BIOPROSPECTING ENDOPHYTIC BACTERIA FOR BIOLOGICAL CONTROL OF COFFEE LEAF RUST

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ABSTRACT: Suppression of plant diseases due to the action of endophytic microorganisms has been demonstrated in several pathosystems. Experiments under controlled conditions involving endophytic bacteria isolated from leaves and branches of *Coffea arabica* L and *Coffea robusta* L were conducted with the objective of evaluating the inhibition of germination of *Hemileia vastatrix* Berk. & Br., race II, urediniospores and the control of coffee leaf rust development in tests with leaf discs, detached leaves, and on potted seedling of cv. Mundo Novo. The endophytic bacterial isolates tested proved to be effective in inhibiting urediniospore germination and/or rust development, with values above 50%, although the results obtained in urediniospore germination tests were inferior to the treatment with fungicide propiconazole. Endophytic isolates TG4-Ia, TF2-IIc, TF9-Ia, TG11-IIa, and TF7-IIa, demonstrated better coffee leaf rust control in leaf discs, detached leaves, and coffee plant tests. The endophytic isolates TG4-Ia and TF9-Ia were identified as *Bacillus lentimorbus* Dutky and *Bacillus cereus* Frank. & Frank., respectively. Some endophytic bacterial isolates were effective in controlling the coffee leaf rust, although some increased the severity of the disease. Even though a relatively small number of endophytic bacteria were tested, promising results were obtained regarding the efficiency of coffee leaf rust biocontrol. These selected agents appears to be an alternative for future replacement of chemical fungicide.
Key words: *Hemileia vastatrix*, *Coffea* spp., *Bacillus,* biocontrol

BIOPROSPECÇÃO DE BACTÉRIAS ENDOFÍTICAS COMO AGENTES DE BIOCONTROLE DA FERRUGEM DO CAFEEIRO

RESUMO: Supressão de doenças de plantas por microrganismos endofíticos tem sido demonstrada em diversos patossistemas. Neste trabalho foram selecionados isolados de bactérias endofíticas de folhas e ramos de cafeeiro com potencial para o controle biológico da ferrugem do cafeeiro, pois é conhecido que esses microrganismos podem possuir essa característica. Bactérias endofíticas isoladas previamente de folhas e ramos de *Coffea arabica* L e *Coffea robusta* L foram avaliadas quanto ao seu potencial de biocontrole da ferrugem do café causada pelo fungo *Hemileia vastatrix* Berk. & Br., raça 2. As bactérias foram testadas para a inibição da germinação de urediniosporos do fungo e em bioensaios para o controle do desenvolvimento da ferrugem alaranjada do cafeeiro em discos de folhas, folhas destacadas e mudas da cv. Mundo Novo. Os isolados de bactérias endofíticas testados demonstraram eficácia na inibição da germinação de urediniosporos e/ou no desenvolvimento da ferrugem, com valores acima de 50%, embora os resultados obtidos nos testes de germinação de urediniosporos tenham sido inferiores ao tratamento com propiconazole (testemunha padrão). Nos testes em discos de folhas, folhas destacadas e em plantas de cafeeiro, os isolados endofíticos TG4-Ia, TF2-IIc, TF9-Ia, TG11-IIa e TF7-IIa demonstraram melhor controle da ferrugem do cafeeiro. Os isolados endofíticos TG4-Ia e TF9-Ia foram identificados como *Bacillus lentimorbus* Dutky e *Bacillus cereus* Frank. & Frank., respectivamente. De acordo com os resultados verifica-se que alguns isolados foram eficientes em controlar a ferrugem do cafeeiro, embora alguns tenham aumentado a severidade da doença. Apesar do número relativamente baixo de bactérias endofíticas testadas, resultados promissores foram obtidos em relação ao controle biológico da ferrugem, sendo que esses poderão no futuro apresentar uma alternativa aos fungicidas.
Palavras-chave: *Hemileia vastatrix*, *Coffea* spp., *Bacillus,* controle biológico

INTRODUCTION

Beneficial endophytic microorganisms comprise especially fungi and bacteria that colonize internal plant tissues without causing visible damage to their hosts (Petrini, 1991). They are different from phytopathogenic microorganisms because they are not detrimental, do not cause diseases to plants, and are distinct from epiphytic microorganisms which live on the surface of plant organs and tissues (Hallmann et al.,

1997). Endophytic bacteria are able to penetrate and become systemically disseminated in the host plant, actively colonizing the apoplast (Quadt-Hallmann et al., 1997b), conducting vessels (Hallmann et al., 1997), and occasionally the intracellular spaces (Quadt-Hallmann et al., 1997a). This colonization presents an ecological niche, similar to that occupied by plant pathogens, and this endophytic bacteria can, therefore, act as biological control agents against pathogens (Hallmann et al., 1997).

