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Bacillus spp. and *Pseudomonas putida* as inhibitors of the *Colletotrichum acutatum* group and potential to control Glomerella leaf spot

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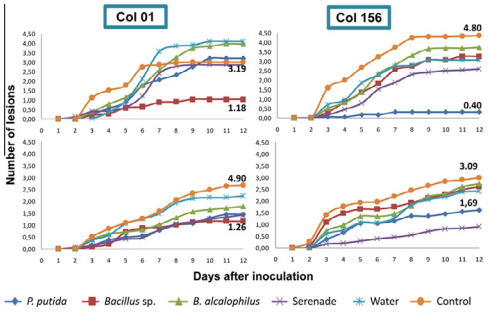
HIGHLIGHTS

- *Bacillus* isolates have potential for biocontrol of *Colletotrichum acutatum* group.
- Differences in the mode of action of *Bacillus* isolates were observed.
- The *C. acutatum* group has different sensitivity to the tested antagonists.
- Serenade® produces antifungal thermostable metabolites against Glomerella leaf spot.

GRAPHICAL ABSTRACT



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ABSTRACT

The control of Glomerella leaf spot (GLS) in Brazil is solely based on fungicide sprays and new alternatives are needed. In apple, few biological control methods have been evaluated, and most have focused on post-harvest pathogens. Therefore, the objectives of this work were to study the mode of action of three bacterial strains and the commercial product Serenade® (*Bacillus subtilis*) against the *Colletotrichum acutatum* group, the causal agents of GLS, and to evaluate the influence of bacterial isolates and Serenade® on the development of the first cycle of infection disease under controlled conditions. To assess the mode of action of the bacterial isolates against strains of the *C. acutatum* group, *in vitro* tests were performed. It was tested the effect of the bacteria on conidial germination and mycelial growth, using three methodologies, (i) fungal-bacterial co-cultivation, (ii) bacterial thermostable metabolites and (iii) bacterial volatile compounds. The influence of the bacterial isolates on the GLS development was assessed using apple seedlings. The seedlings were first sprayed weekly with bacterial suspension for 5 weeks, and were then inoculated with conidia suspensions (10^4 conidia mL^{-1}) of *C. acutatum* group isolates. Seedlings were maintained in chambers (CONVIRON) at 25 °C and a 12-h light regime. Disease severity of GLS was evaluated daily by counting typical lesions caused by *C. acutatum* group on all leaves during 12 consecutive days. The disease progress curve was fitted to nonlinear models for incidence and severity data. The treatments were compared by contrasting epidemiological parameters. *Bacillus* sp. isolated from the apple phylloplane inhibited more than 60% of the *C. acutatum* group conidial germination. The mode of action of *Bacillus* sp. and *Bacillus alcalophilus* on the *C. acutatum* group was through the production of fixed and volatile compounds, which inhibited mycelial growth. The primary mode of action of Serenade® on the *C. acutatum* group was the production of thermostable metabolites capable of completely inhibiting mycelial growth. In the GLS disease cycle, it was possible to adjust the monomolecular model for incidence and the number of lesions. There were significant differences between the epidemiological parameters of GLS

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in seedlings treated with apple phylloplane bacteria or with Serenade® as compared to the controls, indicating a potential for the use of biological control to manage GLS in apple orchards.

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1. Introduction

The Glomerella leaf spot (GLS) disease caused by species of the genus *Colletotrichum* affects apple (*Malus domestica* Borkh.) and occurs with greater intensity in regions with humid subtropical climate, including the Southeast of the United States (González et al., 2006) and the South of Brazil (Becker et al., 2000). GLS usually develops during rainy summers and has become a major concern in recent years, especially in areas where the highly susceptible cultivar 'Gala' is widely planted. Under such conditions, GLS can cause severe defoliation of more than 75%, thereby reducing yield and weakening the nutritional reserves of the plants (Becker et al., 2000; González et al., 2006).

Control of GLS is based on fungicides (Becker et al., 2000). Frequent applications are effective, but also increase production costs and can have damaging effects on the environment and human health. Moreover, plant pathogens can become resistant to fungicides and it can occur fail in the control (Stadnik et al., 2009).

An alternative to the use of fungicides is the application of epiphytic bacteria, which compete with the leaf pathogens for the same niches and thereby reduce disease severity. Leben (1965) recommended that microorganisms isolated from the phylloplane of plants could reduce the inoculum potential of pathogens. Studies pointed out some of the under lying causes, including competition for nutrients between the antagonist and the pathogen, and the production by the antagonist of substances that are potentially harmful to the pathogen (Blakeman and Brodie, 1977; Trotel-Aziz et al., 2008).

Biological control of plant pathogens is mediated by different antagonistic mechanisms, which can be divided into competition, parasitism, antibiosis, predation, hypovirulence and induction of host defense (Cook and Baker, 1983), but it is likely that different mechanisms act synergistically during an antagonistic interaction (Punja and Utkhede, 2003). Knowledge of the nature of these mechanisms is crucial to maximizing the effectiveness of biological control in each system (Blakeman and Fokkema, 1982).

