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Biological control of *Pseudomonas syringae* pv. *garcae* in coffee crop with *Bacillus* spp. isolates

Controle biológico de *Pseudomonas syringae* pv. *garcae* em cafeeiro com isolados de *Bacillus* spp.

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Abstract

Among the diseases that affect the coffee crop, bacterial blight caused by *Pseudomonas syringae* pv. *garcae* (Psg) stands out due to production losses. This study evaluates the antagonistic effect of *Bacillus* spp. against Psg both *in vitro* and in coffee seedlings. *In vitro* antagonism was evaluated by the double layer method for the inhibition of Psg growth. Subsequently, *in vitro* selected antagonists were tested for their ability to inhibit bacteriosis in coffee seedlings (cv. Mundo Novo) under greenhouse conditions. It was evaluated nonspecific metabolic production by antagonistic bacteria and identified the isolates that stood out in the *in vitro* experiment. Isolates considered as the most promising *in vitro* experiments (23% of the total) were chosen for the antagonistic efficiency test in a greenhouse. Isolates B04, B05, B22, B31, B53, B202, B208, B264, B266, and B294 showed ammonia production. Isolates B04, B33, and B294 showed hydrocyanic acid production. Isolates B04, B05, B18, B22, B33, B53, B105, B202, B208, B264, and B294 showed siderophores production. In addition, isolates B18 (*B. subtilis*), B22 (*B. thuringiensis serovar israelensis*), B05 (*B. methylotrophicus*), B53 (*B. safensis*), B202 (*B. subtilis*), and B33 (*B. subtilis*) reduced more than 80% the severity of bacterial blight under greenhouse conditions. Field experiments should be conducted to confirm the potential of these isolates for bacterial blight management.

Additional keywords: bacteria; bacterial blight; *Coffea* spp.

Resumo

Dentre as doenças que incidem na cultura do cafeeiro, a mancha-aureolada, causada por *Pseudomonas syringae* pv. *garcae* (Psg), destaca-se devido às perdas provocadas na produção. Objetivou-se avaliar o efeito antagonístico de isolados de *Bacillus* spp. contra Psg *in vitro* e em mudas de cafeeiro. O antagonismo *in vitro* foi avaliado pelo método da camada dupla, pela inibição do crescimento de Psg. Os antagonistas selecionados *in vitro* foram testados quanto à sua capacidade de inibir a bacteriose em mudas de cafeeiro (cv. Mundo Novo), em casa de vegetação. Foi avaliada a produção de metabólicos inespecíficos pelas bactérias antagonistas e identificados os isolados que mais se destacaram no experimento *in vitro*. Um total de 23% dos isolados foram mais promissores *in vitro* e foram escolhidos para o teste de eficiência antagônica em casa de vegetação. Os isolados B04, B05, B22, B31, B53, B202, B208, B264, B266 e B294 apresentaram produção de amônia. Os isolados (B04, B33, B294) apresentaram produção de ácido cianídrico. E os isolados B04, B05, B18, B22, B33, B53, B105, B202, B208, B264 e B294 apresentaram produção de sideróforos. Os isolados B18 (*B. subtilis)*, B22 (*B. thuringiensis serovar israelensis)*, B05 (*B. methylotrophicus)*, B53 (*B. safensis)*, B202 (*B. subtilis)* e B33 (*B. subtilis*) reduziram em mais de 80% a severidade da mancha-aureolada em casa de vegetação. Experimentos em condições de campo devem ser conduzidos para corroborar o potencial desses isolados para o manejo da mancha-aureolada.

Palavras-chave adicionais: bactéria; *Coffea* spp.; mancha-aureolada.

Introduction

Coffee (*Coffea* spp.) belongs to the family Rubiaceae, and its most cultivated species are *Coffea arabica* L. and *Coffea canephora* L. It is a crop of extreme importance for domestic (green, roasted, and ground grain) and foreign (green grain) market supply. Brazil is the largest producer of this commodity, which generates income for countless people who participate in its production chain (Ortega & Jesus, 2011).

