computer), at the LASEM laboratory – INIQUI (Laboratory of Electron Microscopy and Microanalysis).

2.8. Evaluation of the mode of action of PGPBacCA1 on F. solani 2 and S. sclerotiorum Puesto Viejo

To determine if bacilli cells produced a fungicidal or fungistatic action, samples from the fungal inhibition assays were recovered and studied according to Torres et al. (2016).

2.9. Statistical analysis

A descriptive statistical analysis of the antifungal activity was performed; results were indicated as the average of the determined values ± standard deviation (SD). The parameters indicating GP were analyzed via the non-parametric Kruskal Wallis test ($p < 0.05$) using InfoStat statistical software version 2012.

3. Results

3.1. Impact of cell culture of PGPBacCA1 on the natural mycobiota of white and black common bean seeds

After treatment with PGPBacCA1, only *Cladosporium* spp. survived on white bean seeds, while on black bean, no fungal development was detected (Fig. 1 A and B) (see Fig. S1, Supplementary material). In contrast, control seeds of each bean variety presented the following fungi: *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp. and *Cladosporium* spp. in white beans; while, *Rhizopus* spp., *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp. and *Rhizoctonia* spp. grew from black beans.

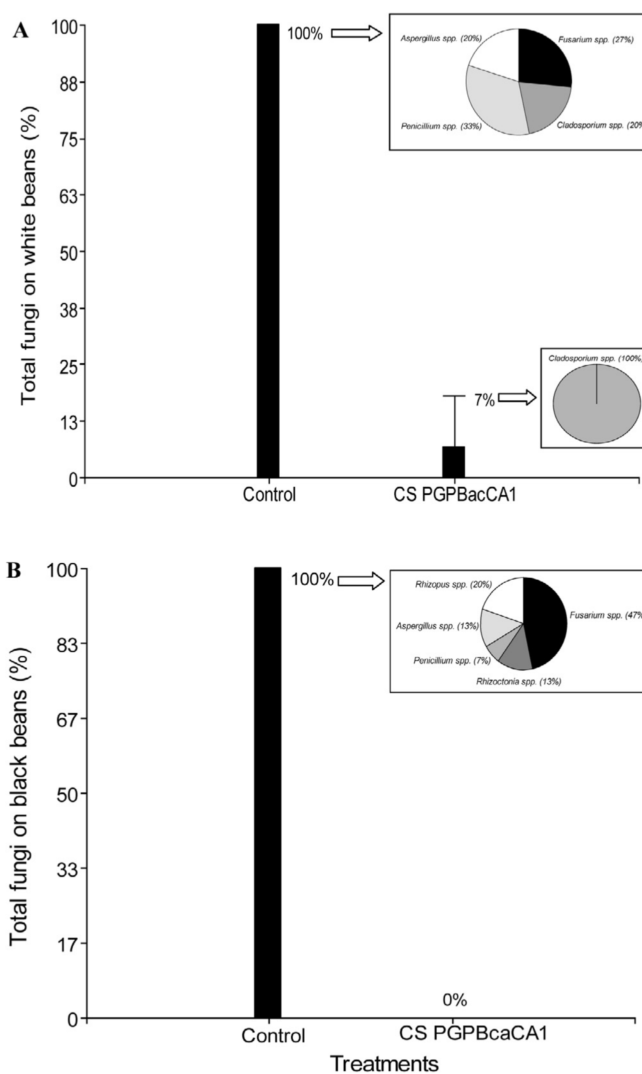


Fig. 1. Phytosanitary status of common bean seeds after application with the cell suspension (CS) *B. amyloliquefaciens* PGPBacCA1. (A) Total fungi on white bean (%), (B) Total fungi on black bean (%).

3.2. Greenhouse experiment

The cells of PGPBacCA1 did not have a significant influence on the germination capacity of either bean variety (Table 1).