GLS is the main disease during summer in all production areas of Brazil, reaching high severity levels due to the extremely favorable climate, and resulting in damage and high inoculum pressure in the subsequent season (Kowata et al., 2010). Under these conditions, control by fungicides is often ineffective, and there are no fungicides available for use during the harvest period. Studies of the potential of biological control agents against GLS will show whether biocontrol may provide apple growers with an important alternative. Biocontrol may also reduce the 10% cost increase due to the need for frequent fungicide applications, which nonetheless are not completely effective and are still associated with losses of approximately 20% (Katsurayama and Boneti, 2012).

In the present study, we tested the commercial product Serenade® that is a formulation of *Bacillus subtilis* strain QST 713 and three additional bacterial strains that were obtained from the apple tree phylloplane from different apple-producing areas in the state of Paraná (Rollemberg, 2008). The objectives of this work were to study the mode of action of the three bacterial strains and of Serenade® against the *Colletotrichum acutatum* group, and to evaluate the influence of bacterial isolates and Serenade® on the development of the first cycle of infection disease under controlled conditions.

2. Materials and methods

Three bacterial isolates were selected from a mass selection (142 isolates) made by Rollemberg (2008). These three selected isolates were obtained from leaves of healthy apple plants from the State of Paraná, these included isolate A68 from Porto Amazonas, isolate C02 from Campo Largo and isolate C12 from Quatro Barras. Isolates A68 and C12 were identified by fatty acid gas chromatography as *Pseudomonas putida* and *Bacillus alcalophilus*, respectively. Isolate C02 was identified by amplification of the 16S rRNA gene by PCR as *Bacillus* sp. and showed 100% similarity with sequences of the 16S rRNA gene of strains of *Bacillus amyloliquefaciens*, *Bacillus methylotrophicus*, *B. subtilis*, *Bacillus vallismortis* and *Bacillus siamensis* contained in data bases. In some experiments, Serenade®, that is a formulation of *B. subtilis* strain QST 713, was also tested. We used two strains of the *C. acutatum* group, Col01 and Col156, which were obtained from leaves of apple 'Gala' displaying symptoms of Glomerella leaf spot. The pathogenicity of the isolates was confirmed by inoculation of a conidia suspension on detached leaves (data not shown). The identity of the *C. acutatum* group isolates was confirmed by molecular analysis (data not shown).

2.1. Mode of action of Bacillus sp., P. putida and B. alcalophilus against the C. acutatum group

2.1.1. Effect on conidial germination

To evaluate the effect of bacterial isolates on the germination of conidia of the *C. acutatum* group, a drop of a 40 μL *C. acutatum* isolate Col01 conidia suspension adjusted to 1×10^4 conidia mL^{-1} was placed in the center of an empty polystyrene Petri dish. Different concentrations of bacteria suspensions were added in 40 μL volumes depending on the treatment, these included *Bacillus* sp. (C02), *P. putida* (A68) and *B. alcalophilus* (C12) at concentrations 1×10^4, 5×10^4 and 2.5×10^5 CFU mL^{-1}, and sterile distilled water as a control treatment.

For the preparation of the bacteria suspensions, isolates were streaked onto Petri dishes containing Nutrient Agar medium with sodium chloride (NaCl) 28 g L^{-1} (AES Laboratoire ®, Combourg, France) and maintained at 25 °C with a photoperiod of 12 h. After 24 h, 40 μL of the bacteria were transferred, with a platinum loop, to Erlenmeyer flasks containing Nutrient Broth liquid medium (13 g L^{-1}) (Himedia ®, Curitiba, Brazil), and the flasks were maintained for 48 h under constant agitation (120 rpm).

The Petri plates containing the fungal-bacterial suspensions were incubated at 25 ± 2 °C in the dark in a completely randomized experimental design with factorial treatments (4 treatments × 3 concentrations). Four replications in each Petri dish constituted an experimental unit. Conidial germination was determined by counting 50 conidia for each repetition after 24 h of incubation following the addition of lactoglycerol as a fixative.

2.1.2. Effect on mycelial growth by bacterial co-cultivation

To study the antagonistic effect of bacterial isolates on mycelial growth of the *C. acutatum* group isolate Col01, we paired fungal and bacterial colonies in Petri dishes on PDA culture medium (39 g L^{-1}) (Himedia ®) as described by Dennis and Webster (1971). Agar plugs of 5 mm in diameter were obtained from the edge of actively growing, eight-day-old *C. acutatum* group colonies, and one agar plug was placed in the center of each Petri dish

containing PDA medium. A day later, four drops (every drop with 40 μL) of the bacterial suspension standardized in a spectrophotometer at D.O. (540 nm) = 0.2 (2×10^5 CFU/mL) were deposited on the agar equidistantly 3 cm from the edge of the fungal colony. The treatments included *Bacillus* sp. (C02), *P. putida* (A68) and *B. alcalophilus* (C12), Serenade® (40 mL L^{-1}) and controls consisting only of the pathogen in culture medium. The plates were incubated at 25 ± 2 °C under a photoperiod of 12 h. We used a completely randomized design with four replications in each petri dish constituting an experimental unit. To assess mycelial growth, two perpendicular fungal colony diameters were measured 4, 7 and 11 days after addition of the bacteria, respectively.

