Antifungal compounds as a mechanism to control *Hemileia vastatrix* by antagonistic bacteria

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ABSTRACT

Pseudomonas putida P286 and *Bacillus thuringiensis* B157 controlled coffee rust under greenhouse and field conditions. Now we studied the mechanism(s) of antagonism of both bacteria to *Hemileia vastatrix*. Bacterial effects on urediniospore germination and disease severity were evaluated in experiments with six treatments: (i) growth broth, (ii) supernatant centrifugation, (iii) bacterial cell centrifugation and re-suspension, (iv) UV inactivation of the bacteria, (v) 523 medium, and (vi) saline solution. The treatments i to iii reduced both spore germination and disease severity. Each isolate was applied at different concentrations and time periods before inoculation of *H. vastatrix*. The efficiency in controlling rust was reduced at the lowest concentrations and at the largest intervals between applications. In spatial separation experiments, both bacteria did not induce systemic protection, whereas protected locally the coffee plants against *H. vastatrix*. Therefore, the mechanism of both isolates against *H. vastatrix* is attributed to the production of antifungal compound (s).
Key words: *Bacillus thuringiensis*, *Coffea arabica*, *Pseudomonas putida*, biocontrol, coffee, rust

INTRODUCTION

Brazil is the largest coffee producer and exporter worldwide (MAPA, 2013). There is an increasing demand for "specialty coffees", including those that are produced organically. Organic coffees have additional value, and their production contributes to the reduction of the environmental damage caused by pesticide use (Caixeta, 2002). The most important coffee disease in Brazil is leaf rust, caused by *Hemileia vastatrix* Berk. & Br, which can reduce coffee yield by 60% (Gouveia et al., 2005). Similar to many rusts, disease epidemics in the field are caused by the uredinial phase of the pathogen.

In organic coffee cropping systems, rust can be managed using resistant cultivars and copper-based products (Carvalho et al., 2002). However, it is difficult to achieve durable resistance because of the variability of *H. vastatrix* (Van der Vossen, 2005). In addition, in Brazil, there is a number of restrictions to the use of copper-based products, whereas the number of organic coffee crop systems is increasing (Carvalho et al., 2002). In these crop systems, biological control is a viable alternative for disease management (Harman, 2000). Using this approach, we have investigated the biocontrol of coffee rust and demonstrated that *Pseudomonas putida* P286 and *Bacillus thuringiensis* B157 control leaf rust in coffee plants under both greenhouse and field conditions (Haddad et al., 2004; Haddad et al., 2009).

Bacillus and *Pseudomonas* species are known biocontrol agents of plant diseases (Han et al., 2006; Melnick et al., 2008; Weller et al., 2012). The mechanisms most frequently associated with the antagonistic effect of these species are the production of compounds with antimicrobial activity (De La Fuente et al., 2006; Ryan et al., 2008; Weller et al., 2012) and induced systemic resistance (ISR) (Saravanan et al., 2004; Romeiro et al., 2005; Ongena et al., 2007). *Bacillus* isolates produce a wide variety of antifungal metabolites, mainly lipopeptides, such as surfactin, itaurine, and fengicine (Lanna Filho et al., 2010).

In *Pseudomonas* spp., the antibiotic 2,4-diacetylphloroglucinol is responsible for natural suppression in the soil (Raaijmakers et al., 1999; Weller et al., 2012). Other compounds produced by *Pseudomonas* spp., such as pyoluteorin (Duffy & Défago, 1999) and pyrrolnitrin (Cui & Harling, 2006; Upadhyay & Srivastava, 2009), are also important for plant disease control. Other *Pseudomonas* species were reported to induce resistance against *H. vastatrix* in coffee plants (Porras et al., 1999). The protective effect and the induction of resistance in coffee seedlings were demonstrated when *P. putida* was applied 4 hours before inoculation with *H. vastatrix* or when *B. subtilis* was applied 8 days before inoculation with *H. vastatrix* (Costa, 2007). The germination of *H. vastatrix* was reduced by the application of a *B. thuringiensis*-based commercial product, and the protective effect of this application to

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coffee-leaf discs inoculated with the pathogen lasted up to 16 days (Cristancho, 1995). Although the results were not conclusive, it was suggested that the application of the product induced a resistance to rust.