In this sense, the suppression of plant diseases due to the action of endophytic microorganisms has been demonstrated in several pathosystems (Narisawa et al., 1998; Lima et al., 1994). Several mechanisms may control this suppression, either directly on the pathogen inside the plant by antibiosis (Sturz et al., 1998) and competition for nutrients (Mari et al., 1996), or indirectly by induction of plant resistance response (M'Piga et al., 1997).

The coffee leaf rust caused by *Hemileia vastatrix* is the main disease in coffee, causing yield losses of 35 to 40%, on average. Control is basically achieved by fungicides. In 2000, in Brazil, the use of fungicides in coffee stood for 3,680 t of active ingredient (Campanhola & Bettiol, 2003). Therefore, alternatives to control coffee leaf rust must be sought. The objective of this work was to select endophytic bacteria isolates from coffee leaves and branches, with biocontrol potential against coffee leaf rust, by means of inhibition assays of urediniospores germination and control of coffee leaf rust in tests with leaf discs, detached leaves, and on potted seedling of cv Mundo Novo.

MATERIAL AND METHODS

Endophytic bacteria

Isolates from leaves and branches (Nunes, 2004), of *Coffea arabica* and *Coffea robusta* plants from Pedreira, Mococa, and Pindorama counties, State of São Paulo, Brazil (Table 1), were maintained in the culture colletion of the Laboratório de Microbiologia Ambiental, Embrapa Meio Ambiente, in sterile distilled water (Castellani, 1967). Forty bacterial isolates were evaluated regarding their capacity to inhibit the germination of *H. vastatrix* urediniospores, and 44 isolates were used to control coffee leaf rust in leaf discs, detached leaves, and seedlings of *C. arabica*, cv. Mundo Novo.

Urediniospore germination

The endophytic bacteria isolates were cultivated on nutrient agar medium (Peptone 5 g; meat extract 3 g; agar 15 g; distilled water 1000 mL) for 24 hours at 28 ± 2ºC, and then transferred with to slants containing sterilized, distilled water. Samples were then shaken vigorously to obtain a homogeneous cell suspension, which was standardized to an optical density of A_{550} = 0.1. Urediniospores of *H. vastatrix*, race II, were collected from coffee leaves containing lesions obtained from plants in "Centro de Café e Plantas Tropicais, Instituto Agronômico de Campinas", and stored in a container with sodium dichromate (relative humidity 52%; 7 ± 2ºC). Urediniospores were suspended in water at a concentration of 1.0 mg mL^{-1} using a magnetic stirrer for 5 minutes. A 15.0 µL aliquot of this suspension, and a 15.0 µL aliquot of the endophytic bacteria suspension were then transferred to microscope slide, mixed and enclosed within plastic boxes containing a layer of foam saturated with water, and sealed with glass plates to maintain high relative humidity. After incubation for six hours (22 ± 1ºC) in the dark, the germination was interrupted by adding 15.0 µL of lactophenol cotton blue dye onto each droplet, and examined under light microscope. The percentage of germinated urediniospores (10 fields at 200 × magnification) was calculated. Urediniospores with germ tubes of at least one half of the length of their larger diameter were considered germinated. Trials were set up in a completely randomized experimental design (n = 4). The experiment was repeated and the means were used for statistical analysis. Sterilized water and propiconazole (Tilt® CE; 1.2 µL of the commercial product per mL of water) were used as controls.

Leaf discs

Discs of young and completely developed leaves of *C. arabica* cv. Mundo Novo plants were removed with a 2.0 cm diameter cork punch and placed into plastic boxes, abaxial surface facing up, over a layer of foam saturated with water (Eskes, 1989). Using a micro-pipet, 25.0 µL of the endophytic bacteria suspension were applied on the leaf discs, 72 and 24 hours before, after, and simultaneously with the same volume of *H. vastatrix* urediniospores suspension (1.0 mg mL^{-1}). After inoculation, boxes were covered with glass plates and incubated in the dark for 24 hours. Then the boxes were maintained under 12h photoperiod, 500-1000 lux, 22 ± 2ºC, and approximately 100% relative humidity. The experiment was set on completely randomized design (n = 3), represented by nine leaf discs each. Severity of the disease was evaluated 30 days after inoculation, using a rating scale from 1 to 5, according to the percentage of leaf area with lesions (1 = 0%; 2 = 1-25%; 3 = 26-50%; 4 = 51-75%; and 5 ≥ 75% of leaf area with lesions). The Waller-Duncan (α = 0.05) test was used to compare the lowest and the highest mean values of lesions percentage for each treatment.