2.1.3. Effect of thermostable metabolites on mycelial growth

Erlenmeyer flasks containing 100 mL of Nutrient Broth liquid medium were inoculated with 40 μL bacteria taken from a Petri dish. After incubation under laboratory conditions for 48 h under constant agitation (120 rpm), aliquots of 10 mL were transferred to vials containing 90 mL PDA medium, and then autoclaved for 20 min at 121 °C. The medium was poured into Petri dishes (25 mL each), and after solidification, each dish was inoculated with one 5 mm agar plug from the edge of an eight-day-old *C. acutatum* group isolate Col01 colony. The treatments were *Bacillus* sp. (C02), *P. putida* (A68) and *B. alcalophilus* (C12), Serenade® (40 mL L^{-1}), and the negative controls were composed of inoculated PDA plates without the presence of metabolites. The plates were incubated at 25 ± 2 °C under a photoperiod of 12 h. We used a completely randomized design, with four replications in each petri dish constituting an experimental unit. Two perpendicular fungal colony diameters were measured 11 days after inoculation.

2.1.4. Effect of antifungal volatile compounds

To study the effect of antifungal volatile compounds, the methodology was adapted from Dennis and Webster (1971). Bacteria were transferred onto one side of a bipartite Petri dish containing Nutrient Agar (28 g L^{-1}). After 24 h, 5 mm diameter mycelial plugs from the edge of eight-day-old *C. acutatum* group colonies were transfered to the other side of the bipartite Petri dish containing PDA medium. The treatments were *Bacillus* sp. (C02), *P. putida* (A68) and *B. alcalophilus* (C12), Serenade® (40 mL L^{-1}), and the negative controls consisted of plates containing water as a treatment. The plates were incubated at 25 ± 2 °C under a photoperiod of 12 h. We used a completely randomized design with three replications. To assess mycelial growth, two perpendicular fungal colony diameters were measured 4, 7 and 11 days after addition of the bacteria, respectively.

2.2. Interference on the development of the first cycle of infection disease under controlled conditions

Apple seedlings 'Gala' held in a greenhouse were sprayed weekly with bacteria suspended in sterile distilled water and standardized in a spectrophotometer at D.O. (540 nm) = 0.2 (2×10^5 CFU/mL) for 5 weeks from the onset of leaf sprouting using a 700 mL manual atomizer (Guarany, São Paulo, Brazil). The treatments included *Bacillus* sp. (C02), *P. putida* (A68) and *B. alcalophilus* (C12), Serenade® (40 mL L^{-1}), water and no application. The spray volume was calibrated to achieve run off, requiring an average of 20 mL per plant.

Bacterial suspensions were generated as described above followed by the addition of 8.5 g NaCl and 10 μL Tween 80/L of suspension. Plants were inoculated 24 h after the last bacterial treatment by spraying with fungal conidia suspension adjusted to 1×10^4 conidia mL^{-1} using *C. acutatum* group isolate Col01 or Col156. Fungi were grown in Petri dishes on oatmeal agar (40 g L^{-1} oatmeal, 16 g L^{-1} agar, 1000 mL distilled water) at 25 ± 2 °C for 8 days.

After inoculation, the individual plants were covered with wetted plastic bags that were attached and sealed with rubber bands to generate humid chambers. The seedlings were maintained in growth chambers Conviron® at 25 °C and a photoperiod of 12 h.

The trials were set up in a completely randomized design with four seedlings per treatment. Typical *Colletotrichum* type lesions were counted daily on all plants for 12 days, this time corresponds to one cycle of the disease, in other words, the number of lesions stabilized and, thereafter, new lesions would be generated by secondary infections. The experiment was repeated twice.

2.3. Data analysis

The conidial germination data was used to calculate the percentage of germination inhibition caused by the treatments compared to the controls. For the fungal-bacterial co-culturing experiment and the antagonistic volatile compounds experiment, index mycelial growth rates (IMGR) were calculated according to the formula described by Maguire (1962) as follows: IMGR = $\Sigma (D - Dp)/N$, with D = current average diameter of the colony, Dp = average diameter of the colony from the previous day, N = number of days after de deposition of de mycelium plug.

For the co-cultivation, the thermostable metabolite and the volatile compound experiments, the diameter of the pathogen colonies was measured at day 11. For all *in vitro* tests, the homogeneity of variances was verified by Bartlett's test. The data were then subjected to analysis of variance by verifying the effect of the treatments using the F test, significant means were compared by Tukey's test ($P < 0.05$).

For the incidence and the number of lesions data in the disease development under controlled conditions experiment were calculated the final incidence (I_f), the number of lesions late (NL_f) (last evaluation), areas under the curve of incidence progress (AUCPinc) and the number of lesions (AUCPnl), summing the trapezoidal areas (Campbell and Madden, 1990) based on the formula: $AUDPC = \sum_{i=1}^{n-1} [(t_{i+1} - t_i)(y_i + y_{i+1})/2]$, where: "$t$" is time in days of each reading, "y" is the percentage of incidence or number of lesions at each reading and "n" is the number of readings. The data were then subjected to analysis of variance by verifying the effect of the treatments using the F test, significant means were compared by Tukey's test ($P < 0.05$).

A monomolecular non-linear model ($y = 1 - (1 - y_0)$. Exp $(-rt)$) was fitted to the incidence and the number of lesions, where 'y' represents the incidence/number of lesions of the disease, 'y_0' the initial inoculum, 'r' the rate of disease progression and 't', the time in days after the first evaluation. The estimated model parameters between treatments were compared by a t-test ($p < 0.05$) as proposed by Madden et al. (2007). The analyses were performed in R software, version 2.13.2 (R Development Core Team, 2011).