Among the pathogens that affect the coffee crop, three bacteria of the genus *Pseudomonas* (*P. syringae* pathovars *garcae* and *tabaci* and *P. cichorii*) cause leaf spot symptoms in plants. Bacterial blight caused by *P. syringae* pv. *garcae* stands out due to the damage caused to coffee production (Rodrigues et al., 2015). Bacterial blight epidemics have increased in growing and newly pruned crops, especially in high altitude regions or regions subject to constant cold winds. Symptoms are characterized by brown leaf lesions whether or not accompanied by a yellowish halo. This disease also causes defoliate and dieback of branches and lesions in rosettes, inflorescences, and young fruits. In nurseries without adequate control measures, under conditions favorable to the disease, this bacteriosis can cause damage of up to 70% in seedlings (Zoccoli et al., 2011).

Chemical control is currently the main measure used for the management of bacterial blight, using inefficient methods such as spraying antibiotics and cupric fungicides (hydroxides, sulfates, oxychlorides, and cuprous oxide) (Belan et al., 2014). Difficulty in diagnosing the disease is another aggravating factor for its management, as the symptoms resemble those of Phoma leaf spot, caused by *Phoma costaricensis* (Matiello & Almeida, 2013). Another important factor for the management of bacterial blight is the appropriate choice of cultivars resistant to this disease for crop establishment (Belan et al., 2014).

Biological control of coffee bacterial blight can be an important tool within an integrated management program for this disease in conventional and/or organic cultivation system.

According to Lanna et al. (2010), *Bacillus* spp. isolates produce a resistance structure called endospore, which enables their longer survival in the environment and a longer shelf life of bioproducts based on this bacterium. In addition, bacteria of the genus *Bacillus* may have multiple antagonistic mechanisms against phytopathogens, such as antibiosis, systemic resistance induction, competition for space and nutrients, synthesis of antimicrobial substances, and production of nonspecific compounds such as ammonium, hydrocyanic acid, and siderophores. However, the use of bacterial isolates of the genus *Bacillus* for biological control of *P. syringae* pv. *garcae* in coffee is not yet established in Brazil.

Due to the importance of coffee crop in the Brazilian agriculture, the search for sustainable agriculture, and the lack of studies and information on the biological control of *P. syringae* pv. *garcae*, the present study evaluated the *in vitro* antagonistic potential of *Bacillus* spp. against this bacterium. Moreover, it was tested the selected isolates for their ability to inhibit bacterial infections in coffee seedlings under greenhouse conditions and identified the most promising species of *Bacillus* spp. for the management of bacterial blight, also analyzing some metabolites produced by these isolates.

Materials and methods

Experimental site

The experiment was carried out in the Laboratory of Microbiology and Phytopathology (LAMIF) and in a greenhouse of the Federal University of Uberlândia, Monte Carmelo Campus, MG, Brazil.

Bacterial isolation from soil

Bacteria of the genus *Bacillus* were collected from the soil in native forests in Monte Carmelo city, Minas Gerais State, Brazil. One gram of each soil sample was diluted in 10 mL of sterile saline solution, and vortexed for one minute. Then, a 1 mL aliquot was removed for pasteurization (12 minutes at 80 °C in a water bath and subsequent thermal shock in crushed ice for 5 minutes), aiming at the selection of sporulating bacteria. The suspension obtained was diluted twice, reaching a concentration of 10^{-2}. From the last dilution, 100 µL of the suspension was applied to a Petri dish containing solid culture medium 523 (Kado & Heskett, 1970), followed by incubation at 28 °C for 24 hours. The fifty-six isolates obtained were transferred to test tubes and kept at 4 ºC, being preserved in mineral oil (Alfenas & Mafia, 2007) and incorporated into the LAMIF Bacterial Culture Collection.