In coffee plants, the application of *B. thuringiensis* B157 and *P. putida* P286 was shown to control rust efficiently (Haddad et al., 2004; Haddad et al., 2009). To define disease management strategies using these isolates, it is necessary to understand their mechanisms of action against *H. vastatrix*. Therefore, we examined these mechanisms and evaluated the criteria proposed by Steiner & Schömbeck (1995) to determine whether ISR is related to the effects of the isolates.

MATERIAL & METHODS

General procedures

To define the antagonistic mechanism(s) of *B. thuringiensis* B157 and *P. putida* P286, the following criteria were evaluated: (i) the lack of toxic effects of the inducing agent on the pathogen; (ii) the lack of a relationship between the resistance magnitude and the application of increasing quantities of the inducer; (iii) the need for a time interval between the exposure of the plant to the inducing agent and the resistance expression; and (iv) the resistance response pattern, whether local or systemic (Steiner & Schömbeck, 1995).

The urediniospores were brush-collected from pustules of leaves of 'Catuaí' plants grown in greenhouse, sieved and suspended in sterilized distilled water (SDW) at a concentration of 1 mg urediniospores/mL. The suspension was shaken for 40 min in a magnetic stirrer. The collection-agitation procedures were executed immediately before inoculation with *H. vastatrix*.

The bacterial isolates were stored in potato-dextrose-agar (PDA) in culture tubes at 5°C. Before application, each isolate was grown in 523 liquid medium (Kado & Heskett, 1970) in Erlenmeyer flasks that were shaken in an orbital agitator for 24 h at 150 rpm and 30°C.

Urediniospore germination in presence of bacterial cells and possible cell-produced metabolites

For each bacterial isolate, the following four treatments were performed: (i) culture broth collected after bacterial growth in 523 liquid medium; (ii) supernatant collected by centrifugation of the culture broth at 14.000 rpm for 20 min; (iii) bacterial cells collected (after the centrifugation treatment described in ii) and resuspended in 0.85% NaCl saline solution; and (iv) cells collected from treatment iii and inactivated by exposure to ultraviolet radiation for 30 min. The following three control treatments were included: C1, 10 μL saline solution + 10 μL urediniospore suspension; C2, 10 μL copper hydroxide (2.2 g i.a./L) + 10 μL urediniospore suspension; and C3, 10 μL 523 medium + 10 μL urediniospore suspension.

A 10 μL sample of each treatment + 10 μL of the urediniospore suspension were placed in Petri dishes (5-cm diameter) containing 1.5% agar-water. The Petri dishes were maintained at 22°C in the dark. After 16 h, 5 μL of lactophenol was added to each dish to terminate the urediniospore germination. The germination of 100 urediniospores was assessed using light microscopy. A spore was considered germinated when the length of the germination tube was equal to or larger than the spore length. The ability of each treatment to reduce the germination efficiency compared to the corresponding control treatment was evaluated. Treatments iii and iv were compared to C1, while treatments (i) and (ii) were compared to C3.

Each experiment was performed twice using a completely randomized design with three replications (one Petri dish = one experimental unit).

Rust severity in presence of bacterial cells and possible antagonist-produced metabolites

The treatments described in the previous section were applied by spraying, up to the running point, the abaxial face of the second and third pair of leaves from the apex of six-month-old 'Catuaí' plants that were grown in plastic bags in the greenhouse. The plants were maintained in a dew chamber (relative humidity >90%) at 25°C in the dark. After 48 h, the *H. vastatrix* was inoculated at the treatment site, and the plants were transferred back to the dew chamber. 48 hours after inoculation (hai), the plants were transferred to the greenhouse. After 40 days, the inoculated leaves were collected and photographed, and their images were scanned and analyzed to estimate the rust severity using the Quant software (Vale et al., 2003). The efficiency of each treatment at reducing rust severity was calculated relatively to the corresponding control treatment as described above.

The experiment was performed twice using a completely randomized design with three replications (one plant = one experimental unit).

Urediniospore germination in coffee plants seedlings in presence of antagonistic bacteria

Cell suspensions of each bacterial isolate, grown in 523 medium, were prepared in 0.85% NaCl saline solution + 0.05% Tween 20, and the concentration was adjusted to $OD_{540} = 0.2$ using a spectrophotometer. The 523 medium + 0.85% NaCl saline solution + 0.05% Tween 20 was used as control treatment. Each treatment was applied to the abaxial face of the second pair of leaves, followed by an immediate inoculation with *H. vastatrix* at the same site. The plants were maintained in a dew chamber at 25°C in the dark.