3. Results

3.1. Mode of action of Bacillus sp., P. putida and B. alcalophilus against the C. acutatum group

Bacillus sp. (C02) was the only antagonist capable of inhibiting germination of conidia of the *C. acutatum* group at the three concentrations tested as compared to the control. At concentrations of 5×10^4 and 2.5×10^5 CFU mL the inhibition was higher (67.5% and 66.5%, respectively) than at concentration 1×10^4 - CFU mL (27.00%). *P. putida* (A68) and *B. alcalophilus* (C12) significantly inhibited conidia germination but only at concentration 2.5×10^5 CFU mL, by 28.5% and 22.5%, respectively. However, at

Table 1
Inhibition of conidial germination of the *Colletotrichum acutatum* group isolate Col01 after 24 h in contact with biological antagonists at different concentrations.

Treatment	Inhibition of conidial germination (%)[a] Concentration (CFU mL^{-1})		
	1×10^4	5×10^4	2.5×10^5
Pseudomonas putida (A68)	6.5 abB	20.0 bAB	28.5 bA
Bacillus sp. (C02)	27.0 aB	67.5 aA	66.5 aA
Bacillus alcalophilus (C12)	12.0 abA	20.0 bA	22.5 bA
Control	0.0 bA	0.0 bA	0.0 cA

[a] Original data without processing, means followed by the same lowercase letter in columns and uppercase letters in rows do not differ significantly based on Tukey's test at $p > 0.05$, CV (%) = 48.48.

the same concentration germination inhibition by *Bacillus* sp. (C02) was 38% and 44% higher, respectively (Table 1).

In the co-cultivation experiments, *B. alcalophilus* (C12), *Bacillus* sp. (C02) and Serenade® reduced the rate of mycelial growth rates (IMGR) and the maximum diameter of colonies of the *C. acutatum* group compared to the control. *P. putida* (A68) did not inhibit mycelial growth of the *C. acutatum* group when paired in culture (Table 2).

The thermostable metabolite experiment showed that Serenade® did produce such antifungal compounds which completely inhibited mycelial growth. Mycelial growth of the *C. acutatum* group was stimulated by the presence of the thermostable compounds produced by *P. putida* (A68), *Bacillus* sp. (C02) and *B. alcalophilus* (C12) (Table 2).

The volatile compound experiment showed the production of this type of compound by *B. alcalophilus* (C12) and *Bacillus* sp. (C02). These two antagonists reduced the *C. acutatum* group IMGR by 16% as compared to the control. Serenade® did not produce any volatile compounds capable of inhibiting the mycelial growth of the *C. acutatum* group (Table 2).

3.2. Interference on the development of the first cycle of infection disease under controlled conditions

There was no significant difference between treatments for the final incidence (I_f) and the area under the curve of incidence progress (AUCPinc) data for both isolates tested in any of the experiments (Table 3).

The number of injuries late (NL_f) and the area under the curve of the number of lesions progress (AUCPnl) observed in apple seedlings 'Gala' sprayed with biological antagonists, was only significantly different between treatments for the *C. acutatum* group isolate Col156 in experiment 2. However, the only treatment

Table 2
Index mycelial growth rates (IMGR) of the *Colletotrichum acutatum* group and maximum colony growth (MMGR) in cultures paired with bacterial isolates, in culture medium containing thermostable metabolites of bacterial isolates and in colonies under the influence of volatile compounds produced by bacterial isolates.

Treatment	Cultures paired		Thermostable metabolites	Volatile compounds	
	IMGR[a]	MMGR[a]	MMGR[a]	IMGR[a]	MMGR[a]
Control	0.72 a	3.75 a	4.77 a	0.54 a	3.05 a
Pseudomonas putida (A68)	0.57 ab	3.14 ab	4.39 a	0.51 ab	3.00 a
Bacillus alcalophilus (C12)	0.41 b	2.54 bc	4.31 a	0.49 abc	2.78 ab
Serenade®	0.37 bc	2.11 bc	2.80 b	0.45 bc	2.62 b
Bacillus sp. (C02)	0.16 c	1.40 c	0.00 c	0.45 c	2.53 b
CV (%)	24.50	20.63	19.25	4.84	4.23

[a] Original data without processing, means followed by the same letter in the column do not differ significantly by Tukey's test at $p > 0.05$. Serenade® = Commercial product based on *B. subtilis*. CV = coefficient of variation.

Table 3
Final incidence[a] (I_f), area under the progress curve of incidence (AUPCinc), number of injuries[b] late (NL_F) and area under the progress curve for number of lesions (AUPCnl) in apple seedling 'Gala' sprayed weekly with biological antagonists for 5 weeks and inoculated with *Colletotrichum acutatum* group isolates Col01 or Col156. Seedlings were kept in growth chamber Conviron® at 25 °C.

