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Biological Control and Microbial Ecology

e-Xtra*

Effects of Fengycins and Iturins on *Fusarium oxysporum* f. sp. *physali* and Root Colonization by *Bacillus velezensis* Bs006 Protect Golden Berry Against Vascular Wilt

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ABSTRACT

Bacillus velezensis Bs006 has shown antagonistic activity on *Fusarium oxysporum* f. sp. *physali* and biocontrol activity against Fusarium wilt (FW) in golden berry (*Physalis peruviana*). We hypothesized that strain Bs006 has the ability to synthesize antimicrobial cyclic lipopeptides (CLPs) like other members of the same species. However, if so, the real effects of CLPs on *F. oxysporum* f. sp. *physali* and their potential as a biocontrol tool against *Physalis*-FW have not been elucidated. In this study the CLPs profile of Bs006 in liquid culture and antagonist–plant–pathogen interactions were characterized. Also, the potential effects of supernatant free of bacteria against *F. oxysporum* f. sp. *physali* and FW were explored and compared with the effects of pure CLPs. Ultraperformance liquid chromatography–electrospray ionization–mass spectrometry analysis revealed the capacity of Bs006 to synthesize homologous compounds of iturins, surfactins, and fengycins in liquid culture and on the inhibition zone against *F. oxysporum* f. sp. *physali* in dual confrontation tests. Bs006 supernatant reduced the germination and growth of *F. oxysporum* f. sp. *physali* and caused vacuolization, swelling, and lysis of *F. oxysporum* f. sp. *physali* cells in a concentration-dependent manner. Pure fengycins affected the development of *F. oxysporum* f. sp. *physali* from 11 mg/liter and iturins from 21 mg/liter. In a gnotobiotic system, Bs006 colonized the root surface of golden berry, inhibited the growth of *F. oxysporum* f. sp. *physali*, and produced CLPs. Individual application of Bs006 and supernatant protected the plants from *F. oxysporum* f. sp. *physali* infections by 37 to 53%, respectively. Meanwhile, fengycins reduced the disease progress by 39%. These results suggest further studies to select an optimum combination of Bs006 and supernatant or CLPs, which might be a good option as biofungicide against *F. oxysporum* f. sp. *physali*.

Keywords: antibiosis, biological control, biotechnology, Cape gooseberry, disease control and pest management, fungal pathogens, PGPR, *Physalis peruviana*

Golden berry (*Physalis peruviana*) is an important crop for the Colombian economy, with an annual value of exports amounting to US$32 million in 2019 (Leal 2020). This fruit has been cultivated for approximately 30 years, but the yield has shown a decreasing trend. Although it is still the primary world producer, Colombia's status is at risk because of the incidence of vascular wilt disease caused by *Fusarium oxysporum* f. sp. *physali* (Simbaqueba et al. 2018) and the increase in cultivated area in other countries, because this fruit has become an economic alternative for Kenya, Zimbabwe, Australia, New Zealand, India, and Ecuador. Fusarium wilt (FW) affects >40% of plants in a plantation (Liberato et al. 2014). Because it is a new crop and the cropped area is small (<6,000 ha), there are no registered phytosanitary products to use in this crop against soilborne plant pathogens. So traditional management of this disease has been focused on use of fungicides that have been applied in other crops, it being necessary to develop and register different control measures or to validate existing control methods such as soil solarization.

Although currently knowledge among farmers about biological control as an alternative measure is broader than it was a few years ago, its implementation is still limited by the lack of consistency in field trials, the low technical and biological quality of many bioproducts, and their low commercial availability. These factors could be alleviated by the elucidation of modes of action and factors affecting the efficacy of biological control agents during its interaction with the host and pathogen and could contribute to the success of this control method and to expanding its use.

Biological control agents such as *Trichoderma* spp., *Bacillus* spp., and *Pseudomonas* spp. are the most used genera against FW in many hosts (Khan et al. 2017). *Bacillus subtilis* species complex are recognized for their antagonistic effects against soilborne phytopathogens, including several *formae speciales* of *F. oxysporum* (Fira et al. 2018). The biocontrol effects of *Bacillus* spp. are due mainly to the biosynthesis of cyclic lipopeptides (CLPs) from the iturin, fengycin, and surfactin families, which are involved in direct effects of destabilizing cell membranes of phytopathogens (Bonmatin et al. 2003; Han et al. 2015; Zhang et al. 2013) and indirect effects by inducing defense responses in the host plant (Jourdan et al. 2009; Rabbee et al. 2019). Furthermore, fengycins and iturins have been consistently reported as causing effects such as swelling and lysis of cells on a broad spectrum of filamentous fungi, including *Fusarium* spp. (Chitarra et al. 2003; Hu et al. 2007; Li et al. 2007; Vanittanakom et al. 1986; Yu et al. 2002). However, as far as is known in most studies about mechanisms of action, the characterization of biocontrol effects by *Bacillus* spp. had been based on one or two components of the biocontrol system, but the potential of biological control

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based on CLPs production during the tritrophic host–pathogen–*Bacillus* interaction has been poorly studied.

Although few studies have been carried out to select natural antagonists of *F. oxysporum* f. sp. *physali*, strains from *Pseudomonas* spp. and *Bacillus* spp. have shown the highest potential between plant growth–promoting rhizobacteria isolates (Díaz et al. 2013; Toloza-Moreno et al. 2020; Urrea et al. 2011). Particularly, the potential of the native strain *B. velezensis* Bs006 (formerly *B. amyloliquefaciens* [Gamez et al. 2015] and relocated in *B. amyloliquefaciens* operational group [Fan et al. 2017]) as a biocontrol agent against FW in golden berry has been demonstrated, and it is known that its efficacy can be influenced by both the absence of native microbiota in the soil and the relationship between the concentration of the biocontrol agent and *F. oxysporum* f. sp. *physali* (Moreno-Velandia et al. 2019). Bs006 also has plant growth–promoting traits (Díaz et al. 2013; Gamez et al. 2019; Moreno-Velandia et al. 2019), suggesting that it is an ideal microorganism to be used in agriculture. Nevertheless, the role of the CLPs against *F. oxysporum* f. sp. *physali* and the antagonistic potential of Bs006 during simultaneous interaction with the host and the pathogen have not been determined.

Because it is well known that several beneficial *Bacillus* spp. produce CLPs, we hypothesized that CLPs are involved in antibiosis effects shown by Bs006 against *F. oxysporum* f. sp. *physali*. Thus, the aims of this study were to determine the CLP profile synthesized by Bs006, determine the potential effects of its supernatant on *F. oxysporum* f. sp. *physali*, measure the antagonistic capacity of Bs006 against *F. oxysporum* f. sp. *physali* during the interaction with both the host and the pathogen, and study the effects of pure CLPs on both the pathogen and the vascular wilt progress.

MATERIALS AND METHODS

Biological material. Golden berry seeds were used for gnotobiotic system and greenhouse bioassays. Seed surface disinfection and seedling preparation were performed as described by Moreno-Velandia et al. (2019). Native strains of *B. velezensis* (Bs006) and *F. oxysporum* f. sp. *physali* (Map5) were provided from the Working Collection of the AGROSAVIA Microorganisms Germplasm Bank. Reactivation and growth of both microorganisms for production of inocula were carried out according to the method of Moreno-Velandia et al. (2019).

Liquid fermentation of Bs006. *B. velezensis* Bs006 was grown in 125-ml Erlenmeyer flasks by triplicate in Landy medium (Landy et al. 1948) for 48 h according to the detailed procedure described in the Supplementary Materials. First, 10 ml of fermented medium from each flask was sampled after incubation, and growth of Bs006 was measured through optical density ($OD_{600\ nm}$), the remaining volume was centrifuged (12,000 rpm, 10 min), and the recovered supernatant was filtered with 0.22-µm filters (Millipore) and then used for CLPs extraction.

Extraction and characterization of CLPs. Sterile supernatant was subjected to solid phase extraction in C_{18} cartridges (10 g, Alltech) according to the report by Nihorimbere et al. (2012) with the modifications described in the Supplementary Materials. Detection and quantification of CLPs in extracted samples were performed via the ultraperformance liquid chromatography–electrospray ionization–mass spectrometry (UPLC-ESI-MS) method described in the Supplementary Materials. CLPs were identified based on the comparison of retention times and molecular masses with those from known standards provided by Lipofabrik, Cité Scientifique, Bât. Polytech-Lille, France (Supplementary Figs. S1, S2, and S3). The concentration of each CLP family present in strain Bs006 supernatant was calculated based on the calibration curves of standard iturins, fengycins, and surfactins (Supplementary Fig. S4).