After 0, 3, 6, 12, 18, and 24 h, the spore germination was evaluated under light microscopy on glass slides prepared in accordance with Silva et al. (1999). The application/inoculation sites were covered using colorless enamel. After drying, enamel was removed, and a foil-formed film was set on a slide using a drop of blue lactophenol. Six microscope fields, each containing 100 urediniospores, were examined, and the number of germinated spores was determined. The

efficiency of each isolate at reducing germination relatively to the control was calculated.

Considering the results of the *B. thuringiensis* B157 experiment, a second experiment was performed to evaluate the urediniospore germination 12 hai using a scanning electron microscope. The leaf discs were transferred into a fixing solution (25% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) at 5°C. After 24 h, the leaf discs were subjected to three successive 15-min washes in 0.1 M sodium cacodylate buffer (pH 7.2) and were dehydrated in a graded ethanol series (30, 50, 70, 80, 90, 95 and twice in 100% ethanol) for 10 min. The leaf discs were dried at the critical point inside a Balzers apparatus (model TEC-030) and were assembled, abaxial face up, onto aluminum tops for further gold coating inside a Balzers apparatus (model FDU-010). Using a scanning electron microscope, model LEO VP1430, the samples were visualized, the images were digitally recorded, and the antagonist distribution was recorded. In addition, the urediniospore germination in the leaves treated with *B. thuringiensis* B157 was compared to the control treatment.

Dose-response ratio

The extent of the rust reduction was examined following the application of different concentrations of antagonist at different times. Bacteria from each isolate were diluted in 0.85% NaCl saline solution + 0.05% Tween 20 at seven different concentrations with the following OD_{540} readings measured using a spectrophotometer: 0 (no bacteria), 0.001, 0.01, 0.05, 0.1, 0.5, and 1.0. For each treatment, the bacteria were atomized onto the second and third pairs of leaves of the coffee plant seedlings. Immediately following the application of the bacterial treatments (time 0) or at 15 or 30 days after the application of the treatments, *H. vastatrix* inoculation was performed. At 40 days after the initial application, the severity of the rust on the leaves was evaluated, as described above. The efficiency of each treatment at reducing rust was calculated relative to the control treatments.

The experiment was performed twice using a completely randomized design, in a factorial scheme (7 concentrations x 3 application times), and with three replications (one plant = one experimental unit)

Space-time separation between the antagonists and *Hemileia vastatrix*

For each isolate, an experiment comprising the following seven treatments was performed: (i) application of the antagonist to the second pair of leaves and inoculation of *H. vastatrix* on the third pair of leaves; (ii) application of the antagonist to the third pair of leaves and inoculation of *H. vastatrix* on the second pair of leaves; (iii) application of the antagonist to the second and third pairs of leaves on one side of the plant and the inoculation of *H. vastatrix* on the same pairs of leaves on the other side of the plant; (iv) application of the antagonist in the soil (30 mL/plant) and

the inoculation of *H. vastatrix* on the second and third pairs of leaves; (v) application of the antagonist and inoculation of *H. vastatrix* on the second and third pairs of leaves; (vi) the application of a copper hydroxide spray (2.2 g i.a./L) and inoculation of *H. vastatrix* on the second and third pairs of leaves; and (vii) application of 523 medium + saline solution and inoculation of *H. vastatrix* on the second and third pairs of leaves. To avoid contamination, the unapplied/ uninoculated leaves were coated with plastic film.

Hemileia vastatrix was inoculated 0 (immediately), 3, 6, 9, or 12 days after the application of the antagonist. The plants were maintained in a dew chamber at 25°C in the dark. After 48 h, the plants were transferred into a growth chamber maintained at 22ºC with a 12-h photoperiod. At 40 days after inoculation, rust severity was assessed, as previously described. The efficiency of each treatment at reducing rust severity was calculated relatively to treatment vii.

The experiment was performed twice using a completely randomized design, in a factorial scheme (7 treatments x 5 application times), and with three replications (one plant = one experimental unit).