Treatment	Col01		Col156		Col01		Col156	
	I_f (%)	AUC Inc[c]	I_f (%)	AUPC Inc	I_f (%)	AUPC Inc	I_f (%)	AUCP Inc
Experiment 1								
P. putida (A68)	47.71 ns	373.16 ns	52.96 ns	388.24 ns	65.00 ns	433.48 ns	58.45 ns	449.94 ns
Bacillus sp.(C02)	30.56	221.53	48.61	382.84	65.42	475.00	74.11	558.93
B. alcalophilus (C12)	49.13	416.47	45.42	371.25	58.21	426.79	63.12	481.80
Serenade®	49.04	369.95	49.68	378.31	66.67	411.59	60.71	353.30
Water	53.70	396.05	41.88	354.06	81.93	541.67	76.74	558.51
Control	56.55	487.80	46.51	409.60	76.29	592.56	81.11	696.11
	NL_f (%)[b]	AUPCnl[d]	NL_f (%)	AACP nl	NL_f (%)	AUPC nl	NL_f (%)[d]	AUPC Nl[d,e]
Experiment 2								
P. putida (A68)	6.25 ns	32.13 ns	2.38 ns	13.19 ns	1.50 ns	9.00 ns	1.50 ab	10.88 ab
Bacillus sp. (C02)	3.25	23.13	1.50	9.63	0.88	5.81	4.50 a	32.00 a
B. alcalophilus (C12)	3.13	21.94	4.88	33.94	1.75	8.38	2.75 ab	17.00 ab
Serenade®	4.50	32.38	3.00	19.75	0.63	3.44	0.75 b	3.80 b
Water	3.50	22.63	2.63	22.81	1.25	8.38	2.88 ab	17.56 ab
Control	5.38	41.31	2.88	20.06	1.13	9.19	4.25 ab	34.88 a

[a] Percentage of seedlings with symptoms.
[b] Number of lesions per seedling.
[c] Transformed data $y = \sqrt{x}$.
[d] Means followed by the same letter in the column do not differ significantly by Tukey's test at $p > 0.05$.
[e] Transformed data $y = 3\sqrt{x}$. ns = not significant. Serenade® = commercial product based on *B. subtilis*.

where AUCPnl was significantly different from the control was Serenade® (Table 3).

The monomolecular model fitted to the data of incidence progress (Fig. 1) and the number of lesions of GLS in apple seedlings 'Gala ' resulted in a determination coefficient (R^2) greater than 88% for incidence data and above 92% for the number of lesions data.

The maximum asymptote (y_{max}) for the incidence data was significantly lower for C. *acutatum* isolate Col01 in treatments with *Bacillus* sp. strain C02 as compared to the controls in both experiments. The rate (r) in both experiments was significantly lower for Serenade® than for the controls. For the same *C. acutatum* isolate (Col01), when comparing the estimates for the number of lesions data parameters, the y_{max} in both experiments was significantly lower than for the control in seedlings treated with *Bacillus* sp. (C02) (Table 4).

Estimates of the effect of the initial inoculum (y_0) of *C. acutatum* isolate Col156 were not significantly different from the controls, except for *Bacillus* sp. (C02) in experiment 1. For *C. acutatum* isolate Col156, comparing the estimates for the number of lesions data parameters, the y_{max}, y_0 and the r in experiment 1 were significantly lower than the controls on seedlings treated with Serenade®. In experiment 2, the curve of the monomolecular model did not fit the number of lesions data (Table 5).

4. Discussion

The *Bacillus* isolates used in this study, including *Bacillus* spp., *B. alcalophilus* and the commercial product Serenade®, have potential for biological control *in vitro* and *in vivo* of the *C. acutatum* group. *In vitro*, the bacteria were capable of reducing the germination of the pathogen and the rate of mycelial growth in culture. *In vivo* the bacteria reduced the number of lesions in the disease cycle and the maximum amount of disease (y_{max}) compared with the control. The *Bacillus* isolates differed by their mode of action and by their effectiveness to control the different isolates of the *C. acutatum* group.

The main mode of action of Serenade® was through the production of thermostable metabolites, which completely inhibited mycelial growth of the *C. acutatum* group isolates. Indeed, strains of *B. subtilis* are well-known producers of a wide range of antifungal compounds (Asaka and Shoda, 1996; Ferreira et al., 1991; Leifert, 1995; Swinburne et al., 1975). *B. subtilis* QST 713 is known to produce over 30 different lipopeptides, this mode of action are