Black bean seedlings treated with PGPBacCA1 showed significant differences respect to the control in both parts aerial and root parts of the seedlings (Fig. 2A). Besides, the root part was positively favored by PGPBacCA1 cells (11.90%) respect to the control (1.02%), whereas differences in aerial parts respect to control not were as significant as that observed in roots parts (7.87% and 5.70%, respectively) (Fig. 2A).Unlike the results obtained with black beans, there were no significant differences in the dry matter of white beans plants treated with PGPBacCA1 and controls, neither aerial (6.70% and 6.67%) nor radicular (4.23% and 5.40%)

3.3. Antifungal activity

B. amyloliquefaciens PGPBacCA1 exhibited antagonistic behavior against the eight fungal strains evaluated. *Penicillium* spp., *R. solani* 15 and *S. sclerotiorum* Puesto Viejo were the most sensitive to the bacterial cell suspension (71.5, 73.0 and 76.5%, respectively).

Table 1
Effects of *B. amyloliquefaciens* PGPBacCA1 on the emergence and plant height of two varieties (white and black) of common bean.

Treatments	Growth parameters	Common bean variety	
		WB	BB
Control	Emergence (%)	73.67 ± 5.13 a	71.33 ± 0.58 a
PGPBacCA1		65.33 ± 3.10 a	70.67 ± 0.58 a
	P[2]	0.0751	0.100
Control	Plant height (cm)	9.36 ± 0.61 a	9.92 ± 1.25 a
PGPBacCA1		8.29 ± 0.20 a	10.15 ± 0.32 a
	P[2]	0.100	0.700

WB: White common bean cv. Alubia. BB: Black common vean cv. Nag 12.
Different letters indicate significant differences according to Kruskal Wallis test (P < 0.05).