Data analysis

Using Levene's test, it was observed homogeneity in the error variances for both runs of each experiment.. Therefore, the results of both runs were analyzed jointly using analysis of variance and means comparison by Tukey's test (α = 0.05). For the dose-response ratio experiment, the data were log transformed before regression analysis to better fit the determination coefficients (R^2), error mean squares, parameter significance, normality and unbiased distribution of residuals. The statistical analyses were performed using SAS v. 9.1 software.

RESULTS

Urediniospore germination in presence of bacterial cells and possible cell-produced metabolites

The culture medium and saline solution did not affect urediniospore germination and rust severity (data not shown). The bacterial treatments reduced urediniospore germination, except by when the inactivated bacterial cells were used (Table 1). For *B. thuringiensis* B157, the supernatant and culture broth were more effective than the bacterial cells at reducing the germination; for *P. putida* P286, the germination was reduced equally by the three treatments (Table 1).

Rust severity in presence of bacterial cells and possible antagonist-produced metabolites

As previously mentioned, neither the control culture medium nor the saline solution affected germination and rust severity (data not shown). However, for both bacterial isolates, the supernatant, culture broth and bacterial cells significantly reduced the severity of the disease. The

inactivated bacteria were not effective at reducing rust severity (Table 1).

Urediniospore germination in coffee plant seedlings in presence of antagonistic bacteria

Both bacterial isolates reduced the urenidiospore germination by 100%. Electron microscopy demonstrated that the B157 isolate prevented the formation of germination tubes and inhibited the urediniospore germination (Figure 1).

Dose-response ratio

For both bacterial isolates, there was a significant correlation between the concentration and time of application. The efficiency of the antagonists increased with increased concentrations and decreased as the time of the application increased (for models A and B, *P<0.0001*) (Figure 2).

Space-time separation between the antagonists and *Hemileia vastatrix*

The bacterial isolates did not produce systemic protection. For the two bacterial isolates, a significant effect

was detected only for the treatment factor (P<0.0001). The bacterial isolates were as efficient as copper hydroxide at reducing rust severity when they were applied at the inoculation site (Table 2).

DISCUSSION

Both inhibition of *H. vastatrix* urediniospore germination and reduction in rust severity were attributed to substance(s) exhibiting antifungal activity produced by the antagonistic bacteria. This hypothesis is based on the observation that the supernatant, which does not contain bacterial cells, was as efficient as the bacteria-containing culture broth at reducing both germination and the disease. In the inactivated bacterial cells, the production of antifungal compounds ceased, and when these cells were used to treat the plants, no antagonistic effects were detected. *Pseudomonas* and *Bacillus* species produce different secondary metabolic products, including antibiotics and volatile organic antifungal and antiviral compounds (Raaijmakers et al., 2002; Ryan et al., 2008).

TABLE 1 - Efficiency (%) of the treatments with *Bacillus thuringiensis* B157 and *Pseudomonas putida* P286 relatively to the control treatments (urediniospore suspension + saline solution or 523 medium as positive control, and urediniospore suspension + copper hydroxide as a negative control) in reducing both *Hemileia vastatrix* urediniospore germination and coffee leaf rust severity. Copper hydroxide reduced germination in 94.41% and severity in 98.66%

Treatment	Germination		Severity	
	B157	**P286**	**B157**	**P286**
Culture broth	95.53a[a]	76.53a	99.71a	98.25a
Supernatant	93.02a	73.74a	99.24a	95.29a
Bacterial cells	78.61b	76.11a	94.65a	95.03a
Inactivated bacterial cells	0.83c	0.00b	0.00b	6.18b