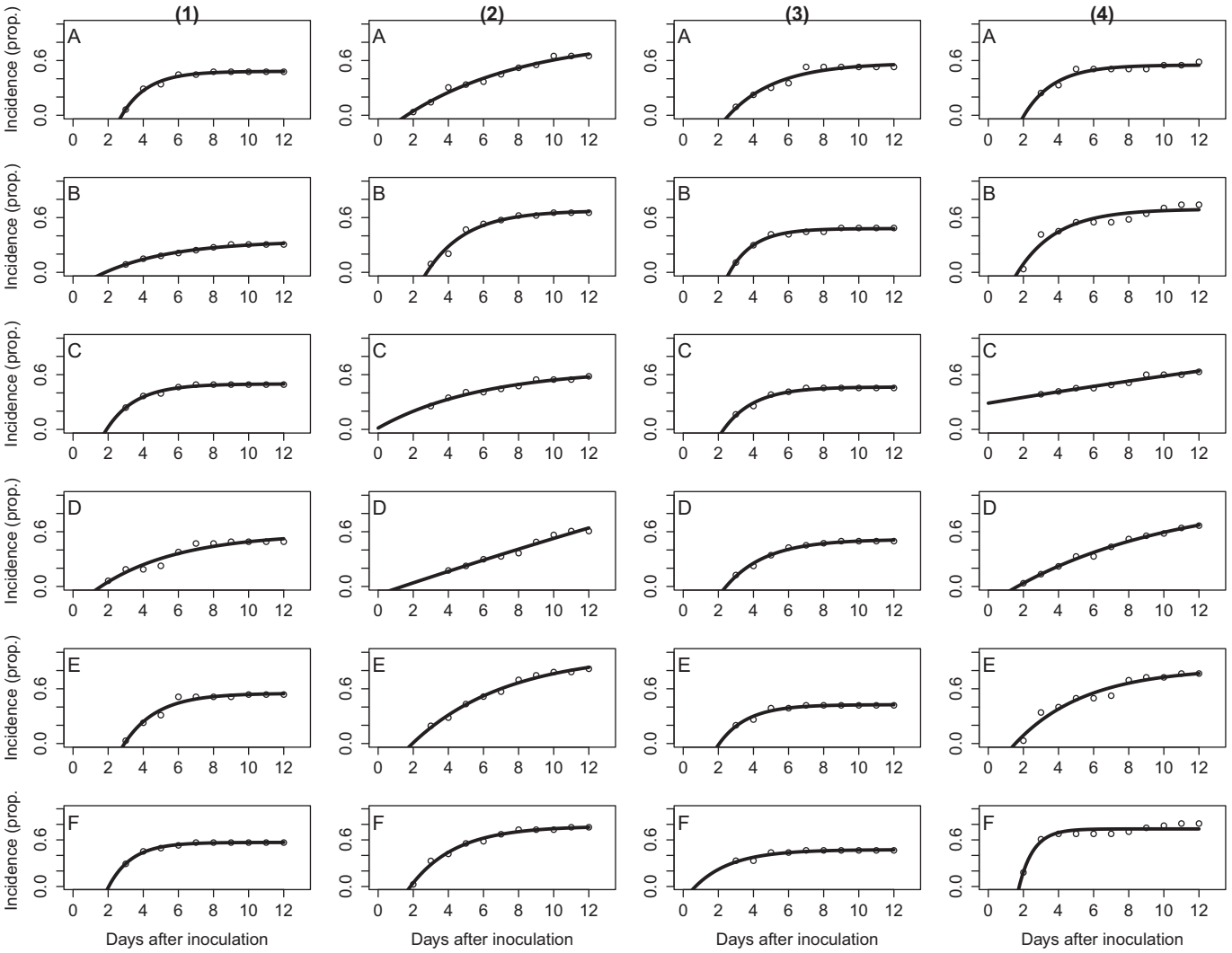

Fig. 1. Progress curves of incidence (% of leaves with symptoms) of GLS in apple seedlings 'Gala' sprayed with biological antagonists weekly for 5 weeks and inoculated with isolates of *Colletotrichum acutatum* group. Value of 1 on the *y* axis of the graph refers to 100% of the seedlings with symptoms. Points represent average incidence in 4 seedlings obtained by evaluation from the pathogen inoculation, and lines represent the fit to monomolecular model. 1 = Experiment 1, Col01; 2 = Experiment 2, Col01; 3 = Experiment 1, Col156; 4 = Experiment 2, Col156. A = *Pseudomonas putida*, B = *Bacillus* sp., C = *Bacillus alcalophilus*, D = Serenade, E = Water, F = Control. Curitiba/PR.

Table 4
Comparison of epidemiological parameters of the monomolecular model fitted to the data of incidence and number of lesions of GLS on apple seedlings 'Gala' treated with biological antagonists weekly for five weeks and inoculated with *Colletotrichum acutatum* group isolate Col01 from Curitiba/PR.

Treatment	Epidemiological parameters estimated					
	Incidence			Number of lesions		
	y_0	r	y_{max}	y_0	r	y_{max}
Experiment 1						
Control	−1.640	0.756	0.567	−3.620	0.374	3.188
Water	−1.640 ns	0.535 ns	0.550 ns	−5.960 ns	0.230 ns	5.132 ns
P. putida (A68)	−2.150 ns	0.676 ns	0.481*	−2.200 ns	0.130 ns	4.877 ns
Bacillus sp. (C02)	0.457*	0.247*	0.345*	−1.150 ns	0.264 ns	1.181*
B. alcalophilus (C12)	−0.580 ns	0.602 ns	0.498*	−2.770 ns	0.131 ns	6.107 ns
Serenade®	0.175*	0.219*	0.582 ns	−1.430 ns	0.103 ns	5.101 ns
Experiment 2						
Control	−0.590	0.387	0.777	−0.820	0.083	4.903
Water	−0.420 ns	0.186*	0.986*	−1.630 ns	0.186 ns	2.798 ns
P. putida (A68)	−0.090*	0.146 ns	0.860 ns	−0.320 ns	0.014 ns	11.29 ns
Bacillus sp. (C02)	−1.320 ns	0.443 ns	0.677*	−2.180 ns	0.340*	1.256*
B. alcalophilus (C12)	0.351*	0.167*	0.663 ns	−0.260 ns	0.042 ns	5.089 ns
Serenade®	−0.200*	0.107*	1.003 ns	−0.360 ns	0.023 ns	7.165 ns

ns = estimated parameters were not significantly different compared to the control by *t*-Student test at $p > 0.05$. y_{max} = maximum asymptote. y_0 = initial amount. r = rate of disease progress. Estimated values by the monomolecular model ($y = 1 - (1 - y_0)$. exp $(-rt)$).
* Estimated parameters showed significant difference compared to the control by *t*-Student test at $p > 0.05$.

