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Biocontrol activity of *Bacillus subtilis* EA-CB0015 cells and lipopeptides against postharvest fungal pathogens

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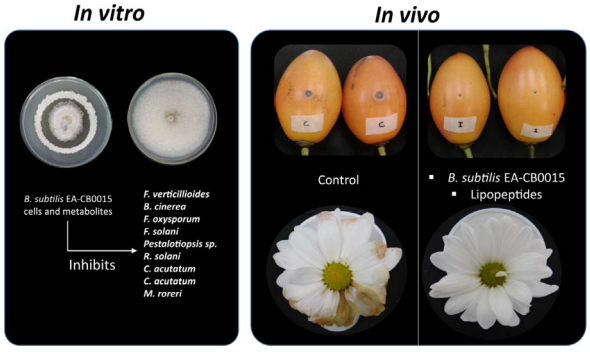
GRAPHICAL ABSTRACT



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ABSTRACT

Post-harvest diseases are responsible for significant losses worldwide, especially the plant pathogenic fungi *Botrytis cinerea* and *Colletotrichum* sp. are particularly severe and devastating. In this study, nine fungal pathogens were screened for growth inhibition by *Bacillus subtilis* EA-CB0015 strain and its metabolites. *In vitro* inhibitory assays showed that *B. subtilis* EA-CB0015 cells and the cell free supernatant (CFS) inhibited the growth of the tested fungal pathogens with different susceptibilities. Therefore, the antifungal activities of lipopeptides iturin A and fengycin C contained in the CFS, were tested against *C. acutatum* and *B. cinerea*. *C. acutatum* was more susceptible with minimal inhibitory concentrations (MIC) of 32 ppm (iturin A) and 128 ppm (fengycin C). Fruit and flower trials confirmed that *B. subtilis* EA-CB0015 cells and its lipopeptides reduced postharvest disease development but to differing degrees. Anthracnose symptoms caused by *C. acutatum* in tamarillo fruits were completely abolished by CFS, iturin A and fengycin C and reduced by 76% when treated with *B. subtilis* cells. In contrast, grey mold disease symptoms caused by *B. cinerea* in chrysanthemum flowers were inhibited by 72% when treated with lipopeptides and by 39% when applied with *B. subtilis* EA-CB0015 cells. Our results indicate that lipopeptides and cells of *B. subtilis* EA-CB0015 have a broad antifungal spectrum and control postharvest diseases caused by susceptible fungal pathogens. Our findings open the possibility of incorporating this biological control agent into different disease management programs.

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1. Introduction

Plant pathogenic fungi generate losses equivalent to 200 billion dollars each year (Horbach et al., 2011) and without the methods currently available for crop protection, their economic impact would be even higher. In the United States and developing countries, 20%–50% of those losses in revenue come from postharvest diseases (Nunes, 2012), mainly those caused by saprophytic fungi such as *Botrytis cinerea* and *Colletotrichum* sp. These fungi have a wide host range and have been ranked within the ten most devastating plant pathogenic fungi (Dean et al., 2012).

Before infection, these fungi remain on their hosts in a saprophytic phase and once the environmental conditions become suitable (i.e. during post-harvest storage), they switch to a pathogenic phase and generate their characteristic symptoms (Cannon et al., 2012; Williamson et al., 2007). Integrated pest management programs are available; however, growers rely heavily on chemical control for mitigating these diseases. As a consequence, the number of resistant fungal strains as well as the number of prohibited molecules in the exportation policies are increasing, and generates a decrease in the public acceptance (Ecobichon, 2001; Schirra et al., 2011; Sivakumar and Bautista-Baños, 2014; Zhan et al., 2014). Trying to mitigate the negative impacts, growers are starting to incorporate biological control agents into their management programs. However, biological products only account for 2.5% of the agricultural supplies market and are mostly used when the chemical control is not suitable such as in organic crops (Ongena and Jacques, 2008; Mehrotra et al., 2017).

Bacteria from the genius *Bacillus* are known for their ability to produce antimicrobial compounds and resistant endospores that allow them to survive environmental stresses (Emmert and Handelsman, 1999). They inhibit the growth and mitigate the symptoms of plant pathogens both *in vitro* and *in vivo* (Ongena and Jacques, 2008). In particular, lipopeptides contribute to *Bacillus* spp. antimicrobial activity. These antimicrobial compounds are active against a wide range of microorganisms including bacteria, fungi, oomycetes and viruses (Raaijmakers et al., 2010). Out of many examples, lipopeptides reduced the severity of *B. cinerea* on apples (Ongena et al., 2004) and *Alternaria citri*, *C. gloesporoides* and *Peniciliium crustosom* in citrus trees, with the iturin lipopeptide having a major role in preventing disease symptoms (Arrebola et al., 2010).