[a]In each column, the means followed by the same letter do not differ (Tukey's test, α=0.05).
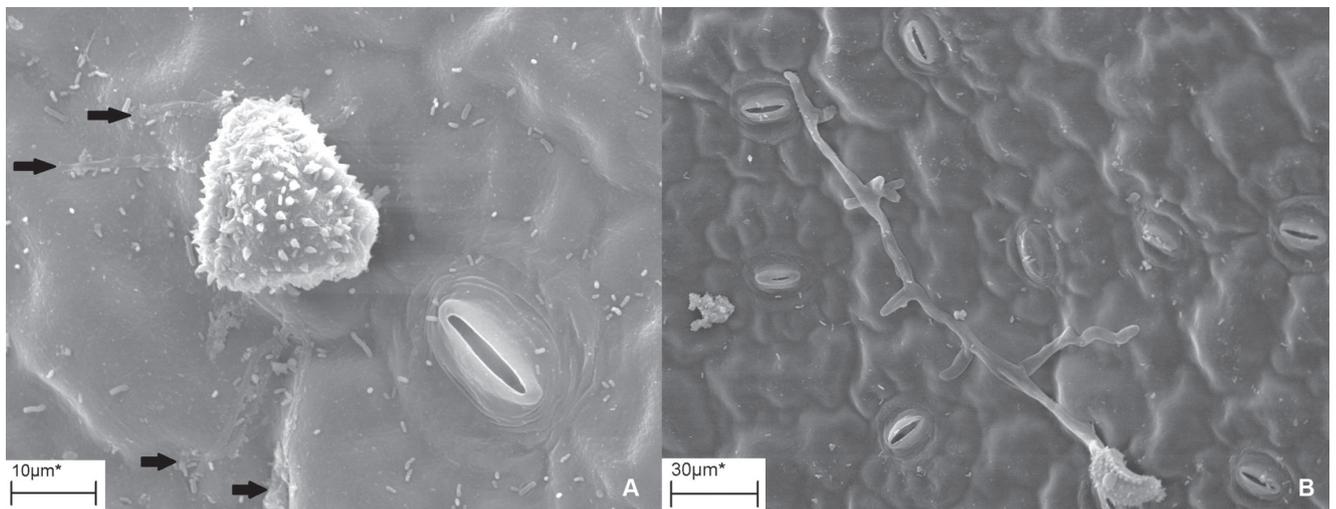
FIGURE 1 - **A.** Inhibition of *Hemileia vastatrix* urediniospore germination in presence of bacterial cells and possible metabolites produced by *B. thuringiensis*. Arrows indicate concentration of bacterial cells close to *H. vastatrix* germination tubes; **B.** Urediniospore germination in absence of the antagonist.

A Efic = 122.68 + 44.76C - 3.64T + 8.59C^2 + 0.074T^2 + 0.44CT
r^2 = 0.74

B Efic = 123.64 + 52.08 C -2.24 T + 9.47 C^2
r^2 = 0.81
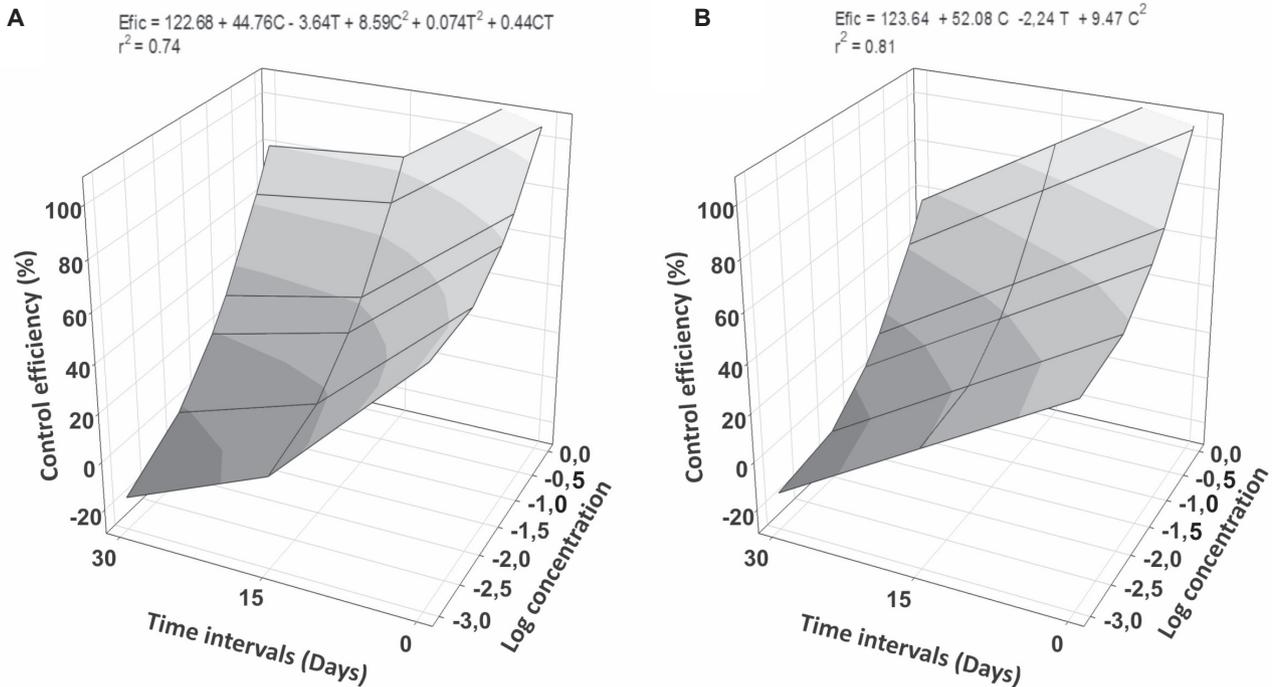
FIGURE 2 - Control efficiency (Efic) of coffee plant rust in combinations of bacterial cells concentration (C) and time intervals (T) between cell application and *Hemileia vastatrix* inoculation. **A.** *Bacillus thuringiensis* B157; **B.** *Pseudomonas putida* P286. Intercepts and slopes of both models differed significantly from zero (*P*<0.05).