Table 1 - Effect of endophytic bacterial isolates from coffee plants on the germination of *Hemileia vastatrix* urediniospores.

Isolate	Source(Species-plant part-loction)	% of germinationof H. vastratrix
Water	-	54.4 a[1]
T F11-III a	*Coffea arabica* - leaf - Pindorama, SP	48.6 ab (10.7)
T F7-I a	*Coffea robusta* - leaf - Pindorama, SP	48.2 ab (11.4)
T F9-I b	*C. robusta* - leaf - Pindorama, SP	48.1 ab (11.5)
T G6-I b	*C. robusta* - stem - Mococa, SP	47.7 abc (12.3)
A F2-I b	*C. robusta* - leaf - Pedreira, SP	47.3 abc (13.1)
T G10-II d	*C.arabica* - leaf - Pindorama, SP	46.2 abcd (15.1)
T F2-II a	*C. robusta* - leaf - Mococa, SP	41.2 bcde (24.2)
T G5-II b	*C. robusta* - stem - Mococa, SP	40.8 bcde (25)
T F9-I a	*C. robusta* - leaf - Pindorama, SP	39.4 bcdef (27.5)
T F3-II a	*C. arabica* - leaf - Pedreira, SP	39.3 bcdefg (27.8)
T G5-III c	*C. robusta* - stem - Mococa, SP	39.2 bcdefg (27.9)
T F12-I a	*C.arabica* - leaf - Pindorama, SP	37.3 cdefgh (31.5)
T F4-II a	*C. robusta* - leaf - Mococa, SP	36.3 defgh (33.2)
T G7-III e	*C. robusta* - stem - Pindorama, SP	33.6 efghi (38.2)
T G7-I c	*C. robusta* - stem - Pindorama, SP	33.3 efghi (38.8)
A F7-III a	*C. robusta* - leaf - Pindorama, SP	33.2 efghi (39)
T G8-III b	*C. robusta* - stem - Pindorama, SP	33.1 efghi (39.1)
T F7-II b	*C. robusta* - leaf - Pindorama, SP	32.6 efghij (40)
T F5-III a	*C. robusta* - leaf - Mococa, SP	31.7 efghij (41.6)
T F10-III a	*C.arabica* - leaf - Pindorama, SP	30.2 efgijk (44.5)
T F8-I a	*C. robusta* - leaf - Pindorama, SP	30.2 fghijkl (44.5)
T F9-I c	*C. robusta* - leaf - Pindorama, SP	29.9 fghijkl (44.9)
T F7-I b	*C. robusta* - leaf - Pindorama, SP	29.6 fghijkl (45.5)
T G10-III c	*C.arabica* - leaf - Pindorama, SP	29.5 fghijkl (45.8)
T F10-II a	*C.arabica* - leaf - Pindorama, SP	29.4 fghijkl (46)
T F7-III a	*C. robusta* - leaf - Pindorama, SP	29.1 fghijkl (46.5)
T F7-II a	*C. robusta* - leaf - Pindorama, SP	28.8 ghijkl (47.1)
T G4-II a	*C. robusta* - stem - Mococa, SP	28.6 hijkl (47.4)
T G12-III a	*C. arabica* - stem - Pindorama, SP	28.3 hijkl (48)
T G4-I a	*C. robusta* - stem - Mococa, SP	28.1 hijkl (48.2)
T F7-III b	*C. robusta* - leaf - Pindorama, SP	27.9 hijkl (48.6)
T G12-II c	*C. arabica* - stem - Pindorama, SP	27.4 hijkl (49.6)
T F2-I c	*C. arabica* - leaf - Pedreira, SP	24.9 ijkl (54.1)
T G8-II a	*C. robusta* - stem - Pindorama, SP	23.9 ijklm (54.3)
T F9-I d	*C. robusta* - leaf - Pindorama, SP	23.8 ijklm (56)
T F4-III a	*C. robusta* - leaf - Mococa, SP	23.6 ijklm (56.6)
T G11-II a	*C. arabica* - stem - Pindorama, SP	22.1 jklm (59.3)
T F9-I a F	*C. robusta* - leaf - Pindorama, SP	21.3 klm (60.8)
A F7-II b	*C. robusta* - leaf - Pindorama, SP	20.7 lm (61.9)
T F2-II c	*C. arabica* - leaf - Pedreira, SP	14.3 m (73.7)
Propiconazole	-	2.5 n (95.4)

[1]Means followed by the same letter do not differ among themselves (Waller-Duncan 5%). Values refer to means between two assays with three replicates, analyzed jointly. Values between parentheses indicate % of germination inhibition.