Pathogen Isolation

The isolate of *P. syringae* pv. *garcae* was obtained from typical bacterial blight lesions in young coffee leaves and isolated in solid culture medium 523 (Kado & Heskett, 1970) at 28 °C.

A pathogenicity test was performed to confirm the virulence of the isolate obtained (Alfenas & Mafia, 2007).

In vitro antagonism of Bacillus spp. against P. syringae pv. garcae (Psg): double layer method

The antagonistic effect of *Bacillus* spp. isolates on the growth of *P. syringae* pv. *garcae* was evaluated by the double layer method. To this end, each *Bacillus* spp. isolate was placed on five Petri dishes containing culture medium 523 (Kado & Heskett, 1970) at four equidistant points, being kept at 28 °C for 16 hours in the dark (Romeiro, 2007; Vidaver et al., 1972). Subsequently, 2 mL of chloroform was added to the inner surface of the lid of each plate. The plates were inverted and the colonies exposed to chloroform

vapors for one hour. The plates were then kept ajar for chloroform volatilization and exposed to ultraviolet light (254 nm wavelength) for 30 minutes. Each plate then received an overlay of the semisolid medium 523 (45 ± 1 °C), to which 0.1 mL of a suspension containing Psg bacterial cells and incubated at 28 °C in B.O.D. After 48 hours, the diameter of the Psg growth inhibition halo was measured for each *Bacillus* spp. isolate (Romeiro, 2007; Vidaver et al., 1972). Isolates were classified according to the scale proposed by Silva et al. (2008).

A completely randomized design with five replicates was adopted considering each Petri dish as an experimental unit. The data obtained were subjected to the Shapiro-Wilk and Bartlett tests to verify the normality of residue distribution and the homogeneity of variance, respectively. Analysis of variance (ANOVA, F test at 5% probability) was performed and the treatments were compared using the Scott-Knott test at 5% probability. Data were analyzed using the R statistical software (R development core team, 2018).

Antagonism of Bacillus spp. against P. syringae pv. garcae under greenhouse conditions

The antagonists selected in the double layer test were sprayed separately on pathogen-susceptible coffee plants (cv. Mundo Novo) with 3 pairs of true leaves. For that purpose, suspensions were obtained by culturing each isolate separately in Petri dishes containing culture medium 523. Each suspension was obtained by washing the surface of the culture medium with sterile water and scraping the bacterial colonies with the aid of a Drigalski spatula. The concentration of each antagonist and Psg suspension was adjusted on a spectrophotometer (absorbance at 540 nm = 0.2) to a concentration of 10^9 CFU mL^{-1} for antagonists and 10^8 CFU mL^{-1} for Psg. Antagonistic bacteria were inoculated after four leaves of each seedling were previously injured using multineedles, being then sprayed with a hand-held sprayer until syrup dripping. After two hours, the pathogen (Psg) was inoculated with a hand-held sprayer, also until syrup dripping. In this experiment, one of the control plants was sprayed only with water and the other only with the pathogen (Psg) suspension. Then, seedlings were kept for 48 hours in a humid chamber inside an B.O.D. (32 ºC temperature and 70% relative humidity), being subsequently transferred and kept in a greenhouse for 28 days for evaluation (Belan et al., 2014).

Evaluations were performed at 7, 14, 21 and 28 days after spraying and followed the severity scale of Belan et al. (2014), which goes from grade 0 (0.0% - no symptoms) to grade 8 (≥45.1% - very severe infection).

A randomized block design with ten replicates was adopted, considering each seedling as an experimental unit. Data were integrated in the area under the disease progress curve (AUDPC), calculated by the formula: AUDPC = ∑ ((Yi + Yi + 1) / 2) (ti+1 - ti), where: Y is the disease intensity (grade given according to the diagrammatic scale used) (Belan et al., 2014); t is the time (interval between evaluations, in days); and i represents the number of evaluations. The Shapiro-Wilk and Bartlett tests were performed to verify the normality of residue distribution and the homogeneity of variance, respectively. Analysis of variance (ANOVA, F test at 5% probability) was performed and the treatments were compared using the Tukey test at 5% probability. Data were analyzed using the R statistical software (R development core team, 2018).