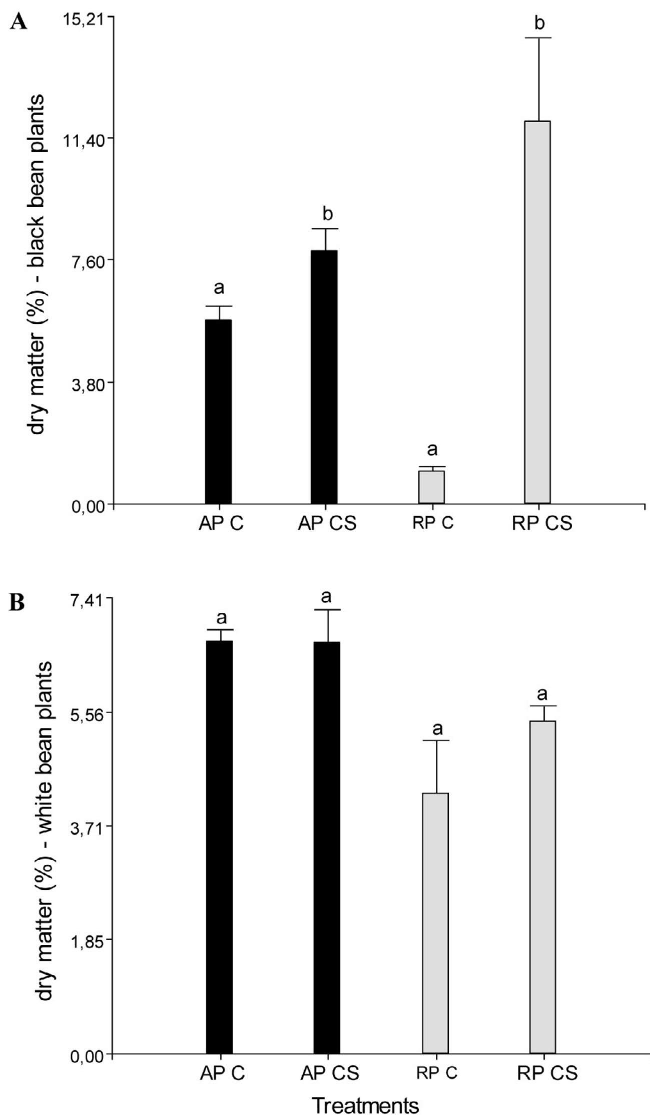


Fig. 2. Dry matter of aerial parts (AP) and radicular parts (RP) of black (A) and white (B) bean plants, which grew with and without the presence of the bacterial cell suspension (CS) of *B. amyloliquefaciens* PGPBacCA1. Different letters indicate significant differences according to Kruskal Wallis test (P<0.05). Black bean plants: of AP, P = 0.012; of RP, P = 0.002 and White bean plants: of AP, P = 0.926; of RP, P = 0.089.

Whereas, both *F. solani* strains were affected more than a 50% (Table 2).

3.4. Study of lipopeptides involved in the antifungal activity by UV MALDI TOF MS

The MS analysis of the lipopeptides synthesized by PGPBacCA1 in solid medium against *F. solani* 2 and *S. sclerotiorum* Puesto Viejo showed several signals in both inhibition zones (ZBC and ZFM) as shown in Figs. S2–S5 (see Supplementary material), respectively. The signals observed were identified as surfactin, iturin and fengycin homologues (Table 3). Some homologues were detected simultaneously as protonated $[M+H]^+$ and/or sodiated $[M+Na]^+$ and/or potassiated $[M+K]^+$ adduct.

A similar number of homologues for surfactin in the ZBC and the ZFM zone were detected in solid medium against *F. solani* 2. Moreover, the signals correlated with the lipopeptides presents in both samples showed similar absolute intensity (Fig. S2). Four surfactin homologues, two homologues of iturin (Fig. S2 and Table 3) and four fengycin homologues (Fig. S3 and Table 3) were detected in both regions under study. Fig. S4 shows homologues of surfactin and iturin produced by PGPBacCA1 in solid medium against *S. sclerotiorum*. No significant differences were observed between ZBC and ZFM samples. Four homologues of surfactin were detected as protonated and/or sodiated and/or potassiated adduct and together with three homologues of iturin (Fig. S4) and six of fengycin (Fig. S5 and Table 3). These samples also showed that the number of homologues of fengycin detected and the intensity of the signals were similar between ZBC and ZFM samples (Fig. S5). In general, these results revealed an efficient diffusion of the lipopeptides synthesized by PGPBacCA1 into the agar medium.

3.5. Scanning electron microscopy (SEM) evaluation

SEM registers clearly showed the suppression of mycelial growth of *S. sclerotiorum* Puesto Viejo by PGPBacCA1 (Fig. 3 I, D). Degenerative changes in the morphology of the fungus hyphae were also identified; with hyphae appearing wrinkled and crushed (Fig. 3 I, E). Moreover, the surface of recent sclerotia formation appeared desiccated; showing lysis of the inner cortex of globular cells (Fig. 3 I, F). This pattern was particular if we compare it with the control fungus growth, which only showed a thick and long mycelium (Fig. 3 I, A-B-C).

The SEM observation of the control sample of *F. solani* 2 allowed us to identify hyaline, elongated and septate hyphae (Fig. 3 II, A). Besides, phialides (Fig. 3 II, B) and microconida were observed at the end of hyphae (Fig. 3 II, C). By contrast, the sample treated with PGPBacCA1 exhibited an abundant and extensive formation of chlamydospores concentrated at the intersection of the fungus and the bacterial inhibition zone (Fig. 3 II, D–E). In addition, chlamydospores appeared in chains around and entering the bacterium-fungus inhibition zone (Fig. 3 II, D); in some cases, those structures were depressed and collapsed (Fig. 3 II, E-F).

Table 2
Antifungal activity of *B. amyloliquefaciens* PGPBacCA1 against different phytopathogenic fungi strains.