Within this genus, the bacterial strain Bacillus subtilis EA-CB0015 (Bs EA-CB0015) was isolated from the phyllosphere of banana plants from Uraba (Colombia), selected for its ability to inhibit the growth of Mycosphaerella fijiensis (Ceballos et al., 2012) and for producing high amounts of the lipopeptides fengycin C, iturin A and surfactin (Villegas-Escobar et al., 2013, Mosquera et al., 2014). Furthermore, a formulation containing Bs EA-CB0015 and its lipopeptides was tested in greenhouse and field conditions on banana plants, showing a reduction in Black Sigatoka disease severity comparable to the chemical fungicides chlorotronil and mancozeb (Gutierrez-Monsalve et al., 2015; Villegas-Escobar et al., 2016). Our previous results have suggested that lipopeptides fengycin C and iturin A have a mayor role in controlling the disease, but it has also been shown that cells of *Bs* EA-CB0015 also affected the development of Black Sigatoka.

To determine if pathogenic fungi differ in susceptibilities to the biological control agent *Bs* EA-CB0015, we evaluated its activity *in vitro* against 9 fungal strains and in two post-harvest pathosystems. We found that *Bs* EA-CB0015 and the CFS have differential inhibitory activities on the fungal pathogens *in vitro*, choosing *C. acutatum* as highly susceptible and *B. cinerea* as more resistant. The difference in susceptibility was also evaluated by determining the MIC of the lipopeptides iturin A and fengycin C, finding lower MIC values for *C. acutatum*. Finally, two different pathosystems were evaluated to determine the effect of *Bs* EA-CB0015 and its lipopeptides to reduce the symptoms caused by *C. acutatum* on tamarillo (*Cyphomandra betacea*) fruits and by *B. cinerea* on chrysanthemum flowers. Tamarillo fruit is the third most important export fruit in Colombia and it has a large local market (Afanador-Kafuri et al., 2003). Chrysanthemum flowers represents 11% of the Colombian flower production (Ascosflores, 2015), which is highly important, as Colombia is one of the world's foremost producers of flowers (https://www.rabobank.com, World Floriculture Map 2015). Therefore, finding new biocontrol strategies to control post-harvest diseases for these agricultural industries is also one of the main objectives of this study.

Table 1
Bacterial and fungal strains used in this study.

Microorganism	Strain	Isolation source	Reference
B. subtilis	EA-CB0015	Banana leaves	GenBank accession number KC006063, Ceballos et al. (2012)
F. verticillioides	EA-HP013	Sugar cane	This study
B. cinerea	IBUN Bc001	Black berry	Dr. Alba Marina Cotes (Corpoica)
F. oxysporum	EA-HP005	Carnation flowers	This study
F. solani	EA-HP003	Chrysanthemum flowers	This study
Pestalotiopsis sp.	EA-HP010	Avocado	This study
R. solani	EA-HP002	Potato	This study
C. acutatum	EA-HP012	Tamarillo	This study
C. acutatum	EA-HP008	Tamarillo	Afanador-Kafuri et al. (2003)
M. roreri	EA-HP007	Cocoa fruits	This study

2. Material and methods

2.1. Microorganisms and culture conditions

Bacillus subtilis EA-CB0015 (Table 1) was stored at −80 °C in Tryptic Soy Broth (TSB, Oxoid) with 20% glycerol and was activated on half-strength Tryptic Soy Agar (TSA; Oxoid) at 30 °C for 48 h before any experimental use. The fungal strains *Rizoctonia solani* EAHP-002, *Botrytis cinerea* IBUN Bc001 (*Bc* IBUN Bc001), *Fusarium solani* EAHP-003, *F. oxysporum* EAHP-005, *Moniliophthora roreri* EAHP-007, *Pestalotiopsis* sp. EAHP-010, *C. acutatum* EAHP-012, *Colletotricum acutatum* EAHP-008 (*Ca* EAHP-008), *F. verticillioides* EA-HP013 (Table 1) were stored in filter papers at room temperature and activated in Potato Dextrose Agar (PDA, Oxoid) at 20 °C before any experimental use. Fungal strain identification was based in the 5.8S-ITS region sequenced with the ITS1 and ITS4 primers (Tapia-Tussell et al., 2008). When necessary, spores were collected in 0.05% tween 80 (Sigma-Aldrich).