Table 5
Comparison of epidemiological parameters of the monomolecular model fitted to the data of incidence and number of lesions of GLS on apple seedlings 'Gala' treated with biological antagonists weekly for five weeks and inoculated with isolate of *Colletotrichum acutatum* group isolate Col156 from Curitiba/PR.

Treatment	Epidemiological parameters estimated					
	Incidence			Number of lesions		
	y_0	r	y_{max}	y_0	r	y_{max}
Experiment 1						
Control	0.339	0.477	0.474	−2.910	0.273	4.802
Water	−0.540 ns	0.625 ns	0.426*	−4.060 ns	0.320 ns	3.351*
P. putida (A68)	−0.470 ns	0.362 ns	0.573*	−3.370 ns	0.129 ns	0.400*
Bacillus sp. (C02)	−2.170*	0.715 ns	0.479 ns	−3.270 ns	0.220 ns	3.888*
B. alcalophilus (C12)	−0.760 ns	0.577 ns	0.465 ns	−3.670 ns	0.213 ns	4.595 ns
Serenade®	−0.500 ns	0.436 ns	0.516*	−2.870*	0.210*	3.209*
Experiment 2						
Control	−5.400	1.223	0.741	−1.240	0.259	3.090
Water	−0.210 ns	0.247*	0.829 ns	−0.540 ns	0.058*	5.528 ns
P. putida (A68)	−0.800 ns	0.581*	0.550*	−1.160 ns	0.258 ns	1.689*
Bacillus sp. (C02)	−0.460 ns	0.443*	0.693 ns	−1.230 ns	0.263 ns	2.568 ns
B. alcalophilus (C12)	−0.950 ns	0.016*	2.241 ns	0.182 ns	−0.030*	−6.720 ns
Serenade®	−4.410 ns	0.012*	5.321 ns			

ns = estimated parameters were not significantly different compared to the control by *t*-Student test at $p > 0.05$. y_{max} = maximum asymptote, y_0 = initial amount, r = rate of disease progress. Estimated values by the monomolecular model ($y = 1 - (1 - y_0)$. exp $(-rt)$).
* Estimated parameters showed significant difference compared to the control by *t*-Student test at $p > 0.05$.

different from other fungicides and therefore represent an opportunity for resistance management and synergy with single-site fungicides (Serrano et al., 2013). Everett and Machin (2005) tested the efficiency of six products in the control of *Glomerella cingulata*, three conventional fungicides and three biological control agents, including Serenade® which significantly reduced symptoms ascribed to *G. cingulata*. *B. subtilis*, formulated as Serenade, has also demonstrated promising control of *Monilinia vaccinii-corymbosi*, the causal pathogen of mummyberry in blueberry (Scherm et al., 2004).

There are several mechanisms *Bacillus* spp. use to control pathogens, including competition for space and nutrients, induction of systemic resistance, production of siderophores and antibiosis (Thomashow and Weller, 1996). For example, the production of *Bacillus* spp. volatile antifungal metabolites may be an important mode of action against diseases caused by *Botrytis cinerea*, *Cercospora kikuchii*, *Alternaria solani* and *Alternaria brassicae* (Wei-Wei et al., 2008) and against *Colletotrichum gloeosporioides* on mangos both *in vitro* and *in vivo* (Zheng et al., 2013). Likewise, this study showed that the *Bacillus* sp. (C02) and *B. alcalophilus* (C12) isolates obtained from the phylloplane of apple trees produced volatile antifungal metabolites which played a major role in inhibiting isolates of *C. acutatum* group in *in vitro* tests.

Leifert (1995) studied two species of *Bacillus*, *B. subtilis* (CL27) and *B. pumilus* (CL45), and reported that the activity exerted *in vivo* against *Bo. cinerea* by *B. subtilis* (CL27) was due to the production of peptide antibiotics, because *B. subtilis* mutants unable to produce the antibiotic, failed to exhibit activity against *Bo. cinerea*. Similarly, Pichard et al. (1995) reported that the *in vitro* activity against *Xanthomonas campestris* pv. campestris, presented by *Bacillus polymyxa*, was due to the production of two peptide antibiotics. Among the various metabolites produced by *Bacillus* spp., peptide antibiotics are primarily responsible for the antagonistic action that control plant pathogenic fungi and bacteria. These include *Erwinia amylovora*, the burning agent of rosettes (Abo-El-Dahab and El-Goorani, 1964), *Uromyces phaseoli*, the cause of bean

rust (Baker and Stavely, 1985), and *Rhizoctonia solani*, *Pythium* spp., *Fusarium* spp. and *X. campestris* pv. campestris, the causal agents of various other diseases (Melo, 1998; Pusey, 1989). No antifungal compounds have yet been isolated from the apple phylloplane bacteria, which is an important study to be conducted in the future.

In addition to *Bacillus* sp. (C02), conidial germination of the *C. acutatum* group that is associated with GLS, was also inhibited by *P. putida* (A68) and *B. alcalophilus* (C12). However, the percentage inhibition was lower and occurred only at the highest concentration tested. More testing with increased concentrations is necessary to determine whether the inhibitory effect can be intensified. Inhibition of conidial germination by resident phylloplane prokaryotes was already observed by Hetherington et al. (1995), who found an isolate that suppressed *C. gloeosporioides* conidia germination by 95%. Parbery (1981) reported that certain *Bacillus* spp. showed lytic action against the walls of various rust spore types. Possibly, this may be due the absorption of the toxic bacterial metabolites together with large amounts of water during the early stages of germination (Alexopoulos et al., 1996).