Detached leaves

Ten isolates of endophytic bacteria used in this experiment were selected according to results observed in the two previous tests. The treatments consisted of a bacterial suspension (A_{550} = 0.1) sprayed on completely developed coffee leaves (*C. arabica* cv. Mundo Novo), 72 and 24 hours before, after and simultaneously with the inoculation of the urediniospore suspension. The coffee leaves were placed in plastic boxes, abaxial surface facing up, over a layer of foam saturated with water, covered with a glass plate and incubated as described. The experimental design was randomized blocks (n = 3), each replicate consisting of three leaves. Inoculation was performed using a

sprayer attached to a compressor, pressure 10 lb in^{-2}. Following inoculation, the boxes were covered and placed in the dark for 24 hours, at 22 ± 2ºC. Treatments were evaluated 21 days after inoculation, by counting the number of lesions per leaf. Means were compared by Tukey test ($\alpha = 0.05$).

Coffee plants

The same endophytic bacterial isolates used in the detached leaves were used in this study. Coffee seedlings (*C. arabica* cv. Mundo Novo) susceptible to all *H. vastatrix* strains were obtained from "Centro de Café e Plantas Tropicais, Instituto Agronômico de Campinas", and transplanted into plastic pots containg 5 L of Red Yellow Latosol, sifted through 1.0 cm^2 mesh sieve and mixed with 2.0 kg of lime, 5.0 kg of simple superphosphate, and 0.5 kg of potassium chloride per m^3 of soil. The bacterial suspensions (A_{550} = 0.1) were manually sprayed to the foliage until run-off. The urediniospore suspension was applied with a sprayer attached to a compressor, pressure 10 lb in^{-2}. After inoculation with the *H. vastatrix* urediniospore suspension (1.0 mg mL^{-1}), plants were incubated in the dark for 48 hours at 22 ± 2ºC, 100% relative humidity, and then transferred to a greenhouse. Plants were irrigated daily and after 30 days the number of lesions per inoculated leaf was evaluated. Sterilized water was used as control. Trial was set up in a randomized blocks design (n = 3), with two plants per pot and, means compared by Tukey test ($\alpha = 0.05$).

The most effective isolates were identified based on cell membrane fatty acid contents, analyzed in a gas chromatograph, using microbial identification software (MIDI, Sherlock® TSBA Library version 5.0, Microbial ID, Newark, DE, USA). Isolates with a similarity index of 0.6 or higher were considered positively identified.

RESULTS AND DISCUSSION

Twenty three out of the 40 endophytic bacteria isolates tested for their capacity of inhibiting *H. vastatrix* urediniospore germination inhibited germination in more than 40% (Table 1), irrespective of source (coffee species or plant organ), in relation to the control (water). In addition, deformations of the germination tube that were detrimental compared to normal development, were observed. All bacterial isolates were statistically inferior to the propiconazole.

In the leaf disc assay, the endophytic bacterium TG4-Ia reduced disease severity in all application intervals tested. Control levels were above 63% when applied at 72 and 24 hours before or simultaneously to fungal pathogen inoculation (Table 2). Other isolates were also effective in reducing rust development in the leaf disc tests, but with less intensity. In general, lower control levels were observed when the bacterium was applied after pathogen inoculation. Some isolates increased severity of the disease, especially TF4-IIa, which increased the severity of lesions by 64% when applied 72 hours before the pathogen. Only TG11-IIa was effective in inhibiting *in vitro* urediniospore germination (Table 1), and in reducing the percentage of leaf area with lesions in coffee leaf discs. Isolates TF2-IIc, TF3-IIa, TF7-Ib, TF7-IIa, TF9-IIa, AF7-IIIa, TG4-Ia, TG4-IIa, TG10-IIIc, and TG11-Iia, were selected for further studies on detached leaves and on coffee plants.

In the detached leaves assay, the most prominent isolates were TF7-Ib, TF9-Ia, TF3-IIa, TG10-IIIc, and TF7-IIa, which showed significant control in all application intervals tested (Table 3). Even though the endophytic bacterium TF9-Ia has yielded the best control (62.0%), its performance was not reproducible on coffee plants. There was a decline in the number of isolates able of reducing severity of the disease, as the interval between the presence of the biocontrol agents and the pathogen decreased.

In the test with coffee plants, the endophytic bacteria were not effective in controlling coffee leaf rust when applied after inoculation of the pathogen (Table 4). Isolates TF2-IIc, TF7-IIa, TG4-Ia, and TG11-IIa were effective when applied either 72 and 24 before or concurrently with the pathogen (Table 4).