Antifungal activity against *F. oxysporum* f. sp. *physali*. *Dual confrontation test.* The antagonistic activity of *B. velezensis* Bs006 against *F. oxysporum* f. sp. *physali* was measured in sterile plastic Petri dishes (90 mm diameter) on solid Landy medium. First, 10 µl of fungal suspension (1×10^7 microconidia/ml), harvested from 2-week-old cultures in potato dextrose agar, was inoculated in the center of the dish. A cell suspension of Bs006, prepared from a 24-h-old culture in Luria-Bertani agar, was centrifuged (10,000 rpm, 10 min), and the pellet was washed three times with sterile distilled water (SDW) and resuspended in SDW. On two opposite sites, 2 cm apart from the edge of the Petri dish, 10 µl of Bs006 suspension (1×10^8 cfu/ml) was inoculated, and the dishes were incubated at 30°C under dark conditions. Seven days after incubation, the diameter of the fungus colony between the bacterium colonies was measured, as was the diameter of the fungus perpendicular to this. Control Petri dishes were inoculated only with *F. oxysporum* f. sp. *physali* or Bs006. There were five replicates for each treatment. The reduction of *F. oxysporum* f. sp. *physali* growth by Bs006 effect was calculated by the following equation: Growth inhibition $= [(C - T)/C] \times 100$, where C = diameter of *F. oxysporum* f. sp. *physali* in control, and T = diameter of *F. oxysporum* f. sp. *physali* in dual culture (Singh et al. 2008).

Quantification of CLPs in inhibition zones. Two agar plugs (5 mm diameter) per dish were sampled with a cork borer from the inhibition zones between *F. oxysporum* f. sp. *physali* and Bs006. Three Petri dishes from dual confrontation and controls were sampled. Agar plugs from a plate were immersed in a 500-µl solution of acetonitrile high-performance liquid chromatography (HPLC) grade (50%) and formic acid (0.1%) prepared in water HPLC grade in Eppendorf tubes of 2 ml. Samples were stirred for 2 min and then incubated overnight in a cold room (4°C). Subsequently, each sample was vortexed for 2 min and centrifuged (10,000 rpm, 10 min), and the supernatant was filtered (Millipore, 0.22 µm). The resulting filtrates were directly injected into a UPLC analyzer, meaning no previous solid-phase extraction of CLPs was made.

Antifungal test of Bs006-supernatant in liquid medium. The effect of Bs006 supernatant (0.5, 1.0, 2.0, 5.0, and 10%) on germination and growth of *F. oxysporum* f. sp. *physali* microconidia was evaluated in potato dextrose broth (PDB) medium. Bs006 was grown in Landy broth according to the procedure described previously. *F. oxysporum* f. sp. *physali* microconidia were harvested from 7-day-old culture in 50 ml of PDB in a 250-ml Erlenmeyer flask (125 rpm, 30°C, 12 h light:12 h dark photoperiod). To prepare the microconidial suspension, the mycelium was discarded by filtering the fermented broth through a double layer of sterile muslin cloth, and the obtained suspension was centrifuged (4,000 rpm for 12 min). The pellet of microconidia was washed two times with SDW and resuspended in SDW. The concentration was adjusted by counting in a Neubauer chamber. The experiment was performed in a set of two sterile flat-bottom 96-well microtiter plates (Falcon, Corning), one to measure the growth of *F. oxysporum* f. sp. *physali* at 620 nm with a microplate reader (Beckman Coulter AD 340) and the other to measure the percentage of germinated microconidia by counting in an Axioscop 2 mot microscopy (Carl Zeiss). In detail, 1 ml of PDB, supplemented with supernatant or with sterile Landy medium as a control, was dispensed in sterile Eppendorf tubes. Then 250 µl of homogenized solution was poured per well, used as blanks in each case (PDB + supernatant or PDB + Landy). The remaining 750 µl was inoculated with *F. oxysporum* f. sp. *physali*, adjusting the concentration to 1×10^5 to measure the growth of fungi and to 1×10^7 microconidia/ml to measure the germination. The inoculated broth was homogenized, and 250 µl of suspension was dispensed to each of three wells for each treatment. The plates were covered and incubated for 48 h under continuous shaking and darkness (125 rpm, 30°C).

Gnotobiotic system for measuring the antagonism against *F. oxysporum* f. sp. *physali*. This experiment was performed to validate the antagonistic activity of Bs006 against *F. oxysporum* f. sp. *physali* in planta. Gelified nutrient solution (45 ml) (Nihorimbere et al. 2012) in 10 sterile square Petri dishes (Greiner Bio-One,

$120 \times 120 \times 17$ mm) was used to place two germinated seeds 3 cm apart from the edge and separated 6 cm from one another. This system was incubated for 7 days at 30°C and 12h light:12 h dark photoperiod in a vertical position. Then 4 µl of Bs006 suspension in SDW (1×10^8 cfu/ml) was inoculated on the crown of one seedling and SDW was inoculated in the other seedling as a control and left to incubate for 4 days. Then, *F. oxysporum* f. sp. *physali* suspension (5 µl - 5×10^5 microconidia/ml) was dropped in the center of the dish, in the space between the two seedlings. Once the inoculum was dried, the system was incubated for 4 days. The distances between the main root of each seedling and the edge of the *Fusarium* colony were measured and were used to calculate the efficacy of the treatment to reduce the growth of the *F. oxysporum* f. sp. *physali* with the following equation: Inhibition (%) = $[1 - (NT/T)] \times 100$, where NT =distance between the fungus and the root of the control plant and T = distance between the fungus and the root of the treated plant.

Quantification of CLPs in the gnotobiotic system. Samples from root (2 cm of the elongation zone of the main root) and inhibition zone (two cylinders of agar 0.5 cm diameter) near the edge of the *F. oxysporum* f. sp. *physali* colony were taken to determine the presence of CLPs and to identify and quantify the compounds produced during antagonist–plant–pathogen interactions. Samples were submerged in acetonitrile (50%) and formic acid (0.1%) solution and were homogenized and kept overnight at 4°C. The samples were then stirred and centrifuged, and the supernatant was filter sterilized (0.22 µm). The resulting solution was analyzed directly by UPLC-ESI-MS.

Antifungal test of pure CLPs in liquid medium. The germination and growth of *F. oxysporum* f. sp. *physali* in PDB supplemented with solutions of pure surfactins, iturins, and fengycins were measured according to the procedure described previously for the effects of supernatant. Purified metabolites, consisting of a mixture of homologous compounds of each CLP family, were reconstituted in methanol, HPLC grade (MeOH) to obtain a stock solution (2 ml) at a concentration of 500 µM (i.e., 718, 522, and 497 µg/ml for fengycins, iturins, and surfactins, respectively). Solutions of CLPs at 7.5, 15, 20, 30, and 50 µM were prepared in 1 ml of sterile PDB. Positive and relative controls consisted of PDB and PDB supplemented with MeOH (same volumes added with treatments), respectively. Blanks consisted of sterile PDB supplemented with each compound (MeOH, surfactins, fengycins, and iturins) free of *F. oxysporum* f. sp. *physali*. Each treated solution was inoculated with *F. oxysporum* f. sp. *physali* after the blank wells were poured, adjusting the concentrations to 1×10^5 and 1×10^7 microconidia/ml, and 250 µl was dispensed by well. The incubation conditions and the measurement of variables were carried out as described previously. Treatments were carried out in triplicate.