TABLE 2 - Efficiency (%) of the treatments with *Bacillus thuringiensis* B157 and *Pseudomonas putida* P286 relatively to the control (application of copper hydroxide spray or 523 medium + saline solution and inoculation of *Hemileia vastatrix* (Hv) on the second and third pair of leaves), in reducing coffee leaf rust severity, with and without space split between the application of the bacteria and Hv inoculation. Copper hydroxide reduced severity in 98.88%

Treatment	B157	P286
Isolates and Hv at the 2nd and 3rd pair of leaves at the same site	99.16a[a]	99.35a
Isolates at the 3rd pair and Hv at the 2nd	3.75b	3.70b
Isolates onto the soil and Hv at the 2nd and 3rd pairs	0.86b	2.30b
Isolates at the 2nd pair and Hv at the 3rd one	-6.79b	-4.62b
Isolates on the right side of the plant and Hv on the left, both at the 2nd and 3rd pairs	-19.64b	-5.01b

[a]In each column, the means followed by the same letter do not differ (Tukey's test, α=0.05).

The broad-spectrum antibiotic 2,4-diacetylphloroglucinol, produced by *Pseudomonas sp.,* is known to be a natural suppressor in soil and has a direct effect on pathogens (Raaijmakers et al., 1999; Weller et al., 2012). This compound, produced by *P. fluorescens,* induced resistance to *Peronospora parasitica* and *Pseudomonas syringae pv. tomato* in *Arabidopsis thaliana* plants (Lavicoli et al., 2003; Weller et al., 2012). *Pseudomonas* species are reported as siderophore producers, e.g. pyoverdin, which are organic compounds of low molecular weight and which sequester iron, especially in environments with limited availability of the element (Neilands et. al., 1986; Flaishman et. al., 1996). However, it is known that

the spores of most biotrofic pathogens do not require any exogenous substance to germinate. *Hemileia vastatrix*, pathogen studied in this paper, is biotrophic and therefore it depends on the living host cells for its nutrition, growth and multiplication. During the pathogen infective process it occurs sequentially urediniospore deposition on the leaf abaxial face, germination and posteriorly appressoria formation on the plant stomata (Coutinho et al., 1993; Martins & Moraes, 1996; Silva et al., 1999, 2002). The only requirements reported yet in order to occur urediniospores germination are free water and a leaf topography that directs the germ tube tip to an stomata opening (Mendgen & Voegele, 2005). In the case

described in this paper, the pathogen's inhibition occured at the spore germination stage. There is no report of needs of iron or any other nutrient to *H. vastatrix* infective process, the formation of the pre-penetration structure of the pathogen is only associated with free water and leaf topography (Azinheira et al., 2001; Azinheira, 2005). Based on this fact, the compound with antifungal activity produced by P286 isolate was not considered as being siderophore.