Phytopathogenic fungi	Antifungal activity of PGPBacCA1 CS[a] (%)
Penicillium spp.	71.5 ± 0.6[b]
R. solani N° 15	73.0 ± 0.9
F. solani 1	54.0 ± 4.2
F. solani 2	56.5 ± 3.5
S. rolfsii Sac 2	29.0 ± 5.6
S. rolfsii Sac3	35.5 ± 3.5
S. sclerotiorum Acceso Norte	36.5 ± 9.2
S. sclerotiorum Puesto Viejo	76.5 ± 2.2

[a] CS: Cell suspension of PGPBacCA1 at concentrations of 1×10^7 cells per mL.
[b] Percentage of fungal inhibition ± standard deviation.

Table 3
Lipopeptides produced by *B. amyloliquefaciens* PGPBacCA1. ZBC: zone near bacterial colony; ZFM: zone near fungal mycelium of *F solani* or *S. sclerotiorum* Puesto Viejo; Matrix: nHo; nd: no detected.

Lipopeptides	Chemical formula	Calculated	Experimental			
			F solani		*S. sclerotiorum*	
			ZBC	ZFM	ZBC	ZFM
Surfactin	$[C_{50}H_{86}N_7O_{13}Na]^+$	1015.62	1016.70	nd	nd	nd
Surfactin	$[C_{52}H_{90}N_7O_{13}H]^+$	1021.67	1021.76	1021.67	1021.79	nd
Surfactin	$[C_{51}H_{88}N_7O_{13}Na]^+$	1029.63	1029.67	1030.73	1029.73	1030.82
Surfactin	$[C_{53}H_{92}N_7O_{13}H]^+$	1035.28	nd	nd	1035.81	1035.58
Surfactin	$[C_{52}H_{90}N_7O_{13}Na]^+$	1043.65	1043.67	1043.67	1043.69	1043.71
Surfactin	$[C_{53}H_{92}N_7O_{13}Na]^+$	1057.66	1057.66	1057.69	1057.69	1057.70
Iturin	$[C_{48}H_{74}N_{11}O_{15}Na]^+$	1067.53	1065.67	nd	1066.64	1066.69
Iturin	$[C_{49}H_{76}N_{11}O_{15}Na]^+$	1081.54	1081.62	nd	1081.80	1081.73
Surfactin	$[C_{53}H_{92}N_7O_{13}K]^+$	1073.64	1073.65	1073.76	1073.72	1073.71
Surfactin	$[C_{54}H_{94}N_7O_{13}K]^+$	1087.65	1087.59	1087.74	1087.71	1087.59
Iturin	$[C_{49}H_{76}N_{11}O_{15}K]^+$	1097.51	1098.74	1098.80	1098.72	1098.77
Iturin	$[C_{52}H_{82}N_{11}O_{15}H]^+$	1101.61	1102.72	1102.89	1102.82	1102.72
Not assigned			nd	1112.65	1113.57	nd
Not assigned			1134.74	1134.75	nd	1134.92
Fengycin	$[C_{70}H_{106}N_{12}O_{20}H]^+$	1435.77	nd	nd	1435.55	1435.46
Fengycin	$[C_{71}H_{108}N_{12}O_{20}H]^+$	1449.78	nd	1449.51	1451.63	1449.47
Fengycin	$[C_{72}H_{110}N_{12}O_{20}H]^+$	1463.80	1463.44	1463.60	1463.47	1463.39
Fengycin	$[C_{71}H_{108}N_{12}O_{20}Na]^+$	1471.77	1471.42	nd	1472.45	1472.47
Fengycin	$[C_{73}H_{112}N_{12}O_{20}H]^+$	1477.82	1477.30	1477.50	1477.45	1477.37
Fengycin	$[C_{72}H_{110}N_{12}O_{20}Na]^+$	1485.78	1485.39	1485.46	1485.34	1485.37
Fengycin	$[C_{74}H_{114}N_{12}O_{20}H]^+$	1491.83	1491.36	1491.39	1491.53	1491.61
Fengycin	$[C_{73}H_{112}N_{12}O_{20}Na]^+$	1499.80	1501.23	1501.38	1499.56	1500.37
Fengycin	$[C_{75}H_{116}N_{12}O_{20}H]^+$	1505.85	1505.59	1505.72	1505.48	1505.40
Fengycin	$[C_{74}H_{114}N_{12}O_{20}Na]^+$	1513.82	1514.33	1515.40	1513.44	1514.41
Fengycin	$[C_{75}H_{116}N_{12}O_{20}Na]^+$	1527.83	1528.30	1529.44	1527.32	1527.42
Not assigned			1543.29	1543.79	1543.42	1544.33
Not assigned			nd	nd	1558.33	1558.41