In vivo experiments. *Experimental conditions and inoculation of microorganisms.* The effect of Bs006 cells, sterile supernatant, and pure CLPs on the progress of FW disease was evaluated. Golden berry plants were grown in pots (plastic trays of 750-cm^3 cones) with a substrate consisting of a mix of soil (Andosol, pH 5.7, OM 12.90, P 11 mg/kg, K 0.69 cmol(+)/kg) and rice husk (3:1 ratio) (hereafter referred to as soil). Moistened soil (60% moisture-holding capacity) was inoculated with a suspension of *F. oxysporum* f. sp. *physali* microconidia reaching a concentration of 1×10^4 cfu/g of soil. Pots were filled with 700 g of soil, and 60-day-old golden berry seedlings were transplanted (one seedling per cone). Treatments and controls were applied by drench immediately after transplant. The plants were irrigated once a day and fertilized once a week with a commercial nutrient solution (36-10-4 and micronutrients). Average air temperature in the greenhouse was 25°C, 12°C minimum and 42°C maximum, and the average relative humidity was 60%, 45% minimum and 75% maximum. Soil temperature in the pots ranged from 15 to 33°C.

Effect of Bs006 and supernatant on FW development. Bs006 was grown in Landy medium (150 rpm, 30°C, 48 h). Washed cell suspension of the bacterium (1×10^8 cfu/ml), supernatant free of bacteria (10% in tap water), and the mix of Bs006 and supernatant treatments were applied by drenching, 30 ml/plant. Controls consisted of plants growing in soil inoculated with *F. oxysporum* f. sp. *physali* without treatments against FW (negative control) and not treated plants growing in soil free of the pathogen (absolute control).

Effect of pure CLPs on FW development. The effect of surfactins, iturins, fengycins, and their mixture were evaluated against FW. The concentration of CLP solutions corresponded to 3.8 mg/liter (surfactins), 16.9 mg/liter (iturins), and 36.48 mg/liter (fengycins), keeping the concentrations of CLPs in the solution of supernatant of Bs006 (10%) described previously. Each compound was diluted initially in 2 ml of MeOH and subsequently in tap water for a total volume of 1,500 ml. Then, 30 ml of each solution was applied to each plant by drench immediately after transplant. Negative and absolute controls were included.

Experimental design of in vitro bioassays. The in vitro experiments had a completely randomized experimental design. The experiments to measure the effect of the supernatant and pure CLPs on germination and growth of *F. oxysporum* f. sp. *physali* had a factorial design. In both cases, the factors were the type of supplement in PDB (supernatant and sterile medium) and the concentrations of the supplement (0.5, 1.0, 2.0, 5.0, and 10%) for the first experiment. In the second case, the supplements in the broth were the solutions of CLPs and MeOH at concentrations of 7.5, 15, 20, 30, and 50 µM, respectively. There were three wells per treatment, filled with 250 µl of medium inoculated with *F. oxysporum* f. sp. *physali*. The experiment under the gnotobiotic system had a completely randomized design, the experimental unit consisted of a dish, and there were 10 replicates. Six dishes were sampled for CLP analysis. Dual confrontation test, antifungal test of Bs006 supernatant in liquid medium, the gnotobiotic system for measuring the antagonism against *F. oxysporum* f. sp. *physali*, and the antifungal test of pure CLPs in liquid medium were repeated once. Because data from the two repetitions of the experiments were similar, representative results from one experiment are shown.

Experimental design of greenhouse tests. Both experiments under greenhouse conditions had a randomized complete block experimental design with four replicates per treatment and 10 plants per experimental unit. The disease incidence (proportion of plants with typical symptoms of FW) and severity (intensity of symptoms in affected plants) were recorded weekly for 6 to 7 weeks after inoculation. The 0 to 5 disease severity scale described by Moreno-Velandia et al. (2019) was used. Calculations of the incidence, severity, the area under the disease progress curve (AUDPC), and the efficacy of the treatments to reduce the development of disease were made as described by Moreno-Velandia et al. (2019). The entire experiment in which the effect of Bs006 and supernatant on FW development was measured was repeated once. Because the results were similar between the two repetitions, equality of variances was verified, and the average of the two experiments was calculated and analyzed. The experiment to measure the effect of pure CLPs on FW development was carried out once.

Data analysis. Data from each experiment were submitted to a Shapiro-Wilk test ($\alpha = 0.05$) and Bartlett test ($\alpha = 0.05$) to verify normality and homoscedasticity, respectively. Significant effects of the treatments were determined by ANOVA via the general linear model procedure (PROC GLM) in SAS. A two-sample t test ($\alpha = 0.05$) was performed to determine significant differences between the mean of paired treatments in the experiment in which the effect of the supernatant against *F. oxysporum* f. sp. *physali* was measured. A Tukey test ($\alpha = 0.05$) was applied for means-treatment comparison in the pure CLPs versus *F. oxysporum* f. sp. *physali* in vitro experiment. Duncan's multiple range test ($\alpha = 0.05$) was used for means comparisons between treatments for the in vivo experiment of bacterial cells and supernatant against FW, and the

Dunnett test (α = 0.05) was used to compare the means of pure CLPs against the control in the last in vivo experiment. All analyses were conducted in Statistical Analysis Software System (version 9.4; SAS Institute, Cary, NC).

RESULTS

CLPs produced by *B. velezensis* Bs006 in liquid medium. The UPLC-ESI-MS analysis of the supernatant free of bacterial cells from fermentation in Landy medium revealed that Bs006 has the capacity to synthesize various homologous compounds of the most known CLPs families. Mass spectrometry analysis of the obtained peaks showed compounds of iturins (retention time from 1.2 to 1.5 min), fengycins (1.6 to 2.1 min), and surfactins (2.8 to 3.6 min) (Supplementary Fig. S5). Based on previous studies (Argüelles-Arias et al. 2009; Chen et al. 2019; Cho et al. 2003; Leclère et al. 2005; Ongena et al. 2005; Sa et al. 2018) and this analysis of pure compounds, several isoforms of each CLP were detected, with typical mass/charge ratios of 1,043 to 1,071 Da with a lipidic tail of 14 and 16 carbons (C14 to C16), which are isoforms of iturin A; 1,435 to 1,491 Da (C14 to C18) correspond to isoforms of fengycin A and 1,505 Da (C17) to fengycin B; and 993 to 1,050 Da (C12 to C16) were identified as five isoforms of surfactin (Table 1, Supplementary Fig. S5). Concentration of fengycins (364.85 ± 130.2 µg/ml) was the highest between the CLP families, followed by iturins (169.27 ± 26.0 µg/ml) and surfactins (38.00 ± 4.1 µg/ml). These results reveal a broad antagonistic potential for strain Bs006 because overall, CLPs from iturin, fengycin, and surfactin families have shown effects against both bacterial and fungal phytopathogens.

In vitro antifungal activity of *B. velezensis* against *F. oxysporum*. *Dual confrontation test.* The in vitro antagonism test showed that Bs006 significantly reduced growth of *F. oxysporum* f. sp. *physali* ($P < 0.0001$), which was evident in the reduction of the fungal colony and the inhibition zone between the bacterium and the fungus (Supplementary Fig. S6). Whereas the diameter of the *F. oxysporum* f. sp. *physali* colony in the direction of the two bacterial inoculation points was 3.83 ± 2.2 cm, the colony of *F. oxysporum* f. sp. *physali* reached the edge of the plate in the control (i.e., 8.50 cm diameter), representing an inhibition of *F. oxysporum* f. sp. *physali* growth of approximately 55%.

CLP profile in inhibition zones. Bs006 synthesized various homologous compounds of all three CLPs families during the dual confrontation test with *F. oxysporum* f. sp. *physali*, because these were detected in the inhibition zones, showing a similar profile of detected ions to those observed in liquid culture (Supplementary Fig. S7). It was found that the concentration of fengycins and iturins was similar in the inhibition zone, and the concentration of surfactins was the lowest (Fig. 1). With respect to the total amount of CLPs produced in the inhibition zones, the proportion of iturins was 45%, fengycins 43%, and surfactins was the lowest at 12%. The concentrations of CLPs detected both in the inhibition zones of Petri dishes inoculated with the two microorganisms and in the dishes free of the fungus were similar according to the ANOVA (P = 0.8094, 0.4498, and 0.2527 for iturins, fengycins, and surfactins, respectively) (Fig. 1), indicating that the presence of *F. oxysporum* f. sp. *physali* neither stimulates nor represses the biosynthesis of CLPs by Bs006 under these experimental conditions.