2.2. B. subtilis EA-CB0015 biomass, spores, and cell free supernatant production

Bs EA-CB0015 biomass was obtained by transferring one bacterial colony into a 500 mL Erlenmeyer containing 200 mL of culture medium D and incubating it for 5 days at 30 °C and 200 rpm. Medium D was composed of 0.042 g/L $MnSO_4$*$4H_2O$, 0.031 g/L $CaCl_2$, 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 1.0 g/L $(NH_4)2SO_4$, 4.0 g/L $MgSO_4$*$7H_2O$, 32.5 g/L yeast extract and 33.4 g/L glucose (Mosquera et al., 2014). After 5 days of incubation, cells were recovered by centrifugation at 4500 rpm for 15 min and suspended in water to a final concentration of $6.2 \pm 0.6 \times 10^8$ UFC/mL to obtain the cell suspension (CS). For the spore suspension (SS) an aliquot of the CS was heat treated for 20 min at 80 °C and the concentration calculated ($4.6 \pm 0.6 \times 10^7$ UFC/mL). The supernatant obtained from the centrifugation step was passed through cellulose ester membrane (0.2 μm; Advantec MFS, Inc) to obtain CFS.

2.3. B. subtilis EA-CB0015 lipopeptides purification

To purify the *Bs* EA-CB0015 lipopeptides, we used the methodology described by Villegas-Escobar et al. (2013). First, the amberlite pellets were recovered from the *Bs* EA-CB0015 cultures and the absorbed metabolites were eluted with 200 mL of methanol (MeOH). After elution, the solvent was evaporated (50 psig, 50 °C) and the solid fraction suspended in 10 mL of water. The suspension was passed thought a solid phase extraction (SPE) C18 cartridge (Agilent®, 10 g) and successively eluted using 80 mL MeOH/water gradients depending on whether they were going to be used to the biocontrol activity tests or to determine the lipopeptides MIC. For the former, the gradient consisted in 0%, 50% and 100%, and for the latter 20%, 40%, 70% and 100%. The eluates corresponding to 70% and 100% fractions were evaporated (50 psig, 50 °C), and the solid residues were dissolved in either equal volumes of water (200 mL; pH 7.0) or MeOH. The aqueous fractions were used for evaluating the *Bs EA-CB0015* lipopeptides activity as a mixture (LIP) in the biocontrol activity tests, while the methanolic fractions were further purified using reversed-phase high pressure liquid chromatography (RP-HPLC).

For the biocontrol activity tests, 100% methanolic fraction was injected onto a column Zorbax Eclipse XDB C18 (250 mm × 4.5 mm, 5 µm, Agilent®) and eluted using the solvent system 0.1% TFA/water (solvent A) and 0.1% TFA/acetonitrile (solvent B). The system was operated in a gradient mode from 30 to 70% B in 60 min followed by 70–100% B in 15 min and 100% B for 10 min at a flow rate of 1 mL/min, 30 °C and UV detection at 214 nm. The fractions eluted between 16–21 min, 25–48 min and 72–78 min corresponding to iturin A (ITU), fengycin C (FEN) and surfactin (SUR), respectively, were recovered. Then the eluted fractions were evaporated (50 psig, 50 °C) and dissolved in phosphate buffer pH 7.2 to a final concentration of 700 ppm.

For MIC evaluations, the 70% and 100% methanolic fractions were injected and eluted as previously described using a gradient from 30 to 52% B in 35 min, 52 to 100% B in 20 min and 100% B for 10 min. The fractions eluted between 14 and 20 min for the 70% methanolic fraction corresponding to the ITU and the fractions eluted between 11–17.3 and 26.3–31.1 min for the 100% methanolic fraction corresponding to the FEN and SUR, respectively, were recovered. Then, the eluted fractions were evaporated and dissolved in methanol at different concentrations.