3.6. Impact of B. amyloliquefaciens on the viability of S. sclerotiorum and F. solani

The reactivation in fresh medium of the mycelium of *S. sclerotiorun* Puesto Viejo and *F. solani* 2, from the area adjacent to the inhibition zone produced by PGPBacCA1 revealed the fungicidal action of the lipopeptides synthesized by this bacterium against both fungal strains. Particularly, *S. sclerotiorum* did not show any mycelial development, whereas *F. solani* 2 exhibited minimum growth with respect to its control (see Fig. S6, Supplementary material).

4. Discussion

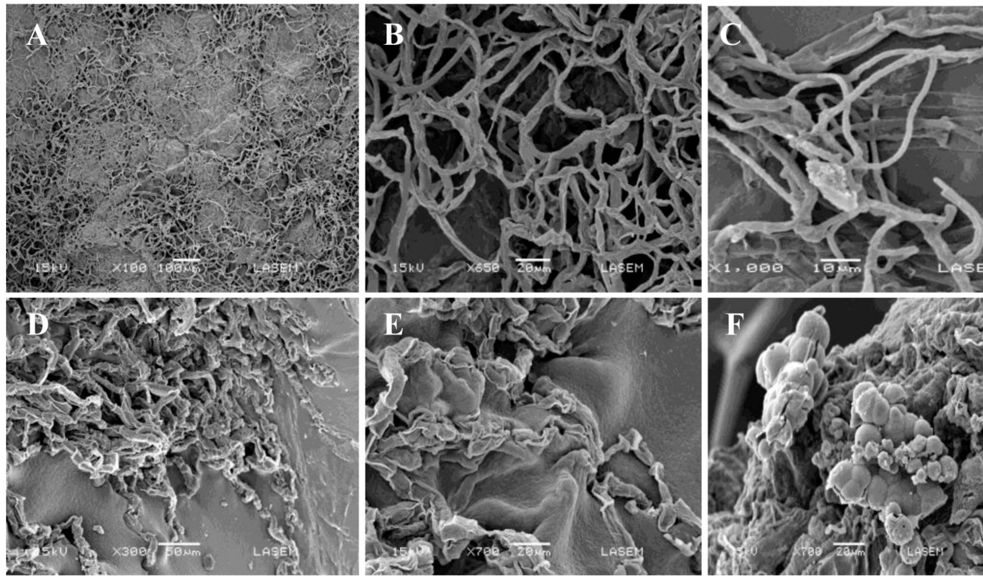
Several *Bacillus amyloliquefaciens* strains have been widely studied and used in agriculture as plant growth promoters due to its inhibitory effects against different phytopathogenic microorganisms (Babalola, 2010; Cawoy et al., 2014; Chowdhury et al., 2015; Pérez-García et al., 2011; Wu et al., 2015). *B. amyloliquefaciens* PGPBacCA1 was isolated in our laboratory and selected for its antifungal action on several *Macrophomina phaseolina* strains, and for its capacity to co-produce lipopeptides of the families of surfactin, iturin and fengycin (Torres et al., 2015, 2016).