Germination and growth of *F. oxysporum* f. sp. *physali* under the effect of CLPs. *Effect of supernatant from Bs006 liquid culture.* Germination of *F. oxysporum* f. sp. *physali* microconidia was reduced by filtered supernatant of Bs006 liquid culture from 19 to 21% at 24 h after incubation as compared with the respective controls (sterile Landy medium) of each concentration, being 2, 5, and 10%, the most effective treatments (Fig. 2A). These observations indicate that some compounds in the supernatant of Bs006 culture are implicated in the delay of the fungus growth, suggesting that this effect is mediated by CLPs produced by the bacterium.

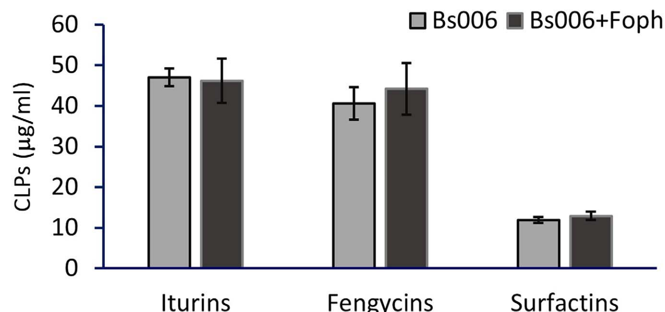

Fig. 1. Pattern of total cyclic lipopeptides (CLPs) produced by *Bacillus velezensis* Bs006 in the growth inhibition zones during dual confrontation with the strain *Fusarium oxysporum* f. sp. *physali* Map5 (Foph) on gelified Landy medium. Samples of agar were taken from plates inoculated only with the bacteria (Bs006) and from those inoculated with both bacteria and fungus (Bs006+Foph). Samples were taken at 7 days after incubation under 30°C and darkness. Bars on the columns represent the standard deviation of CLPs mean concentration from three plates. ANOVA detected no significant effects of treatments on the concentration of each group of CLPs at the significance level of 0.05.

TABLE 1. Summary of cyclic lipopeptides (CLPs) homologous from the iturin, fengycin, and surfactin families produced by *Bacillus velezensis* Bs006 in Landy medium detected by ultraperformance liquid chromatography–electrospray ionization-mass spectrometry

CLP	Mass peak	Assignment	Adducts	References
Iturin A	1,044.1 $[M+H]^+$	C14	1,066.1 $[M+Na]^+$	Cho et al. 2003
	1,058.1 $[M+H]^+$	C15	1,080.1 $[M+Na]^+$	Argüelles-Arias et al. 2009
	1,072.1 $[M+H]^+$	C16	1,093.9 $[M+Na]^+$	Chen et al. 2019
			1,110.0 $[M+K]^+$	
Fengycin A	1,436.4 $[M+H]^+$	C14		Ongena et al. 2005
	1,450.4 $[M+H]^+$	C15		Sa et al. 2018
	1,464.4 $[M+H]^+$	C16		Chen et al. 2019
	1,478.4 $[M+H]^+$	C17	Not detected	
	1,492.4 $[M+H]^+$	C18		
Fengycin B	1,506.4 $[M+H]^+$	C17		
Surfactin	995.2 $[M+H]^+$	C12	1,017.2 $[M+Na]^+$	Leclère et al. 2005
	1,009.2 $[M+H]^+$	C13	1,031.2 $[M+Na]^+$	Argüelles-Arias et al. 2009
	1,023.2 $[M+H]^+$	C14	1,045.2 $[M+Na]^+$	Chen et al. 2019
	1,037.2 $[M+H]^+$	C15	1,059.2 $[M+Na]^+$	
	1,050.3 $[M+H]^+$	C16	1,073.2 $[M+Na]^+$	
			1,087.3 $[M+K]^+$	

Growth of *F. oxysporum* f. sp. *physali* measured by optical density at 48 h after contact with the supernatant from Landy media fermented by Bs006 was also reduced in a concentration-dependent manner. At 24 h, the supernatant significantly reduced the growth of the fungus from 2% supernatant treatment in comparison with its respective controls (PDB supplemented with sterile medium). Although *F. oxysporum* f. sp. *physali* continued growing in all treatments after 24 h (Supplementary Fig. S8), the supernatant added to PDB caused a significant reduction of the fungus growth in all tested concentrations (Fig. 2B), and a significant reduction of the specific growth rate in the log phase was observed under 10% supernatant treatment (Supplementary Table S1).

Effect of pure CLPs. Iturins and fengycins reduced the germination of the fungus in a concentration-dependent manner, whereas surfactins did not affect conidial germination. The presence of iturins in PDB medium significantly reduced *F. oxysporum* f. sp. *physali* germination at 30 and 50 µM (i.e., 31.30 and 52.16 µg/ml, respectively) ($P < 0.05$), whereas in the case of fengycins, all tested concentrations significantly ($P < 0.05$) reduced germination of the fungus (Fig. 3). Efficacy of fengycins at 50 µM (i.e., 71.75 µg/ml) to reduce *F. oxysporum* f. sp. *physali* germination was 78%, and that of iturins at 50 µM was 28%, which suggests greater power of fengycins than iturins to inhibit the germination of *F. oxysporum* f. sp. *physali*.

Consistent with the effects on germination, the evaluation of *F. oxysporum* f. sp. *physali* growth showed that after 24 h of incubation of the pathogen in the presence of CLPs, compounds of iturin and fengycin families reduced the growth of the fungus in a concentration-dependent manner, whereas surfactins did not affect the growth of *F. oxysporum* f. sp. *physali*. The presence of iturins at concentrations of 30 and 50 µM and fengycins at 50 µM in PDB

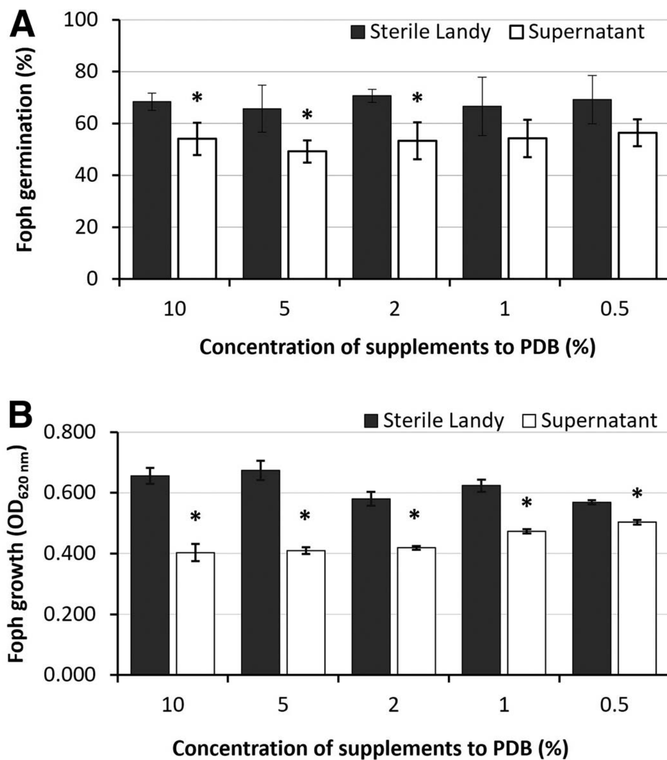

Fig. 2. Effect of filtered supernatant from liquid culture of Bs006 in Landy medium as supplement to potato dextrose broth (PDB) at concentrations of 0.5, 1.0, 2.0, 5.0, and 10% on **A,** the germination of microconidia and **B,** growth of *Fusarium oxysporum* f. sp. *physali* (Foph). Germination was measured at 24 h after incubation, and *F. oxysporum* f. sp. *physali* growth data were recorded at 48 h after incubation. Asterisks on the columns denote significant differences compared with the respective control according to two-sample *t* test (α = 0.05). Bars on the columns represent the standard deviation of data (*n* = 3).