Evaluation of the production of nonspecific compounds by Bacillus spp. isolates selected in vitro

Ammonia production

To evaluate ammonia production, antagonistic bacteria were cultivated in test tubes containing liquid culture medium 523. A litmus paper strip was then attached to one end between the inner edge of the tube and the cotton plug. The plates were kept in B.O.D. at 28 ºC for three days, with a 12/12 h photoperiod. The color change of the indicator paper from purple to bluish within three days indicated ammonia production (Schaad et al., 2001).

Hydrocyanic acid production

Antagonistic bacteria were cultured in Petri dishes on Tryptone Soy Agar (TSA) medium. Then, onto each plate was placed a filter paper soaked in 5 mg of copper ethyl acetoacetate and 5 mg of 4,4'-dimethylene (-N,N-dimethylaniline), dissolved in 2 mL of chloroform. Steel sheets were deposited on the paper and the plates kept at 28 ºC for 72 hours, with 12/12 h photoperiod. Isolates producing hydrocyanic acid (HCN) were identified through brown spots on the indicator paper (Castric & Castric, 1983).

Siderophores production

All glassware used in this procedure was immersed in sulfochromic solution for 48 h and rinsed several times with distilled water (Schwyn & Neilands, 1987). Antagonistic bacteria were cultured in test tubes on Tryptone Soy Agar (TSA) medium and kept in B.O.D. at 28 ºC for 48 hours, with 12/12 h photoperiod. Then, 1 mL of the culture supernatant was collected, transferred to Falcon tubes with 1 mL H_2SO_4 and left for stirring on an orbital shaker for 30 min. Subsequently, 1 mL of 35% sodium acetate solution, 1 mL of 1% sulfanilic acid solution and 0.5 mL of 1.3% iodine solution were added. Excess iodine was removed by adding 2% sodium arsenite solution and 1 mL of 0.15% α-naphthylamine. The color change from dark brown to transparent, formed after 20 minutes, indicates hydroxylamine-producing bacteria (Csáky, 1948) and has also been used to indicate siderophores production (Lanna et al., 2010).

Molecular identification of Bacillus spp. isolates

The most promising *Bacillus* isolates selected *in vitro* were identified. DNA extraction was performed according to Sambrook et al. (1989) with modifications. Primers fD1 (5'-AGA GTTTGATCCTGGCTCAG-3') and rD1 (5'AAGCTTAAGGAGGTGATCCAGCC-3') were used for amplification of the 16S rRNA gene according to Weisburg et al. (1991). Reactions were performed in a thermal cycler, with a cycle of 94 °C for 3 min, followed by 30 cycles at 92 °C for 50 sec, 62.3 °C for 50 sec, and 72 °C for 1 min and 45 sec, and a final cycle at 72 °C for 7 min. The amplified fragments were separated on 1% agarose gel in 1X TBE buffer (1 M Tris base, 1 M Boric Acid and 0.5 M EDTA), stained with fluorescent dyes, and subjected to ultraviolet light in a photodocumenter for visualization of the bands. PCR products were sequenced using the BigDye V3.1 kit and injection into an ABI 3730 device with approximately 500 ng DNA and 10 pmol of primer fD1 (5'-AGA GTTTGATCCTGGCTCAG-3'). The sequences obtained were compared in the Genbank database (National Center for Biotechnology Information - NCBI), available online at the website http://www.ncbi.nlm.nih.gov through the BLASTn program.

Results and discussion

Twenty-three percent (23%) of the isolates (B04, B05, B18, B22, B31, B33, B53, B105, B202, B208, B264, B266 and B294) showed an inhibition halo equal to or greater than 3 cm, being classified as antagonists with high inhibitory capacity against the pathogen under study according to Silva et al. (2008) (Table 1). These isolates were chosen for the antagonistic efficiency test under greenhouse conditions because of their higher inhibition of Psg growth.