Endophytes TG4-Ia, TF9-Ia, TF2-IIc, and TF7-IIa, identified as *Bacillus lentimorbus*, *Bacillus cereus*, *Clavibacter michiganensis* subsp. *michiganensis* Smith, and *Klebsiella pneumoniae* Schroeter, respectively, showed the best performance. The other endophytes were identified as *Bacillus* sp. (TF3-IIa), *Klebsiella pneumoniae* (TF7-Ib), *Pandorae pnomenusa* Coenye at al. (AF7-IIIa), *Kocuria kristinae* Kloos et al. (TG4-IIa), *Cedecea davisae* Grimont et al. (TG10-IIIc), and *Acinetobacter calcoaceticus* Beijerinck (TG11-IIa).

The efficiency of certain endophytic bacteria isolates in controlling coffee leaf rust can vary according to the moment of biocontrol agent application. In general, the endophytes were more effective when applied 72 and 24 hours before and concurrently with the inoculation of *H. vastatrix* urediniospores. Similar results were obtained by Bettiol et al. (1994) and Bettiol & Várzea (1992); these authors registered reductions in the percentage of leaves with lesions and in the number of lesions per leaf rating 60% and 100%, by spraying different concentrations of non-endophytic *Bacillus subtilis*-based products 72 and 24 hours before the application of *H. vastatrix* urediniospores, on coffee

Table 2 - Percentage of leaf area with lesions caused by coffee leaf rust, in cv. "Mundo Novo" leaf discs, submitted to suspensions of endophytic bacteria isolates 72 hours before and after, 24 hours before and after, and concurrently with the inoculation of *Hemileia vastatrix*.