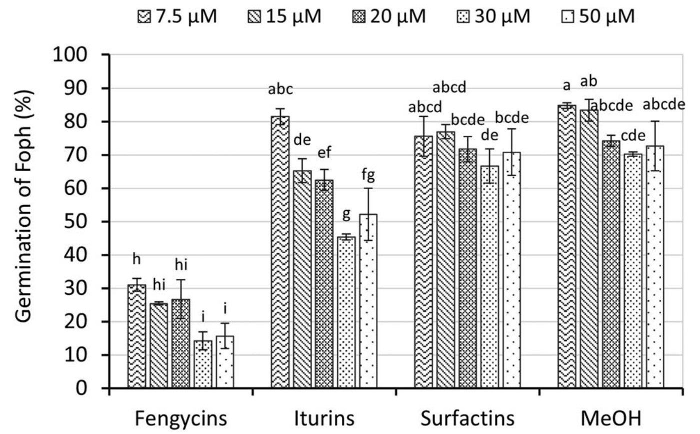

Fig. 3. Effect of pure cyclic lipopeptides (CLPs) (mix of homologous compounds in each CLP family) as supplement to potato dextrose broth (PDB) media at concentrations of 7.5, 15, 20, 30, and 50 µM on germination of *F. oxysporum* f. sp. *physali* (Foph) microconidia. Pure methanol (MeOH) and PDB without supplement were used as controls. Data were recorded at 24 h after incubation. Because germination of microconidia on PDB control was similar to that of MeOH, no effect of the solvent was assumed, and data from the PDB control were not included in the statistical analysis. Columns sharing the same letter are not significantly different according to the Tukey test (α = 0.05). Bars on the columns represent the standard deviation of data (*n* = 3).

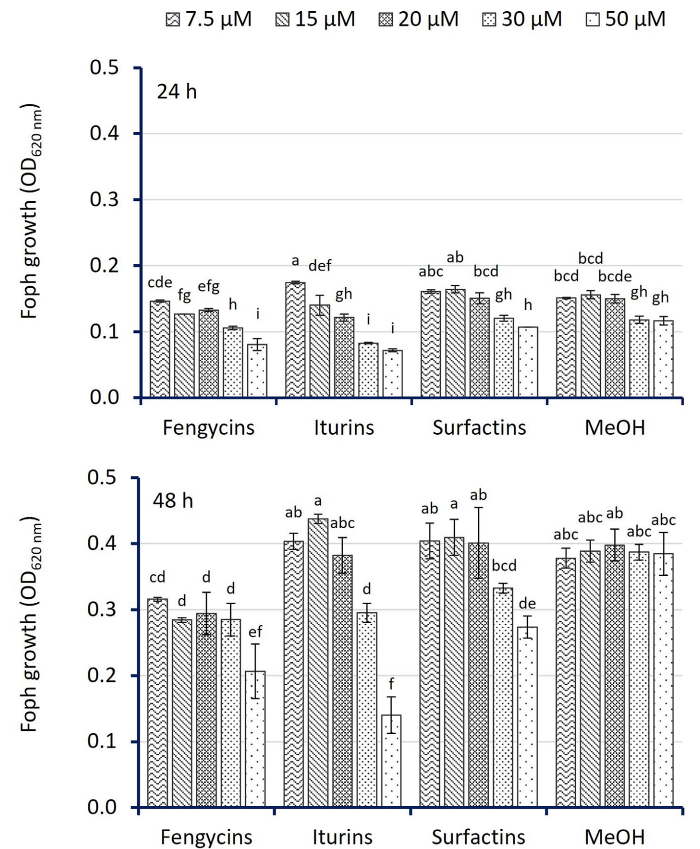

Fig. 4. Effect of pure cyclic lipopeptides (CLPs) (mix of homologous compounds in each CLP family) as supplement to potato dextrose broth (PDB) media at concentrations of 7.5, 15, 20, 30, and 50 µM on growth of *F. oxysporum* f. sp. *physali*. Pure methanol (MeOH) and PDB without supplement were used as controls. Data were recorded at 24 and 48 h after incubation. Because *F. oxysporum* f. sp. *physali* (Foph) growth on PDB control was similar to that of MeOH, no effect of the solvent was assumed, and data from PDB control were not included in the statistical analysis. Columns sharing the same letter are not significantly different according to the Tukey test (α = 0.05). Bars on the columns represent the standard deviation of data (*n* = 3).

medium significantly reduced the growth of *F. oxysporum* f. sp. *physali* ($P < 0.05$) compared with the control (PDB supplemented with methanol) (Fig. 4). Although surfactins at concentrations of 30 and 50 µM reduced fungal growth compared with the absolute control, this effect was not significantly different ($P > 0.05$) from the effect of methanol (Fig. 4), so the effect cannot be attributed to the presence of surfactins in the PDB medium. In terms of efficacy, the highest concentration of iturins and fengycins reduced the growth of *F. oxysporum* f. sp. *physali* by 38 and 31%, respectively. The growth of *F. oxysporum* f. sp. *physali* was also measured at 48 h of exposure to CLPs, and it was observed that the highest concentration of surfactins (50 µM, i.e., 49.65 µg/ml) caused a significant reduction of *F. oxysporum* f. sp. *physali* growth ($P < 0.05$), compared with the controls (Fig. 4). Meanwhile, the negative effect of iturins was maintained at the concentrations of 30 and 50 µM, whereas in the case of fengycins, all concentrations significantly reduced the growth of the fungus (Fig. 4). Iturins at 50 µM reduced growth of the fungus by 63%, whereas the same molar concentration for fengycins and surfactins reduced growth of *F. oxysporum* f. sp. *physali* by 46 and 29%, respectively. Correlation analysis between measured variables of *F. oxysporum* f. sp. *physali* development (germination and growth) and CLPs concentration established consistent effects of fengycins and iturins on germination. However, a stronger correlation was found for all CLPs and growth of *F. oxysporum* f. sp. *physali* measured by optical density ($R^2 = 0.61$ to 0.77). This analysis showed an inverse relationship between the concentration of CLPs and *F. oxysporum* f. sp. *physali* development (Supplementary Fig. S9). Thus, these results indicate that iturins and fengycins are the main CLPs compounds causing effective reduction of *F. oxysporum* f. sp. *physali* growth and allow us to suppose that these types of compounds in supernatant from Bs006 liquid culture also may be involved in the reduction of the fungus growth.

The exposure of *F. oxysporum* f. sp. *physali* to the Bs006 supernatant showed significant reduction of microconidia germination from 2% (5 µl of supernatant in 250 µl of broth), which represents the dosages of 7.2 and 3.2 mg/liter for fengycins and iturins, respectively, which are the main compounds causing significant effects, according to the previously mentioned results. Additionally, exposure of *F. oxysporum* f. sp. *physali* to individual treatments of pure fengycins and iturins showed significant effects from 7.5 µM (approximately 10.8 mg/liter) and 20 µM (approximately 20.9 mg/liter), respectively. Preliminary evaluations showed that concentrations of CLPs <7.5 µM did not significantly reduce either germination or growth of the fungus. These findings suggest that those are the lowest concentrations of single treatments of fengycins and iturins to significantly reduce *F. oxysporum* f. sp. *physali* development.

Description of the damage caused by the supernatant and pure CLPs on F. oxysporum *f. sp.* physali. Microscopic observations to *F. oxysporum* f. sp. *physali* microconidia exposed to the Bs006 supernatant revealed destruction and morphology changes of microconidia and hyphae in a concentration-dependent manner. They consisted of pore formation, cellular swelling, vacuolization, lysis, and inhibition of germination. Damage was stronger and affected a larger population of *F. oxysporum* f. sp. *physali* microconidia when higher concentrations of supernatant were used as a supplement to the PDB medium (Fig. 5). Furthermore, the evaluation of pure lipopeptides showed physical damage to the microconidia and the hyphae exposed to fengycins, which were similar to the damage caused by supernatant (Fig. 5). It was observed that iturins and surfactins caused no physical injuries to *F. oxysporum* f. sp. *physali*.

Antifungal activity of Bs006 in a gnotobiotic system. *B. velezensis* Bs006 colonized the main and secondary roots of golden berry seedlings and formed a biofilm on the surface at 4 days post-inoculation (dpi) (Fig. 6). This biofilm contributed to an inhibition zone, visibly reducing the growth of *F. oxysporum* f. sp. *physali* colonies toward roots after inoculation of the fungus in the center of the Petri dish, whereas in the control plant (free of *Bacillus*), *F. oxysporum* f. sp. *physali* colony growth took place without restriction (Fig. 6). By measuring the distances between the main root of each seedling to the edge of the *F. oxysporum* f. sp. *physali* colony, it was possible to calculate the efficiency of the treatment. Thus, it was found that growth of the fungus was reduced by Bs006 root colonization by 62 ± 21%.