2.4. Antifungal activity screening

We evaluated *Bs* EA-CB0015 inhibitory effect against the fungal strains on agar plates using the methodology described by Sinclair and Dhingra (1995). Briefly, *Bs* EA-CB0015 was inoculated forming a circumference (∅: 6 cm) in PDA plates (∅: 9 cm). Agar plugs (∅: 5 mm) from PDA fungal cultures were placed at the center of the bacterial circumference and incubated at 20 °C. To evaluate the effect of CFS against the fungal strains, 80 µL of CFS were added on each of 8 wells punched forming a circumference into the PDA agar. When fungal colonies growing in axenic cultures reached a diameter of 6 cm, the colony radius of the fungi growing in the presence of *Bs* EA-CB0015 or the CFS were measured at 4 different points and averaged. Three biological replicates were used per fungal strain and the inhibitory percentage was calculated considering a 6 cm radius as the maximum growth and 0% inhibition. Additionally, the growth rate (mm/day) of the different fungi growing in axenic cultures was determined by periodically measuring the colony radius.

2.5. B. subtilis EA-CB0015 lipopeptides minimal inhibitory concentration

To determine the MIC, 100 µL of Sabouraud broth was added to each well of a 96-well plate and then mixed with different concentrations of purified lipopeptides (1–1025 ppm) and fungal spores of *Bc* IBUN Bc001 or *Ca* EAHP-008 at a concentration of 1×10^3 (spores/mL) (Wiegand et al., 2008). Plates were incubated at 22 °C for 6 days. After that incubation period, the optical density was measured using a Microplar Reader iMark™ (Biorad) at 595 nm, and the MIC was determined by the minimum concentration of lipopeptides that completely prevented visible growth.

2.6. Fruit trials

To determine the effect of *Bs* EA-CB0015 and its metabolites on *Ca* EAHP-008 and *C. acutatum* EA-HP012 infection in tamarillo fruits, we used a modified version of the methodology described by Bautista-Rosales et al. (2013). Precisely, tamarillo fruits were surface sterilized with 0.1% bleach for 1 min and 70% ethanol for 2 min, rinsed with water and wounded (1 × 2 mm width and depth). Then, we added 25 µL of either water, CS, SS, CFS, the eluted fractions recovered for each lipopeptide (FEN, ITU, SUR) or the mixture (LIP) and 15 µL of fungal spore suspension (4×10^5 spores/mL) to the wounds. The treatments CS, SS, CFS were added either 24 h before or 24 h after fungal inoculation. The lipopeptide fractions were only evaluated 24 h before fungal inoculation. The inoculated fruits were incubated for 6 days in humid chambers with a 12/12 photoperiod, 90% humidity, and 25 °C. After the incubation period the diameter of the lesions were measured for each fruit. Four replicates of single fruits per each treatment were used. Then experiment was repeated, and the number of fruit per treatment increased to five.

2.7. Flower trials

To evaluate the effect of *Bs* EA-CB0015 and its metabolites on *Bc* IBUN Bc001 infection in chrysanthemum flowers, we used a modified version of the methodology described by Darras et al. (2006). Specifically, chrysanthemum flowers were rinsed with water and placed in containers (12 oz) filled with 20 mL of water. Then, either CS, CFS, LIP or water were sprayed using an Minispray gun with cup K-3® with 25 mL/min follow rate and 60 drops/cm^2 coverage. After 24 h, the sprayed flowers were spray inoculated with 3 mL of a fungal spore suspension (1×10^5 spores/mL) as before. The flowers were incubated for 7–14 days in humid chambers with a 12/12 photoperiod, 90% humidity and 20 °C. To measure disease incidence, the proportion of petals showing symptoms was evaluated. To determine disease severity, a scale was defined as follow: 0 representing symptomless petals, 1 petals with well-defined spots, 2 petals with coalescent spots and chlorotic halo, 3 petals showing necrosis in less than 50% of the petal area and 4 petals showing necrosis in more than the 50% of the petal area. The number of petals in each category was determined and used to calculate the weighted arithmetic mean of severity for each flower. Two independent experiments were done, and 6 and 8 flowers were used as biological replicates for the first and second experiment, respectively.

2.8. Statistical analysis

A complete randomized design was used in all experiments performed. To evaluate differences among treatment means, we used either an analysis of variance (ANOVA) or the Kruskall-Wallis test. For the pairwise comparison of the treatments means, we used the Tukey multiple comparison test. To determine differences in treatment means relative to the control means, we used the Mann-Whitney-Wilcoxon test. The selection of the statistical test was based on whether or not the data fitted the assumptions for each test. To evaluate correlation between inhibitory activity and grow rate, the data was fitted to a lineal model. All the analysis were done in R 3.0.1. (R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.) using the packages lattice and car and a 95% confidence level for all the tests.