In this work, we evaluated its potential as an antagonist against soilborne and seedborne pathogenic fungi. Interestingly, each type of common-bean cultivar studied had a different natural fungal microbiota. Alubia beans have the major variability of fungi, and *Cladosporium* spp. strains were the most resistant. In turn, all the fungi detected in control Nag12 beans disappeared after treatment with the bacilli cells. It is not known *apriori* if the fungi inherent to common beans will grow or not when the seeds are in the soil environment. However, if a simple treatment, such as the one shown in this work, succeeds at inhibiting most of them, then a healthy seed should germinate.

The increase in dry matter observed in root and aerial parts of Nag 12 suggests that PGPBacCA1 can act as a growth promotor agent. Moreover, this work generated information related to the associated mycobiota of the seeds, and interestingly, only few authors have reported similar assays (Barret et al., 2015; Parsa et al., 2016).

Considering the impact of *B. amyloliquefaciens* PGPBacCA1 on fungal and the prior results obtained compared to *M. phaseolina*, in this work we studied the potential of this strain against several soilborned fungi more in depth. *B. amyloliquefaciens* PGPBacCA1 had greater capacity to inhibit *S. sclerotiorum* (76.5% than *F. solani* (56.6%). As well, MALDI TOF analysis revealed that the inhibition of both fungi, of different origins (i.e., seedborne and soilborne), by PGPBacCA1 was mainly related to the co-production of surfactin, iturin and fengycin. Several scientific studies reported that different *B. amyloliquefaciens* strains co-produces the lipopeptides iturin and/or fengycin, and that these are responsible for the antagonistic effect compared to phytopathogenic fungi, such as *S. sclerotiorum*, *R. solani*, and *F. solani* (Alvarez et al., 2011; Arrebola et al., 2010; Cawoy et al., 2014; Li et al., 2014; Souto et al., 2004). Nevertheless, few reports have identified the homologues of each family of the lipopeptides involved in the biological actions. The lipopeptides iturin and fengycin possess antifungal activity, whereas surfactin has mainly antibacterial activity (Ongena and Jacques, 2008). Surfactin also has the capacity to exert a synergistic effect on the activity of iturin (Thimon et al., 1992) or fengycin (Koumoutsi et al., 2004). In this study, we determined not only the co-production of these three lipopeptides, but also the synthesis, in the presence of phytopathogenic fungi, of the different homologues of each family. By using mass spectrometry MALDI-TOF, Cawoy et al. (2014) determined that *Bacillus* strains co-producing the three lipopeptides families can efficiently inhibit a major type of pathogens (i.e. *Cladosporium*, *Botrytis*, *Fusarium* and *Pythium*), compared with those strains that do not produce iturin or are not able to synthesize any lipopeptide at all. On the other hand, these