Isolates	- 72 hours	- 24 hours	0 hours	+ 24 hours	+ 72 hours
Water	10.8 bcdefgh	13.6 ab	10.7 efghij	12.2 abcde	14.3 ab
TF1-IIa	7.8 ghi (27.9)	8.4 abcdefg (37.9)	5.2 klmno (50.7)	12.4 abcde	11.4 abcd (19.8)
TF2-Ic	9.8 defgh (9.0)	9.9 abcdef (26.8)	16.2 abc	12.1 abcde (1.4)	11.0 abcd (11.1)
TF2-IIc	11.2 bcdefgh	3.0 fg (77.6)	2.4 no (77.0)	11.6 abcdef (11.7)	12.6 abcd (12.1)
TF3-IIa	15.3 abc	6.9 bcdefg (49.4)	1.2 o (88.1)	9.3 bcdef (9.3)	14.6 a
TF4-IIa	17.8 a	8.8 abcdefg (35.2)	11.0 defghij	14.0 abcd	14.1 ab (1.2)
TF4-IIIa	12.8 bcdef	9.4 abcdef (31.0)	11.0 defghij	11.2 abcdef (8.4)	-
TF5-IIIa	13.8 abcde	10.1 abcde (25.4)	12.8 abcdefgh	12.7 abcde	12.3abcd (14.2)
TF7-Ia	14.0 abcde	10.7 abcd (21.0)	13.6 abcdefgh	11.4 abcdef (6.9)	13.8 abcd (3.2)
TF7-Ib	9.9 defgh (8.5)	2.1 g (84.1)	6.4 jklmn (39.6)	12.2 abcde	13.2 abcd (7.3)
TF7-IIa	12.0 bcdefgh	7.1 bcdefg (47.9)	4.3 lmno (59.3)	13.6 abcd	14.0 ab (1.9)
TF7-IIb	9.8 defgh (8.9)	9.9 abcdef (26.8)	14.1 abcdefgh	13.6 abcd	14.0 abc (2.1)
TF7-IIIa	13.4 abcde	11.6 abcd (14.5)	17.1 ab	11.8 abcdef (3.3)	13.8 abcd (3.2)
TF7-IIIb	14.6 abcd	7.1 bcdefg (47.3)	12.6 abcdefgh	7.3 def (40)	12.7 abcd (10.7)
TF8-Ia	13.2 abcde	10.4 abcd (23.2)	17.7 a	10.2 abcdef (16.7)	13.2 abcd (7.8)
TF8-IIa	14.0 abcd	11.1 abcd (18.4)	14.1 abcdefgh	11.8 abcdef (3.7)	9.3 abcd (35.1)
TF9-Ia	7.4 hi (31.7)	6.0 cdefg (55.9)	2.3 no (78.1)	12.3 abcde	10.0 abcd (30.0)
TF9-Ia F	9.1 efgh (16.1)	11.6 abcd (14.4)	16.1 abcd	11.6 abcdef (4.8)	10.4 abcd (27.3)
TF9-Ib	8.1 fghi (24.9)	11.4 abcd (15.7)	15.8 abcde	13.0 abcde	12.9 abcd (9.8)
TF9-Ic	11.7 bcdefgh	8.9 abcdefg (34.4)	10.0 fghijk (6.2)	14.5 ab	12.9 abcd (9.5)
TF9-Id	10.5 cdefgh	7.0 bcdefg (48.4)	10.4 fghijk (2.4)	10.5 abcdef (13.6)	9.3 abcd (35.1)
TF10-Ia	12.2 bcdefgh	9.7 abcdef (28.3)	12.0 bcdefghi	12.6 abcde	11.7 abcd (17.9)
TF10-IIa	12.4 bcdefg	12.4 abcd (8.9)	10.3 fghijk (3.3)	16.7 a	10.7 abcd (24.8)
TF10-IIIa	11.7 bcdefgh	12.6 abcd (7.1)	11.3 cdefghij	14.5 ab	10.8 abcd (24.3)
TF11-IIIa	10.9 bcdefgh	14.2 a	15.1 abcdef	14.3 abc	13.3 abcd (6.7)
TF12-Ia	15.3 abc	12.0 abcd (11.3)	11.8 cdefghi	10.0 abcdef (18.0)	9.0 abcd (36.6)
AF2-Ib	12.3 bcdefg	12.9 abc (5)	10.2 fghijk (4.3)	9.4 bcdef (22.5)	8.3 bcd (41.8)
AF7-IIb	10.5 cdefgh (2.7)	10.0 abcdef (26.4)	11.9 cdefghi	11.5 abcdef (6.0)	7.8 cd (7.8)
AF7-IIIa	11.3 bcdefgh	8.6 abcdefg (36.6)	7.7 ijklm (28.1)	9.9 abcdef (18.5)	7.6 d (7.7)
TG4-Ia	3.9 i (63.8)	3.1 efg (76.7)	1.6 no (85)	7.5 cdef (38.5)	8.3 bcd (8.3)
TG4-IIa	10.5 cdefgh (3.3)	13.7 ab	11.5 cdefghij	8.8 bcdef (28.1)	11.3 abcd (11.4)
TG5-IIb	9.95 defgh (8.4)	5.9 cdefg (56.5)	9.2 hijkl (14.1)	10.7abcdef (12.1)	10.2 abcd (10.2)
TG5-IIIc	11.5 bcdefgh	7.6 abcdefg (43.9)	9.8 ghijk (8.3)	9.4 bcdef (23)	11.9 abcd (16.9)
TG6-Ib	11.3 bcdefgh	9.1 abcdefg (32.9)	12.1 bcdefghi	11.0 abcdef (9.9)	12.0 abcd (16.2)
TG7-Ic	13.7 abcde	11.9 abcd (12.7)	11.6 cdefghij	12.8 abcde	11.0 abcd (23.0)
TG7-IIIe	14.1 abcd	9.8 abcdef (27.8)	14.7 abcdefg	11.7 abcdef (4.2)	10.6 abcd (25.5)
TG8-IIa	15.5 ab	9.7 abcdef (28.2)	12.2 bcdefghi	10.9 abcdef (10.5)	9.6 abcd (9.6)
TG8-IIIb	15.1 abc	8.4 abcdefg (38.1)	11.8 cdefghi	9.7 bcdef (20.3)	11.6 abcd (11.7)
TG8-IIIc	13.4 abcde	12.3 abcd (9.2)	10.9 efghij	9.9 abcdef (18.8)	11.5 abcd (11.5)
TG10-IId	15.4 abc	10.0 abcdef (26.2)	10.5 fghij (2.1)	6.3 ef (47.8)	12.0 abcd (12.0)
TG10-IIIc	14.5 abcd	5.7 defg (57.6)	3.7 mno (65.0)	9.2 bcdef (24.6)	10.6 abcd (10.7)
TG11-IIa	12.1 bcdefgh	3.0 fg (77.9)	2.2 no (79.3)	4.9 f (59.3)	11.4 abcd (11.5)
TG11-IIb	13.4 abcde	10.9 abcd (20.3)	11.9 bcdefghi	8.4 bcdef (31.0)	10.0 abcd (10.0)
TG12-IIc	11.3 bcdefgh	8.8 abcdefg (34.9)	11.2 cdefghij	9.8 bcdef (19.6)	10.1 abcd (10.2)
TG12-IIIa	12.4 bcdefg	9.9 abcdef (27.0)	10.2 fghijk (4.6)	8.6 bcdef (8.6)	11.4 abcd (20.4)

[1]Means followed by the same letter do not differ among themselves (Waller-Duncan 5%). Values refer to means of three replicates, with nine discs each. The numbers between parentheses indicate the percentage of control of the disease relative to the control treatment. TF1-IIa and TF10-Ia were isolated from leaves and TG11-IIb from stem of *Coffea arabica* from Pindorama. TG8-IIIc and TF8-IIa were isolated from stem and leaves, from *C. robusta* from Pindorama, respectively.