There are reports that coffee plant endophytic species, *Escherichia fergusonii* and *Acinetobacter calcoaceticus*, produce siderophores (Silva et al., 2012). In this case the authors reported that these species had an effect on the coffee growth promotion and had not a direct effect on the pathogen or on the reduction of the disease severity. Eichhorn et al. (2006) reported the importance of iron capture during the biotrophic development of *Ustilago maydis* hyphae. However, this fact is related to the pathogen development only after the spore germination and host penetration, being associated with the fungus ability to colonize *Zea mays* tissues. In other pathosystems, there is a relationship between the dimensions of the outside edges of the guard cells and chemicals when forming the appressoria (Collins et al., 2001; Wiethölter et al. 2003). Despite these facts, it is necessary to evaluate if the P286 isolate can produce siderophore, like pyoverdin, and if it could influence urediniospore germination.

For *B. subtilis*, the antagonism of *Gloeosporium gloeoesporioides* was attributed to the antibiotic itaurine (Cho et al., 2003). Surfactin, produced by *B. licheniformis*, has a direct effect on spore germination and mycelial growth in *Magnaporthe grisea* (Tendulkar et al., 2007). This compound, when produced by *B. subtilis*, induced resistance to *Botrytis cinerea* in bean plants (Ongena et al., 2007). Therefore, we propose that the observed antagonism was caused by direct compound (s) production.

The disease control efficiency increased when highest concentrations of both bacterial isolates were used. Therefore, it is likely that there is a direct relationship between the efficiency of the pathogen control and the concentration of the antifungal compound. The compound was degraded over time because the pathogen control efficiency also decreased with time, mainly at the lower concentrations of the bacterial isolates. Under field conditions, the bacterial isolates produced a significant reduction in the incidence and severity of rust when applied at an OD_{540} of 0.2 (Haddad et al., 2009). At this concentration in greenhouse, the bacteria were not as effective as in field experiments. This difference is likely related to the *H. vastatrix* inoculum; under greenhouse conditions, spore germination is usually higher than in the field (data not shown). Furthermore, in the greenhouse, the inoculation occurs just once at a high inoculum concentration, whereas in the field, the inoculum is gradually deposited over time and at lower concentrations. Therefore, both the effective

concentration and the application time must be determined for field conditions.

Systematicity is an essential criterion for ISR determination. For both bacterial isolates, only a local effect was observed. Furthermore, when resistance is induced by an antagonist applied at a separate location from the pathogen, it is expected that the time interval between the inducer application and the expression of resistance will be longer than when the antagonist is applied at the inoculation site. For example, when *Lysobacter enzymogenes* was applied at the inoculation site of the *Bipolaris sorokiniana,* the resistance response in tall fescue (*Festuca arundinacea*) was immediate, whereas with the spatial separation of the treatment and inoculation sites, the effect was delayed for two days (Kilic-Ekici & Yuen, 2003). The efficiency of both bacterial isolates to control the disease increased when the time of the application was reduced.

In this study, the findings do not follow the criteria set by Steiner & Schömbeck (1995) to account for the ISR. In addition, we determined that the bacterial isolates did not protect the coffee plants from *C. coffeicola* infections (data not shown). Although both bacterial isolates should be challenged with other pathogens to expand the scope of the protection test, in this study, when systemic resistance was induced, the protection spectrum observed was not as wide as expected. Therefore, the data reinforce that the main mechanism of antagonism of *B. thuringiensis* B157 and *P. putida* P286 is mediated by the production of toxic compounds to the germination of *H. vastatrix* urediniospores.

Organic coffee production is increasing in Brazil. Although the use of a number of copper compounds to protect plants against rust is allowed, the biocontrol of diseases has a promising future in the Brazilian coffee industry. The use of bacteria that produce compounds that inhibit spore germination is an efficient strategy for rust biocontrol in coffee plants. The *Bacillus* species reduced the urediniospore germination by greater than 40% and caused deformation of the germination tubes (Shiomi et al., 2006). It is believed that the efficiency of the biocontrol agent is directly linked to the interference on the initial establishment of the disease stages (Yoshida et al., 2001; Guetsky et al., 2001). Therefore, substance(s) with antifungal action produced by the antagonist bacteria was/were responsible to disease control, and there was a direct effect between the antagonist concentrations and the disease control. The use of *B. thuringiensis* B157 and/or *P. putida* P286, or the compound(s) they excrete, in the field is likely to become a reality in Brazil, particularly for organic coffee production. An important next step will be to identify and characterize the toxic compound(s) with potential to be a new chemical fungicide compound.

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