Table 1 - Evaluation of antagonism of *Bacillus* spp. on the growth of *Pseudomonas syringae* pv. *garcae in vitro*.

Isolates	Inhibition halo (cm)
B02, B05, B12, B15, B16, B45, B57, B63, B95, B97, B99, B101, B104, B111, B115, B132, B144, B170, B190, B193, B211, B212, B256, B263, B267, B268, B269, B272, B292, B316, B340, B432, B436.	(0 – 1.9) c
B03, B13, B212, B221, B226, B258, B271, B416, B436, B451.	(2 – 2.9) b
B04, B05, B18, B22, B31, B33, B53, B105, B202, B208, B264, B266, B294.	(> 3.0) a

Means followed by the same letter in the column do not differ by Scott-Knott test (p > 0,05). Isolates with low inhibition capacity (0-1.9), moderate inhibition capacity (2.0-2.9 cm) and high inhibition capacity (>3.0 cm).

Several isolates of *Bacillus* spp. have been shown to be efficient for *in vitro* inhibition of the growth of various phytopathogens. Silva et al. (2008) found that *Bacillus* spp. isolates succeeded in inhibiting the growth of *Pseudomonas syringae* pv. *tomato*, and Carrer et al. (2015) found that *B. subtilis* isolates reduced the growth of *Clavibacter michiganensis* subsp. *michiganensis*, both being tomato pathogens. Leão et al. (2016) verified that *Bacillus* isolates showed moderate to high inhibition on the growth of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*, a bacterial pathogen of common bean.

Rodrigues et al. (2015) inoculated *P. syringae* pv. *garcae* in coffee seedlings (cultivar Mundo Novo), observing the first symptoms, near the inoculation point, only after the first 10 days. At 20 days, the authors observed greater severity in regions distant from the inoculation point.

As can be seen from the disease progress curves (Figure 1), all treatments (*Bacillus* spp. isolates) differed statistically from the control (Psg), that is, the 13 isolates selected *in vitro* considerably decreased the severity of bacterial blight under greenhouse conditions.

Isolates B05, B18, B22, B33, B53 and B202 were the most efficient in controlling bacterial blight, reducing disease severity by, on average, 82.68% compared to the control, not differing statistically from each other (Figure 1). These isolates decreased the symptoms of bacterial blight in coffee seedlings by 84.3%, 86.3%, 84.3%, 80.4%, 80.4% and 80.4%, respectively.

Isolates B31, B105, B208, B264 and B294 also decreased disease severity, not differing statistically from each other (Figure 1). These isolates decreased the symptoms of bacterial blight by 68.6%, 64.7%, 78.4%, 74.5%, and 68.6%, respectively.

Isolates B04 and B266 also decreased disease severity and did not differ statistically from each other (Figure 1). They decreased the symptoms of bacterial blight by 66.7% and 62.7%, respectively.

Chemical control is currently the main measure employed against bacterial blight, using inefficient methods to control bacteriosis such as spraying antibiotics and cupric fungicides (hydroxides, sulfates, oxychlorides and cuprous oxide) (Belan et al., 2014). The results obtained under greenhouse conditions in the present study may represent an important tool in the management of this important disease in coffee crop.