Table 2
Effect of *B. subtilis* EA-CB0015 cells and CFS on the growth of different plant pathogenic fungi *in vitro*.

Pathogenic fungi	Strain	Growth rate[1] (mm/d)	CFS Growth inhibition[2] (%)	Bacterial cells Growth inhibition[3] (%)
F. verticillioides	EA-HP013	3.6 ± 0.1 **a**[1]	−4.48 ± 2.6 **a**	33.1 ± 1.7 **a**
B. cinerea	IBUN Bc001	11.8 ± 0.2 **b**	20.53 ± 0.8 **b**	33.9 ± 1.5 **a**
F. oxysporum	EA-HP005	3.0 ± 0.1 **ae**	16.78 ± 2.3 **b**	42.5 ± 0.4 **b**
F. solani	EA-HP003	2.3 ± 0.1 **c**	21.12 ± 0.5 **b**	50.8 ± 2.2 **c**
Pestalotiopsis sp.	EA-HP010	4.6 ± 0.1 **d**	39.09 ± 0.5 **c**	51.1 ± 1.1 **c**
R. solani	EA-HP002	8.8 ± 0.3 **f**	57.68 ± 0.3 **e**	54.4 ± 1.0 **c**
C. acutatum	EA-HP012	2.9 ± 0.1 **ec**	23.34 ± 2.2 **b**	64.7 ± 0.7 **d**
C. acutatum	EA-HP008	3.0 ± 0.0 **ae**	34.19 ± 1.8 **c**	67.5 ± 1.7 **d**
M. roreri	EA-HP007	1.7 ± 0.0 **g**	39.97 ± 1.4 **c**	93.3 ± 1.7 **e**

Means followed by the same letter were not significantly different at the 95% confidence level as determine by the Tukey's range test (n = 3). [1]*p*-value = 1.868e−08, [2]*p*-value = 1.89e−12, [3]*p*-value = 2.2e−16. CFS denotes cell free supernatant, Bacterial cells denotes *B. subtilis* cells.

3. Results and discussion

3.1. B. subtilis EA-CB0015 inhibits mycelial growth in different plant pathogenic fungi

Strain EA-CB0015 and CFS showed growth inhibitory activity against all fungal pathogens used in this study. The colony reductions ranged from 33.1% to 93.3% for *Bs* EA-CB0015 cells and from no inhibition to 57.7% for the CFS (Table 2). When the fungal strains were growing on the agar surface with *B. subtilis* EA-CB0015, the mycelium growth was retarded (Table 2, Supplementary material Fig. S1). This growth inhibition was accompanied with an increased swelling and branching of *Bc* IBUN Bc001 hyphae (Supplementary material Figs. S2, S3) and swelling of *Ca* EAHP-008 hyphae (Supplementary material Fig. S2). These results indicate that *Bs* EA-CB0015 produces inhibitory compounds with a broad spectrum in both submerged cultures and agar plates and suggest that the fungi vary in susceptibility. Along with the differences in growth inhibitions, we found variations in fungal growth rate (Table 2). The differences in growth inhibition could be explained due to differences in growth rates or because of differential susceptibility. Specifically, slow-growing fungi could provide the bacterial strain more time to produce antifungal metabolites and therefore, shows higher inhibition. However, we found no correlation between the fungal growth inhibitions and growth rate (bacteria: $R^2 = 0.12$ and *p*-value = 0.19; CFS: $R^2 = -0.10$; *p*-value = 0.63).