In addition to the inhibitory effect of apple phylloplane bacteria on the conidial germination of *C. acutatum* group isolates, there also was a reduction in mycelial growth that differed between bacterial isolates. *Bacillus* sp. (C02) and *B. alcalophilus* (C12) reduced mycelial growth of *C. acutatum* group isolates upon co-cultivation and by production of volatile compounds, where as *P. putida* was not able to reduce mycelial growth as compared to the control. Similarly, Fuga et al. (2011) evaluated the inhibition of mycelial growth of *C. gloeosporioides*, the cause of fruit rots, using various bacteria from soil samples, and concluded that the isolates showed considerable variation in antifungal activity against *C. gloeosporioides in vitro*. The antagonistic potential of *Bacillus* spp. was also observed by Kim and Chung (2004) who reported inhibitory activity against *C. lagenarium* by a strain of *B. amyloliquefaciens* producing subtilisin.

Chen et al. (2008) identified 14 volatile antifungal compounds from *B. subtilis* that inhibited the development of *Bo. cinerea*, the causal agent of gray mold on fruits and vegetables. Among bacteria, *B. subtilis* strains can produce more than 24 antifungal substances with a considerable variety of structures (Stein, 2005). Bacteria isolated from canola and soybean roots, and from straw, produced volatiles that inhibited survival, infection and reproduction of *Sclerotinia sclerotiorum*, by acting on sclerotia, mycelium and ascospores. Of the 14 bacteria tested, 12 isolates produced medium to high levels of antifungal volatiles (Fernando et al., 2005). While studies demonstrated the effectiveness of bacterial volatile compounds on postharvest pathogens and on soil borne pathogens, their potential may be reduced in the phylloplane. The *Bacillus* isolates from the apple tree phylloplane were able to produce antifungal volatile compounds that reduced mycelial growth of *C. acutatum* group isolates *in vitro*. However, the effect of these compounds on orchard can be reduced to their volatile nature, so it is important to study new formulations to get better results with this isolates or to test those isolates to reduce of disease symptoms on fruit during the storage or commercialization.

Kupper et al. (2003) used isolates of *B. subtilis* and *Bacillus* spp. from leaves and flowers of citrus plants and found that all isolates caused inhibition of mycelial growth in *C. acutatum*, possibly due to the metabolites produced by the bacteria. Some isolates also produced thermostable metabolites that retained their antagonistic activity even after autoclaving. But metabolite thermostability was not observed for the isolates obtained from the apple phylloplane. *Bacillus* sp. (C02) and *B. alcalophilus* (C12) produced antifungal compounds, but they were probably heat sensitive, since bacterial culture broth did not inhibit mycelial growth upon autoclaving. Thus, these results suggested that isolates of *Bacillus* spp. produced metabolites with different properties and perhaps different modes of action. The fact that the apple phylloplane isolates produced inhibitory substances towards the *C. acutatum* group, is important for our understanding of the mechanism of action of such compounds, which enables us to control the pathogens by application of the antagonistic substances directly, instead of by the introduction of live bacteria into orchards. Furthermore, the study of the antagonistic potential *in vitro* is important to guide selection of methodologies for future field trials.

In the experiments related to interference on the development of the first cycle of infection disease under controlled conditions, there were no significant differences between the phylloplane bacteria, Serenade®, water, and the control, on AUCPinc, AUCPsev, NL_f and I_f. However, differences between treatments after adjusting the monomolecular model and between the estimated epidemiological parameters could be observed. Jeger (2004) stated that analysis of the progress of a disease provides important information on the development of epidemics and control measures, but stressed that the epidemiological parameter estimates obtained from nonlinear models are the most important aspect for comparative treatments and interpretations of biological effects in a system.

When comparing severity parameter data, an important detail was that the inoculum did not differ from the control treatment and in most cases, even with an increase in inoculum, the amount of disease did not increase. Probably the treatment coverage of the leaf surfaces was not perfect, which may explain the similarities in initial inoculum. The initial inoculum is an important parameter related to the monocycle of a disease, because according Bergamin Filho (2011), the increase in symptoms is mainly due the amount of inoculum that reaches the surface of the plant. However, the GLS is a polycyclic disease and symptom increase is mainly due to secondary cycles of the pathogen on the host, and does not just depend on the initial inoculum (Bergamin Filho, 2011). Thus, based on studies of the interference on the development of the first cycle of infection disease under controlled conditions, it can be seen that the preventive application of biological control agents onto leaf surfaces can be important to prevent primary infection by the pathogen.

5. Conclusions

Bacillus sp. (C02) inhibited germination of the *C. acutatum* group isolates by more than 60%. *Bacillus* sp. (C02) and *B. alcalophilus* (C12) produced fixed and volatile compounds that inhibit the mycelial growth of *C. acutatum* group isolates from apple to a similar degree. The primary mode of action of the commercial product Serenade® on the *C. acutatum* group was by production of thermostable metabolites capable of inhibiting mycelial growth by 100%.

There were significant differences between epidemiological parameters of the first cycle of infection disease in seedlings treated with bacteria isolated from the apple phylloplane and Serenade® compared to controls, indicating a potential for the use of biological control to manage GLS in the field.

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