Table 3 - Effect of endophytic bacteria applied 72 and 24 hours before and after, and concurrently with the inoculation of *Hemileia vastatrix* urediniospores, on the number of rust lesions per leaf in detached leaves of coffee (*Coffea arabica)* cv. Mundo Novo.

Isolate/Species	- 72 hours	- 24 hours	0 hours	+ 24 hours	+72 hours
Water - control	216.8 a1	195.4 a	200.2 a	234.1 a	232.4 a
TF2-IIc - *Clavibacter michiganensis* subsp. *michiganensis*	84.8 b (60)	86.2 b (56)	109.7 ab (45)	162.0 abcd (31)	110.9 c (52)
TF3-IIa - *Bacillus* sp.	90.1 b (58)	87.1 b (55)	8.2 b (56)	113.1 bcd (51)	101.0 c (56)
TF7-Ib - *Klebsiella pneumoniae*	84.6 b (61)	72.7 b (63)	88.3 b (56)	107.7 cd (54)	137.6 bc (41)
TF7-IIa - *Klebsiella pneumoniae*	89.4 b (58)	81.0 b (58)	74.0 b (63)	101.3 cd (56)	97.6 c (58)
TF9-Ia - *Bacillus cereus*	82.9 b (62)	80.9 b (58)	90.4 b (55)	128.9 bcd (45)	119.4 bc (48)
AF7-IIIa - *Pandorae pnomenusa*	110.3 b (49)	85.1 b (56)	124.6 ab (38)	98.1 d (58)	134.8 bc (42)
TG4-Ia - *Bacillus lentimorbus*	99.9 b (53)	112.4 b (42)	113.2 ab (43)	111.8 bcd (52)	228.0 a (2)
TG4- IIa - *Kocuria cristinae*	130.2 b (40)	94.8 b (51)	152.3 ab (24)	207.6 ab (11)	197.1 ab (15)
TG10-IIIc - *Cedecea davisae*	83.7 b (61)	80.8 b (59)	102.1 b (49)	165.1 abcd (29)	140.6 bc (39)
TG11-IIa - *Acinetobacter calcoaceticus*	160.3 ab (26)	145.2 ab (26)	114.1 ab (43)	196.9 abc (16)	234.0 a

[1]Means followed by the same letter do not differ among themselves (Tukey 5%). In detached leaf tests, values refer to means of three replicates, with three leaves each; in seedling tests, values refer to the means of 10 replicates. The numbers between parentheses indicate the percentage of control of the disease relative to the control treatment.

Table 4 - Effect of endophytic bacteria applied 72 and 24 hours before and after, and concurrently with the inoculation of *Hemileia vastatrix* urediniospores, on the number of rust lesions per leaf of coffee plants (*Coffea arabica)* cv. Mundo Novo.

Isolate/Species	- 72 hours	- 24 hours	0 hours	+ 24 hours	+72 hours
Water - control	143.2 abc	155.3 a	148.3 a	130.6 a	134.4 a
TF2-IIc - *Clavibacter michiganensis* subsp. *michiganensis*	76.8 ef (43)	95.8 c (38)	31.1 cd (79)	146.8 a	141.4 a
TF3-IIa - *Bacillus* sp.	134.7 abcd (6)	110.2 abc (29)	79.1 b (46)	162.1 a	136.1 a
TF7-Ib - *Klebsiella pneumoniae*	174.4 a	153.4 ab (1)	71.9 bc (51)	149.7 a	133.9 a
TF7-IIa - *Klebsiella pneumoniae*	88.0 cdef (38)	77.8 c (50)	88.9 b (40)	124.0 a (5)	139.7 a
TF9-Ia - *Bacillus cereus*	110.0 bcdef (23)	99.4 bc (36)	107.5 ab (27)	129.9 a	132.7 a (1)
AF7-IIIa - *Pandorae pnomenusa*	122.9 abcde (14)	107.8 abc (31)	74.9 bc (49)	133.3 a	153.5 a
TG4-Ia - *Bacillus lentimorbus*	61.7 f (57)	73.8 c (52)	22.2 d (85)	117.6 a (9)	120.2 a (10)
TG4- IIa - *Kocuria cristinae*	120.5 abcde (16)	113.6 abc (27)	115.6 ab (22)	151.8 a	144.6 a
TG10-IIIc - *Cedecea davisae*	154.1 ab	80.3 c (48)	91.8 b (38)	134.1 a	144.5 a
TG11-IIa - *Acinetobacter calcoaceticus*	83.2 def (42)	79.6 c (49)	89.4 b (39)	127.2 a (2)	135.2 a