B. subtilis active compounds (i.e. lipopeptides) have species-specific activities (Liu et al., 2014; Loeffler and Vanittanakom, 1986; Winkelman et al., 1983). Fengycin C and iturin A lipopeptides are responsible for most of *Bs* EA-CB0015 inhibition of *M. fijiensis* growth (Mosquera et al., 2014) and might be involved in the activities observed in this work. To determine if *Bs* EA-CB0015 lipopeptides had species-specific activities responsible for the differences in growth inhibitions, we evaluated the lipopeptide concentration that prevented *Bc* IBUN Bc001 and *Ca* EAHP-008 growth in liquid culture. In fact, the MICs of fengycin C and iturin A for *Ca* EAHP-008 were 128 ppm and 32 ppm respectively, while the MICs of fengycin C and iturin A for *Bc* IBUN Bc001 were > 128 ppm and 128 ppm respectively. Therefore, *Ca* EAHP-008 was more susceptible to *Bs* EA-CB0015 lipopeptides than *Bc* IBUN Bc001. Loeffler and Vanittanakom (1986) reported that *B. subtilis* fengycin inhibits *C. acutatum* growth stronger than *B. cinerea* growth, supporting our observations. In addition to fengycin C and iturin A, we evaluated surfactin, but the inhibition obtained at 128 ppm only reached 46 ± 14% for *Ca* EAHP-008 and 0% of inhibition for *Bc* IBUN Bc001. The lack of antifungal activity for *Bs* EA-CB0015 surfactins was in agreement with our previous observations for the banana pathogen *M. fijiensis* (Mosquera et al., 2014).

3.2. B. subtilis EA-CB0015 reduces anthracnose development in tamarillo fruits

To determine if the inhibitory activity observed *in vitro* for *C. acutatum* was maintained *in vivo*, we evaluated the effect of *Bs* EA-CB0015 CS, SS and CFS on the infection development of *Ca* EA-HP008 in tamarillo fruits. Independent of the application time, CFS controlled anthracnose the most. Fruits treated with CFS exhibited almost no lesion development (Table 3). For CS and SS, all the treated fruits showed a delay in lesion development. However, only the fruits treated in advance with CS had lesion diameters significantly smaller (2.0 ± 1.1 cm; *p*-value < 0.05) than the non-treated controls (Table 3). In addition to *Ca* EA-HP008, we evaluated the strain *C. acutatum* EA-HP012 with similar results (Supplementary material, Table S1). This suggests that *Bs* EA-CB0015 control of anthracnose in tamarillo fruits is strain independent and increases the potential of success in controlling diseases in field. All these together are in line with our previous findings reported for *M. fijiensis*-Banana pathosystem (Gutierrez-Monsalve et al., 2015). Also, they strongly suggest that *Bs* EA-CB0015 controlling capacity in tamarillo fruits mostly relys on active compounds (i.e. lipopeptides) produced by the bacterium. The *in vivo* production of *Bs* EA-CB0015 lipopeptides has not yet been proven, however Romero et al. (2007) showed that a *B. subtilis* strain produces fengycins and iturins on detached mellon leaves. We are currently evaluating *in vivo* lipopeptides production for *Bs* EA-CB0015. Active compound production would explain the smaller lesion diameters observed for the fruits treated with CS and SS in advanced (Table 3). In these treatments, *Bs* EA-CB0015 cells had additional time to produce antifungal compounds. However, nutrient and space competition cannot be ruled out. Independent to lipopeptide production, *Bs* EA-CB0015 has increased chances to build a population and colonize. Once *Bs* EA-CB0015 is stablished, *C. acutatum* has to outcompete the bacteria to gain access to the fruit and cause lesions.

To test if *Bs* EA-CB0015 lipopeptides could explain the reductions in

Table 3
Effect of *B. subtilis* EA-CB0015 cells and CFS on anthracnose lesion diameter caused by *C. acutatum* EA-PH008 on tamarillo fruits.

Treatment	Lesion diameter (mm)	
	24 HBP[1]	24 HAP[2]
Control	8.3 ± 0.6	8.5 ± 1.2
CS	2.0 ± 1.1*	5.8 ± 1.7
SS	2.5 ± 2.5	4.8 ± 1.8
CFS	0.3 ± 0.3*	0.3 ± 0.3*

The abbreviations denotes: Cell suspension (CS), spore suspension (SS), cell free supernatant (CFS), [1]hours before pathogen inoculation (HBP), [2]hours after pathogen inoculation (HAP). (*) Denotes statistically significant differences respect to the non-treated control at 95% confidence level as determined by the Mann-Whitney-Wilcoxon test. (n = 4). The Kruskall-Wallis test, [1]*p*-value = 0.05, [2]*p*-value = 0.03.

Table 4
Effect of *B. subtilis* EA-CB0015 lipopeptides on anthracnose lesion diameter caused by *C. acutatum* EA-PH008 on tamarillo fruits.