Pattern of CLPs in the gnotobiotic system. UPLC-ESI-MS analysis showed the presence of compounds of the three CLPs families on both the surface of Bs006-colonized root and the agar of the growth inhibition zone. Homologous compounds of iturins from C13 to C16, fengycins from C15 to C18, and surfactins from C12 to C15 were found. Generally, the compounds found in the root were also found in the inhibition zone (Supplementary Fig. S10). The concentration of iturins and fengycins was higher than the concentration of surfactins in both root and agar. However, the concentration of iturins was higher than the concentration of fengycins in the agar, suggesting greater diffusion of iturins in the medium (Fig. 7). No CLPs were detected in the roots and agar close to control plants. Here, the results indicate that Bs006 was able to use root exudates, colonize the surface of the root, synthesize CLPs, and excrete them. Thus, those were spreading on the agar and probably were implicated in the reduction of *F. oxysporum* f. sp. *physali* growth, considering the results described previously. But because the lowest tested concentration of pure fengycins and iturins causing delay of *F. oxysporum* f. sp. *physali* growth were greater than concentrations in root–agar system and supernatant-microplate bioassays, these results suggest that CLPs are not the only compounds involved, and there may be other Bs006 compounds or plant compounds related to resistance, causing the antibiosis effect.

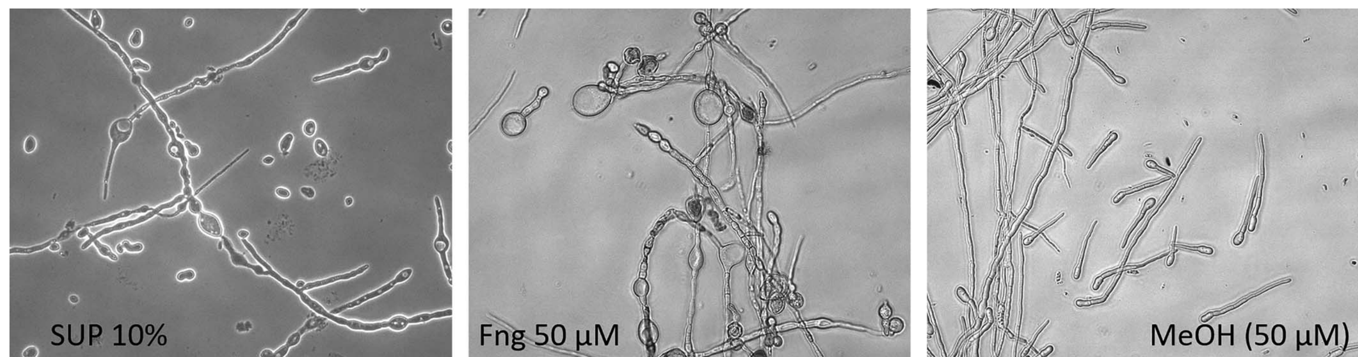

Fig. 5. Effects of supernatant from liquid culture of *Bacillus velezensis* Bs006 added to potato dextrose broth (PDB) at 10% (SUP 10%) and the mix of homologous compounds of fengycins at concentration of 50 µM (Fng 50 µM) on *F. oxysporum* f. sp. *physali* microconidia at 24 h after incubation under 30°C, 125 rpm, and darkness conditions. Pure methanol added to PDB medium at the same volume as fengycins 50 µM treatment (MeOH 50 µM) represents the control.

Biocontrol activity of Bs006 and supernatant under greenhouse conditions. All biological treatments kept the incidence of the disease at a lower level when compared with the negative control throughout the evaluation time (Fig. 8A). On the other hand, the severity of disease was not reduced by the treatments, indicating that once the plants were infected by *F. oxysporum* f. sp. *physali*, none of biocontrol treatments delayed the development of symptoms. In terms of disease incidence progress (proportion of infected plants over time), the treatments showed significantly lower values of the AUDPC compared with the negative control at 46 dpi. The application of the supernatant from Bs006 culture in Landy medium showed the lowest level of disease progress (Fig. 8B), at 53% effectiveness, followed by the effect of the combination of Bs006 cells and supernatant, in which the progress of the disease incidence in plants was reduced by 45%. However, the effect of combining cells and supernatant on the disease progress was not significantly different to the effect of the single application of Bs006 cells (37% effectiveness). The curves describing the disease development (R^2 0.97 to 0.98) followed a linear model during the time of evaluation. Thus, ANOVA to determine the effect of treatments on the slopes of the curves showed significant effects ($P < 0.001$). The slope of the *F. oxysporum* f. sp. *physali* curve in the negative control (2.33) was significantly higher than the slopes of the curves in Bs006 (1.75), supernatant (1.44), and Bs006+supernatant (1.39) treatments. Disease growth rate in the experimental units treated with the combination of Bs006+supernatant was significantly different from those treated only with Bs006. These results indicate that both Bs006 viable cells and supernatant from Bs006 liquid culture, applied to the soil, protected golden berry plants from *F. oxysporum* f. sp. *physali* infections. In the first case, the bacterium must be active in the rhizosphere, expressing its modes of action against the pathogen, and in the second case, some compounds in the supernatant, including cyclic lipopeptides, play an important role against *F. oxysporum* f. sp. *physali*.

Biocontrol activity of pure CLPs under greenhouse conditions. The incubation period of the disease was 3 days longer in plants treated with cyclic lipopeptides than the negative control. However, only the application of fengycins and the mixture of lipopeptides maintained disease incidence at a level considerably lower than those recorded in plants treated with surfactins, iturins, and the control 3 weeks after inoculation of the pathogen (Supplementary Fig. S11A). Therefore, the AUDPC of the incidence was significantly lower with the application of fengycins and the mixture of CLPs as compared with the control (Table 2). The effectiveness of these treatments for reducing the progress of FW incidence was 39 and 35%, respectively. On the other hand, progress of the severity was not significantly reduced by the treatments (Table 2). Additional linear regression analysis of disease incidence on time was performed to compare the effect of the CLPs on the slope of the FW curves. Logistic transformation ($[\ln[y/(1-y)]]$) was used to linearize the incidence progress data, in which y is the proportion of symptomatic plants (Bowers and Parke 1993). Although a 0.01 value was substituted for incidence = 0, data from 31 to 47 days gave a better linear fit (Supplementary Fig. S11B); therefore, these were used for the regression analysis. The R^2 and slope values for the regression of logistically transformed data from 31 to 47 days after inoculation are shown in Table 2. The R^2 values were significant ($P < 0.05$) for all curves. However, the ANOVA showed no significant effects of the treatments on the growth rate of the incidence (Table 2). Accordingly, the findings here indicate that CLPs are active in vivo conditions, but not at the same level, with fengycins being the only group of compounds that worked under greenhouse conditions. Last, compared with controls, reduction in AUDPC for fengycins was lower than that for supernatant, which suggests that fengycins are tightly involved in the biocontrol activity of Bs006, but additional compounds produced by this strain must be involved too.

DISCUSSION

Species of *Bacillus* belonging to the *B. subtilis* complex are known for synthesizing compounds of the three nonribosomal

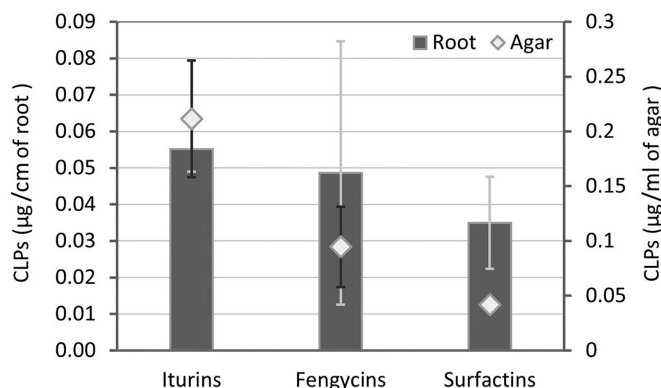

Fig. 7. Concentration of cyclic lipopeptides (CLPs) produced by *Bacillus velezensis* Bs006 on the root surface of golden berry (Root) and inhibition zones between inoculated root and *Fusarium oxysporum* f. sp. *physali* (Agar) in the gnotobiotic system. Samples were taken 9 days after inoculation of Bs006 on the crown root of golden berry seedlings (5 days after inoculation of *F. oxysporum* f. sp. *physali*). Bars on the columns represent standard deviation of the data ($n = 3$).