I) *Sclerotinia sclerotiorum*



II) *Fusarium solani*

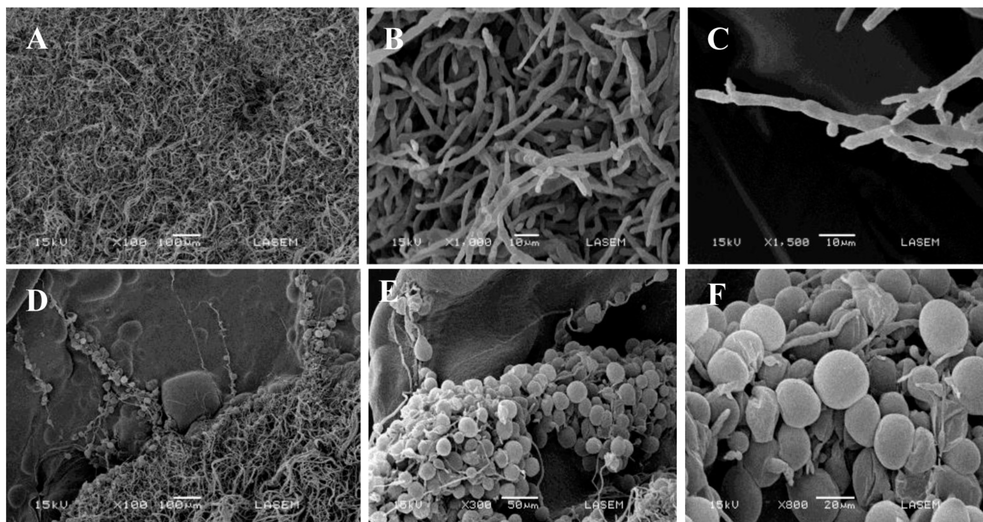


Fig. 3. SEM microphotographs of *S. sclerotiorum* Puesto Viejo (I) and *F. solani* 2 (II) growth without the presence of antagonistic bacteria (control: A, B, C) and faced with *B. amyloliquefaciens* PGPBacCA1 (D, E and F). The figures show: suppression of mycelial growth of *S. sclerotiorum* and *F. solani* (I and II D), degenerative changes in the morphology of the fungus hyphae (I E) and surface of sclerotia desiccated (I F), chlamydospores in chains around and entering the bacterium-fungus inhibition zone (II E) and in some cases, those structures appeared depressed and collapsed (II F).

authors reported that *B. amyloliquefaciens* 98S synthesized different homologues of surfactin, iturin and fengycin, depending on the nature of the pathogenic fungus present. These latter data did not agree with our results: *B. amyloliquefaciens* PGPBacCA1 produces a similar number of homologues of each family of lipopeptides whatever the nature of the fungus it interacts with (i.e., this work and Torres et al., 2016).

Cyclic lipopeptides are known to produce mainly cell-damage by their interaction with the cell membranes, disrupting their integrity (Aranda et al., 2005; Bernheimer and Avigad, 1970; Deleu et al., 2005). This effect mainly depends on both the chemical nature of the lipopeptide and its concentration (Grau et al., 1999; Liu et al., 2014; Peypoux et al., 1999; Sheppard et al., 1991). Different scientific research showed that several *B. amyloliquefaciens* strains can produce damage to the hyphae and survival structures of phytopathogenic fungi, when they come into contact with a solution containing the lipopeptides produced by the bacteria (Alvarez et al., 2011; Souto et al., 2004). The effects of the lipopeptides produced by PGPBacCA1 on the morphology of *S. sclerotiorum* Puesto Viejo and *F. solani* 2 were evaluated in solid media. SEM studies showed changes in hypha and sclerotia morphology of *S. sclerotiorum*; whereas in *F. solani*, an extensive formation of chlamydospores, concentrated in the intersection of the fungus-bacterial inhibition zone and with some injuries, was observed. The type of damage detected in our research coincides with those reported by others authors and confirmed the antifungal effects on

the survival structures of *F. solani* and the presence of deformed formation of sclerotia in *S. sclerotiorum* (Basurto-Cadena et al., 2010). When the reactivation of the treated mycelia of both, *S. sclerotiorun* Puesto Viejo and *F. solani* 2, were analyzed on fresh medium, a fungicidal action of the lipopeptides synthesized by PGPBacCA1 was determined. In particular, *F. solani* 2 exhibited minimum growth, which could also be related to high formation of chlamydospores and conidias showing that these are highly resistant structures resilient to perturbations in their environment. *S. sclerotiorum* did not grow, indicating a greater damage that must be studied in particular. In fact, a ready association and firm anchorage of the lipopeptides synthesized by PGPBacCA1 into lipid layers could have occurred and could thus interfere with biological membrane integrity, causing a destabilizing effect on fungal membranes.

Nowadays, eco-friendly alternatives are welcome in order to manage different crop pathogens or pests, by either being combined with, or directly replacing, the synthetic pesticides presently used. That is why, it becomes relevant to understand and study the dynamics of action of different agro-beneficial strains. *B. amyloliquefaciens* PGPBacCA1 has shown potential as fungicide in two varieties of common bean agriculture of northwestern Argentina and would be an eco-friendly contribution to this crop, which so far has not been addressed in a comprehensive manner.

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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.2016.12.001.

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