[1]Means followed by the same letter do not differ among themselves (Tukey 5%). In detached leaf tests, values refer to means of three replicates, with three leaves each; in seedling tests, values refer to the means of 10 replicates. The numbers between parentheses indicate the percentage of control of the disease relative to the control treatment.

plants, cv. Catuaí. The fact that the endophytic isolates showed activity when applied before the pathogen suggests that these isolates may act by antibiosis, lysis of pathogen structures, competition, or induction of systemic resistance in the host.

Kim et al. (2002) reported that the bacterium *B. lentimorbus* produces the antifungal substances alpha- and beta-glucosidase, with an inhibitory action against the development of *Botrytis cinerea* Pers.:Fr, while Sadfi et al. (2001) reported that this bacterium is capable of releasing volatile substances that contribute to the inhibition of *Fusarium sambucinum* Fuckel in potato tubers. Several authors demonstrated that *B. cereus* can promote growth in various plant species such as tomato (Simon et al., 2001), and wheat (Ryder et al., 1999). In addition, it can actively penetrate the

tissues and disseminate inter- and intracellularly within the host, protecting it from *F. sambucinum*, in potatoes, by producing fungitoxic substances (Chérif et al., 2003). Also, *B. cereus* can produce various chitinases, active against several plant pathogens, such as *F. sambucinum* (Sadfi et al., 2001), *Rhizoctonia solani* Kühn (Ryder et al., 1999), *Helminthosporium solani* Dur. & Mont. (Martinez et al., 2002), *Sclerotium rolfsii* Sacc., *Fusarium oxysporum* Schl., and *Pythium aphanidermatum* (Edson) Fitzp. *B. cereus* has also been reported as endophytic in cotton (*Gossypium hirsutum* L), sweet corn (*Zea mays* L), and citrus plants (*Citrus* spp.) by Di Fiore & Del Gallo (1995).

It could thus be speculated that there were more than one mode of action of those endophytic bacteria in the control of coffee leaf rust. Inhibitory action against *H. vastatrix* in urediniospore germination was shown in specific essays, tests with leaf discs, with detached leaves, and with coffee plants at different application intervals.

The endophytic association of bacteria of the genera *Clavibacter* and *Klebsiella* with some agronomic crops, such as corn, grapevine, rice, cotton, and some crucifers was reported by Lodewyckx et al. (2002). However, reports on the application of species of the above-mentioned genera dealing with control of pathogens are scarce in the literature. *C. michiganensis* subsp. *michiganensis* is a phytopathogenic species (Agrios, 1997), while *K. pneumoniae* can be found in hospitals causing infections in humans (Martins-Loureiro et al., 2001). These characteristics can create barriers to the application of these bacteria in bioassays seeking plant disease biocontrol agents. No reports were found in biological control on the application of the other tested endophytes.

Properties of some endophytic isolates in increasing coffee leaf rust severity shall be highlighted. According to Musson (1994), some endophytic organisms can behave as non-pathogenic in a given host, and as pathogenic in another. Cameron (1970) found that *Pseudomonas* spp. isolates obtained from healthy cherry tissues proved to be phytopathogenic in further tests. This author suggested that the endophyte condition could be one of the forms of survival and escape against surface phytosanitary treatments. Additionally, Whitesides & Spotts (1991) found *Pseudomonas syringae* van Hall isolates from pear tree roots, which were not pathogenic neither to cherry nor to pear trees, and suggested that the internal tissues of pear trees could function as inoculum reservoir to other plants. Thus, the ability of colonizing internal plant tissues could be visualized as a survival mechanism of plant pathogenic bacteria, since they exist in a protected position (Leben, 1981). This fact has biological importance, because demonstrates that the interactions with endophytes could have economic importance related to both the control and to the expansion of plant diseases.

Even though a relatively small number of endophytic bacteria were tested, promising results were obtained regarding the selection of coffee leaf rust biocontrol agents. Further field studies must be conducted to analyze the real potential of endophytic bacteria in field conditions. Studies are also needed to determine the modes of action of those bacteria, the population density of the applied endophytes, and the best form of introduction into the host. The evaluation of the effects of agrochemicals on endophytic bacteria have also to be considered, since those can not only stimulate isolates that are beneficial to rust control but can also select those that increase severity of the disease.

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