Lipopeptide	Lesion diameter (mm)
Control	6.0 ± 0.8
CFS	0.0 ± 0.0*
FEN	0.0 ± 0.0*
ITU	0.0 ± 0.0*
SUR	4.0 ± 0.7

The abbreviations denotes: cell free supernatant (CFS), purified fractions of iturin A (ITU), fengycin C (FEN) and surfactin (SUR) lipopeptides. (*) Denotes statistically significant differences respect to the non-treated control at the 95% confidence level as determined by the Mann-Whitney-Wilcoxon test (n = 5). *p*-value = 0.0001.

Table 5
Effect of *B. subtilis* EA-CB0015 cells and lipopeptides on the incidence and severity of gray mold caused by *B. cinerea* IBUN Bc001 on chrysanthemum flowers.

Treatment	Proportion of symptomatic petals[1]	Disease severity[2]
Control	0.6 ± 0.1 a	1.8 ± 0.2 a
CS	0.4 ± 0.1 b	1.0 ± 0.2 b
LIP	0.2 ± 0.1 c	0.4 ± 0.1 b

The abbreviations denotes: cell suspension (CS), purified fraction of all three lipopeptides (LIP). Means followed by the same letter were not significantly different at the 95% confidence levels as determine by the Tukey's range test (n = 8). [1]*p*-value = 0.0003, [2]*p*-value = 9.9e−05.

anthracnose development observed, we evaluated the effect of the lipopeptide mixture and the purified compounds surfactine, fengycin and iturin lipopeptides. The lipopeptides mixture (LIP, data not shown), the fengycin and iturin lipopeptides completely abolished symptoms development (Table 4). The lipopeptides concentrations (700 ppm) used during these evaluations matched the concentration reported for fengycins in *Bs* EA-CB0015 CFS (Mosquera et al., 2014) while the concentrations for iturin and surfactins in the CFS are lower (300 ppm) (Mosquera et al., 2014), indicating that CFS controlling effect can be fully explained by the fengycins and/or iturins produced in submerged culture.

3.3. *B. subtilis EA-CB0015 reduces gray mold development in chrysanthemum flowers*

To further characterize *Bs* EA-CB0015 action spectrum, we tested the effect of *Bs* EA-CB0015 CS, CFS and LIP on gray mold development in chrysanthemum flowers. Both, CS and LIP reduced the proportion of infected petals from 0.6 ± 0.1 to 0.4 ± 0.1 and 0.2 ± 0.1 and reduced the disease severity from 1.8 ± 0.2 to 1.8 ± 0.2 and 0.4 ± 0.1, respectively (Table 5). Surprisingly, CFS increased gray mold development (Table 5). The difference in activities observed between LIP and CFS could be explained by nutrients not exhausted during *Bs* EA-CB0015 cultures and present in the CFS. The remaining nutrients present in the CFS, might induce disease severity by inducing conidia germination, germ tube growth, appressorium formation and tissue colonization in *B. cinerea* (Akutsu et al., 1981; Schumacher and Tudzynski, 2012).

4. Conclusions

Bs EA-CB0015 and its lipopeptides reduces disease development in *C. acutatum* a necrotroph belonging to the Sordariomycete and *B. cinerea* also a necrotroph belonging to the Leotiomycete. Here we demonstrated that *Bs* EA-CB0015 *in vitro* inhibitory capacity has a broad action range and affects distantly related fungi belonging to the Ascomycota and Basidiomycota phylum. Independent of this broad spectrum, we found that the susceptibility varies from one fungus to another and showed that these differences can be explained by differences in susceptibilities towards *Bs* EA-CB0015 lipopeptides. We also showed that the broad action range observed *in vitro* extrapolates to disease control *in vivo*. In a previous work, we demonstrated that this bacterial strain also reduces growth and disease development in *M. fijiensis* a hemibiotroph belonging to the Dothideomycetes (Gutierrez-Monsalve et al., 2015). These findings suggest that *Bs* EA-CB0015 controlling capacity acts upon distantly related fungi independent of their lifestyle. Finally, we showed that iturin and fengycin lipopeptides produced during *Bs* EA-CB0015 fermentation can explain the reductions in disease development observed for *Bs* EA-CB0015 CFS in tamarillo fruits and chrysanthemum flowers. However, the *in vivo* production of iturin and fengycin lipopeptides needs to be demonstrated.

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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.2017.08.014.

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