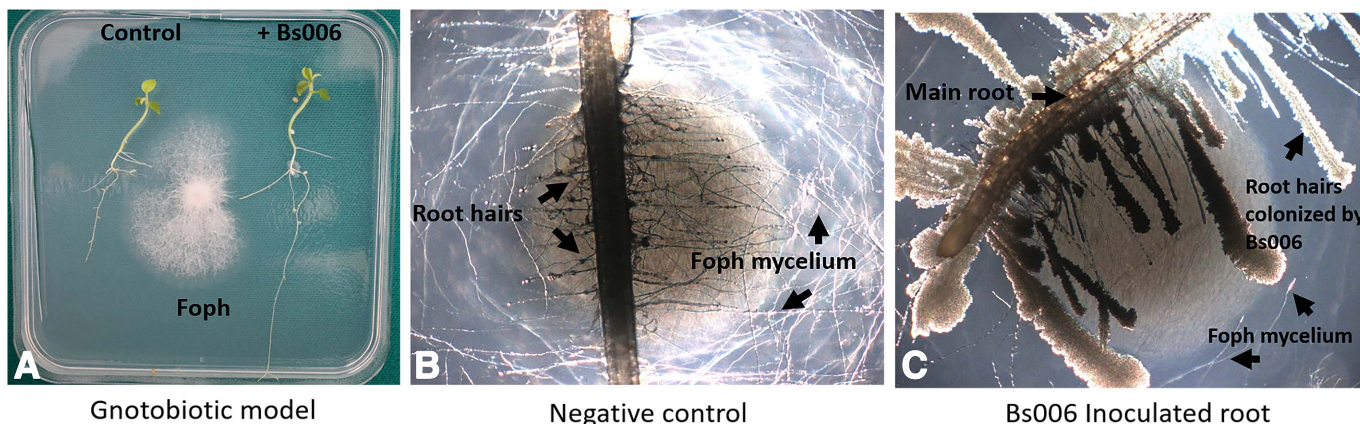

Fig. 6. Gnotobiotic model used to study interaction between *Bacillus velezensis* Bs006, *Fusarium oxysporum* Map5, and *Physalis peruviana*. Control plant was inoculated with sterile distilled water (negative control), and the treated plant was inoculated with Bs006 (+Bs006). **A,** Notice the inhibition zone of growth of *F. oxysporum* f. sp. *physali* (Foph) between fungus and inoculated root with Bs006. **B,** Negative control with dense mycelium of Foph around the root. **C,** Root inoculated with Bs006 with few hyphae approaching the root and biofilm of Bs006 on the root surface. Pictures were taken 5 days after inoculation of Foph in the system.

CLPs families: iturins, fengycins, and surfactins (Fan et al. 2017). Because the secretion of these substances has been shown to be responsible for its protective effect against phytopathogens (Cawoy et al. 2014, 2015), this ability is an important attribute in the context of the biological control of plant diseases. In this context, *B. velezensis* Bs006 has high potential to be used as an alternative to chemical control in managing plant diseases, because the simultaneous production of the three types of CLPs seems to be important for an effective biocontrol activity and is a key factor determining the ability of the strain to control several phytopathogens (Cao et al. 2018; Khan et al. 2017).

In the present study, Bs006 produced CLPs in liquid culture, in the growth inhibition zones against *F. oxysporum* f. sp. *physali*, and on the root surface of golden berry. The profile of CLPs was

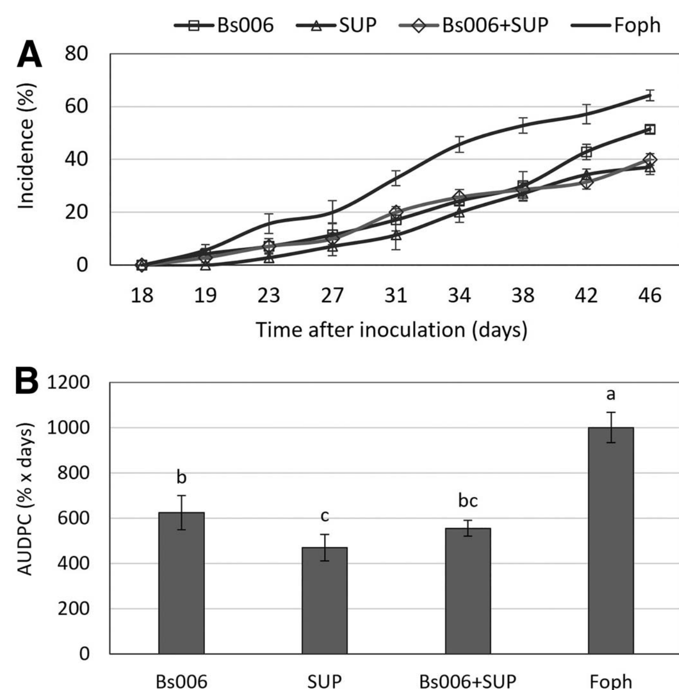

Fig. 8. Effect of individual and mix application of Bs006 cells (Bs006) and filtered supernatant from liquid culture (30°C, 48 h, 150 rpm) of Bs006 (SUP) in Landy medium on the progress of Fusarium wilt incidence in golden berry. **A,** Progress curves of the incidence. **B,** Area under the disease progress curves (AUDPC) for 46 days after inoculation. Grown plants in soil artificially inoculated with the pathogen and without biocontrol treatment represent the negative control (*Fusarium oxysporum* f. sp. *physali* [Foph]). Bars on the curves and columns represent standard error of the pooled data from two independent experiments, which showed equality of variances (n = 7). Treatments sharing the same letter are not significantly different according to Duncan's multiple range test (α = 0.05).

maintained in these three systems, but the concentration was different in each model of study. Thus, the highest concentrations of CLPs were in liquid media, followed by solid media and the lowest levels on the root surface. Moreover, the proportion of CLPs families found in the gelified media was slightly different from the proportion observed in liquid media, indicating an influence of the substrate on the biosynthesis of these compounds.

The profile of CLPs produced by Bs006 in the solid media was similar under both the presence and absence of *F. oxysporum* f. sp. *physali*. By contrast, other works have reported modulation of CLPs biosynthesis by pathogenic microorganisms (Cao et al. 2018; Cawoy et al. 2015). However, we have observed negative effects of *F. oxysporum* f. sp. *physali* on CLP production by Bs006, rather than stimulation, in dual confrontation tests on more nutrient-rich culture media and under liquid coculture conditions (unpublished). This suggests that the fungus can also synthesize compounds that affect the growth of Bs006 and consequently the yield of CLPs. Indeed, antimicrobial compounds from *F. oxysporum* are thought to increase pathogen survival and prevalence when confronted with biocontrol agents (Bacon et al. 2004, 2006; Marzano et al. 2013). Thus, this kind of response between antagonist and pathogen might be a species-specific interaction.

Understanding the modes of action directing the antagonistic activity of a biocontrol agent is essential for developing effective commercial bioformulations and designing application methods to potentiate the biocontrol activity (Guleria et al. 2016). In the present work, Bs006 reduced the growth of the *F. oxysporum* f. sp. *physali* colony, whereas the supernatant free of Bs006 reduced the germination of microconidia and development of mycelium and affected the cellular structure integrity of *F. oxysporum* f. sp. *physali*. This suggests that Bs006 produces substances with fungistatic and fungitoxic potential activities. This result is consistent with those of Kumar et al. (2012), who showed that CLPs from strains of *Bacillus* spp. cause both types of effects on fungal cells in a concentration-dependent manner. However, the final result of interactions between CLPs and phytopathogenic fungi depends on the nature of the target pathogen (Cawoy et al. 2015). Here, we observed no effects on the cellular integrity of *F. oxysporum* f. sp. *physali* when incubated with iturins or surfactins. However, effects such as swelling, lysis, and total degradation of fungi cells have been reported for iturins (Kumar et al. 2012) and fengycins (Vanittanakom et al. 1986) as well as surfactins (Carrillo et al. 2003). These damages on fungi are caused by the destabilizing action on the plasma membrane (Carrillo et al. 2003; Maget-Dana and Peypoux 1994; Patel et al. 2011).

Effects such as formation of pores, formation of ion channels, and loss of cellular integrity caused by CLPs from *Bacillus* spp. might also be the result of cell wall degrading enzymes (Baysal et al. 2013; Chérif et al. 2002; El-Bendary et al. 2016; Inès and Dhouha 2015; Rocha et al. 2014) whereby a synergistic effect

TABLE 2. Effect of pure cyclic lipopeptides (CLPs) supplied to the soil on vascular wilt development in golden berry

Treatment[a]	AUDPC (% × days)[b] Incidence		Severity		r[c]		P[d]	R^{2}[e]
Surfactins	782.50	(112.0)	520.50	(96.2)	0.13	(0.05)	0.0097	0.92
Iturins	717.50	(165.2)	521.50	(169.9)	0.15	(0.01)	0.0004	0.99
Fengycins	537.50	(75.8)[f]	395.50	(69.9)	0.18	(0.03)	0.0030	0.96
CLP mixture	565.00	(123.7)[f]	413.00	(112.3)	0.18	(0.03)	0.0047	0.95
Negative control	875.00	(203.9)	588.25	(171.7)	0.14	(0.05)	0.0013	0.98

[a] Negative control: golden berry plants grown in artificially inoculated soil with *Fusarium oxysporum* f. sp. *physali* (strain Map5) and not treated against the disease.
[b] AUDPC, area under the disease progress curve based on periodically recorded data of incidence and severity for 47 days after transplant. Values represent the average of four replicates with 10 plants each. Values in parentheses are standard deviations.
[c] Estimated rate parameter for logistically transformed curves in the period from 31 to 47 days after transplant. Data in parentheses represent the standard deviation of the estimated parameter.
[d] Probability that the estimated rate is not significantly different from zero (α = 0.05).
[e] Coefficient of determination for linear regressions of logistically transformed data of incidence on time.
[f] Significant differences at α = 0.05 according to the Dunnett test (α = 0.05).

between chitinases and antibiotics could also play a key role during antagonism (Chérif et al. 2002; Di Pietro et al. 1993). However, in this study the damages described previously were observed when *F. oxysporum* f. sp. *physali* was exposed to Bs006 supernatant and pure fengycins, indicating that fengycins have the ability to cause lysis to *F. oxysporum* f. sp. *physali* cells, similar to the action of chitinases. Although CLPs in agar inhibition halos have also been related to mycelial damage of the fungi (Torres et al. 2017), for the hydrolytic action of the enzymes contact between *Bacillus* spp. and phytopathogen is necessary (Chérif et al. 2002). Although the focus of this study was on CLPs, a previous work showed that Bs006 produces cell wall–degrading enzymes, which can also play a role in the control of *F. oxysporum* f. sp. *physali* (Izquierdo-García et al. 2020).

In this study, high concentrations of iturins were detected in the inhibition zones between Bs006 and *F. oxysporum* f. sp. *physali*, rather than fengycins and surfactins, suggesting that iturins play an important role in fungistatic activity against *F. oxysporum* f. sp. *physali*, agreeing with the report of Cawoy et al. (2015). However, the scientific reports are variable, showing both fungistatic and fungicidal effects caused by iturins and fengycins, depending on both the target fungus and the concentration of CLPs (Chitarra et al. 2003; Kumar et al. 2012; Li et al. 2005; Yuan et al. 2012; Zhao et al. 2014).

In the present study, the lowest concentration of fengycins needed to significantly reduce the growth of *F. oxysporum* f. sp. *physali* was 10.8 mg/liter. However, there are variable data and effects in the literature about the minimum inhibitory concentration of fengycins against *Fusarium* spp. (Hu et al. 2007; Vanittanakom et al. 1986; Zhao et al. 2014). In these previous studies, the effect of fengycins inhibited conidia germination and reduced mycelial growth but did not cause cell membrane damage or loss of cytoplasmic content. Moreover, based on the present work, it is important to consider that damages caused by supernatant and pure CLPs were present in a certain proportion of *F. oxysporum* f. sp. *physali* populations, which suggests that unaffected microconidia have some defense mechanism against fengycins and iturins. So tolerant propagules continued growing, as evidenced by a higher value of the optical density 48 h after incubation.

A large number of publications show the ability of *Bacillus* spp. to produce CLPs in vitro, but publications demonstrating the production of these compounds on the root surface or the rhizosphere are still limited. Many publications suggest some role of CLPs in the antagonism based on in vitro studies, but there are few studies about CLPs that involve the interaction of plant, pathogen, and biocontrol agents. Our gnotobiotic system demonstrated the ability of Bs006 to colonize and produce CLPs on the host root and its interaction with *F. oxysporum* f. sp. *physali*, suggesting that root exudates served as a nutritional source for Bs006. Synthesized iturins and fengycins were the most concentrated on the root, and this could be associated with *F. oxysporum* f. sp. *physali* growth prevention, in contrast to previous studies of the secretome of *B. velezensis* strains on roots (Debois et al. 2014; Fan et al. 2011), where surfactins were the most produced CLPs. Although at a lower proportion, Bs006 also synthesized surfactins on the physalis root, which might play an important role in the process of colonization and biofilm formation (Bais et al. 2004). This fact shows a huge potential to use Bs006 and its CLPs to protect golden berry root against *F. oxysporum* f. sp. *physali* infections.

Interestingly, one application of biological treatments to soil artificially inoculated with high concentrations of the virulent strain *F. oxysporum* f. sp. *physali*-Map5 showed a significant reduction of the progress of FW incidence, but this disease control measure was not completely effective. These results suggest an antagonistic role of Bs006 cells and the compounds produced by the bacterium in the culture medium that were applied to the soil. Specifically, 30 ml of supernatant solution (10%) per plant, containing 36.48, 16.9, and 3.8 mg/liter of fengycins, iturins, and surfactins, respectively, achieved the highest effectiveness (53%). Meanwhile, 30 ml of Bs006 cell suspension at 1×10^8 cfu/ml per plant showed an effectiveness of 37%. Comparatively, the efficacy of the application of 30 ml of pure fengycins and the mixture of the three pure CLPs solutions, containing the same concentrations of CLPs, was less than the application of the supernatant, suggesting that additional compounds produced by Bs006 in liquid culture could exert an effect against *F. oxysporum* f. sp. *physali*. However, these results also indicate that further studies need to be done to improve the efficacy of treatments through the evaluation of higher dosages of CLPs than evaluated in this study, the frequency of biocontrol applications, and the combination with other treatments such as other biocontrol agents, chemical fungicides, and cultural practices.

Additionally, the finding that that concentrations of CLPs observed in our gnotobiotic system were lower than the lowest tested concentrations that reduced the growth of *F. oxysporum* f. sp. *physali* found in microplate experiments supports our hypothesis that other compounds from Bs006, or synthesized by the plant under the effect of the rhizobacterium, could be involved in the antibiosis effect. Thus, under our experimental conditions the concentration of inhibitory compounds produced by Bs006 on the root surface or stimulated in the host and diffused to the medium were sufficient to prevent the progress of *F. oxysporum* f. sp. *physali* toward the plant. However, under natural rhizosphere conditions, the situation is probably different, considering that native microflora and environmental conditions may influence the colonization patterns of the bacterium, CLPs synthesis, and the stability of these compounds (Fan et al. 2011; Pertot et al. 2013).

Overall, the results of the present study show that *B. velezensis* Bs006 has the ability to colonize the surface of golden berry roots and to protect it against *F. oxysporum* f. sp. *physali* infection through CLPs synthesis. Among these compounds, iturins reduced microconidia germination of *F. oxysporum* f. sp. *physali* microconidia, whereas fengycins displayed both reduced germination and physical damage on *F. oxysporum* f. sp. *physali* in a concentration-dependent manner. Supernatant or pure CLPs produced by Bs006 could be incorporated as part of the biological treatment to control FW of golden berry. However, additional studies are needed to determine the impact of its application to the soil on the interaction between the native microflora and the development of vascular wilt, thus adjusting the directions for use as a biopesticide.

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