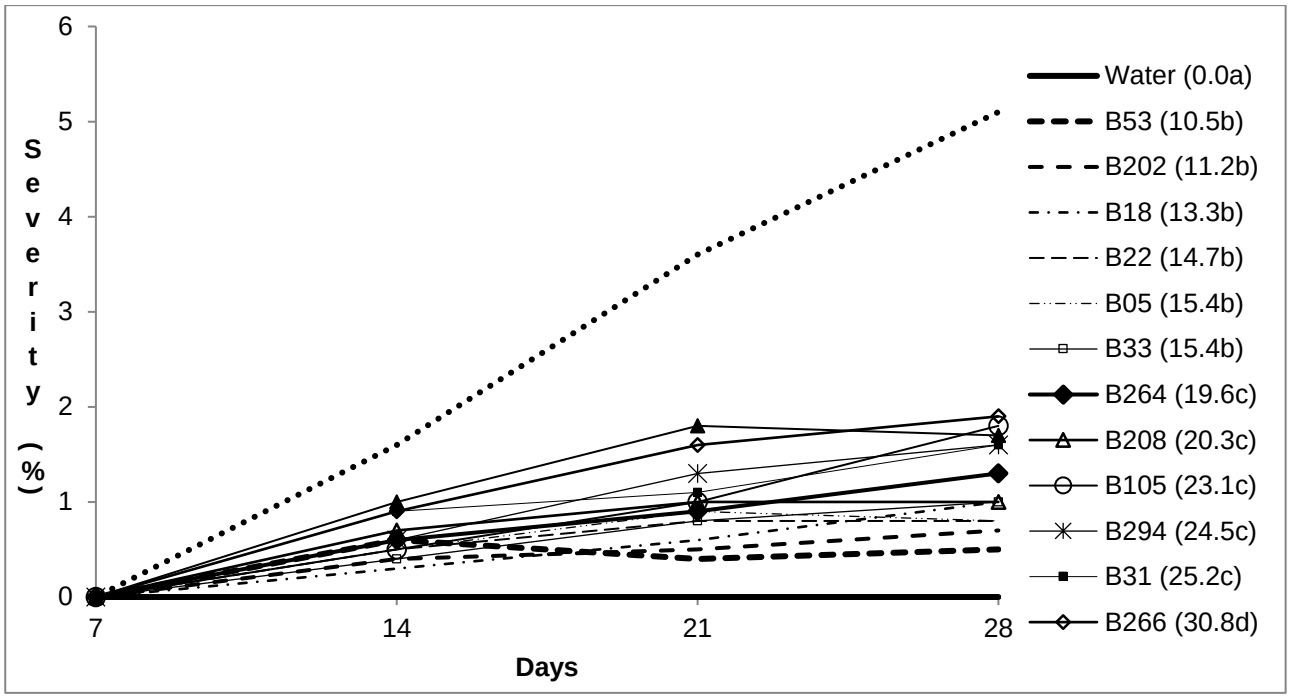

Figure 1 - Curve of progress of severity of bacterial blight in coffee seedlings inoculated with different isolates of *Bacillus* spp. and *Pseudomonas syringae* pv. *garcae*.
(Values in parentheses in the caption represent the area under the disease progress curve (AUDPC), average of 10 seedlings (replicates), after 28 days of artificial inoculation with *P. syringae* pv. *garcae*. Means followed by the same letter do not differ from each other by Tukey's test, p> 0.05).

Some studies, such as that by Lanna et al. (2010), found positive results from the use of *Bacillus* spp. under greenhouse conditions to control several phytopathogens, with a decreased severity of several diseases. In sweet potato (*Beta vulgaris* L.) and tomato (*Solanum lycopersicum*), application of *B. subtilis* reduced the incidence of *Rhizoctonia solani* (Baldotto et al., 2010). Silva et al. (2014) used a *B. pumilus* isolate, antibiotic Kasumin® (kasugamycin), and calcium phosphite to control soft rot in bell pepper caused by *Pectobacterium carotovorum* subsp. *carotovorum*. Calcium phosphite and the *Bacillus* isolate showed lower AUDPC values, and the pathogen was resistant to kasugamycin. Furthermore, Kupper et al. (2003) found that *Bacillus* spp. isolates inhibited the growth of *Colletotrichum acutatum*, the causal agent of premature drop of citrus fruits (*Citrus* spp.), under both laboratory and field conditions.

It was possible to amplify an approximately 1700-bp fragment of the 16S rRNA gene from the thirteen most outstanding bacterial isolates in the *in vitro* experiment. Moreover, BLASTn analyses made it possible to identify *Bacillus* species according to the degree of sequence similarity available in Genbank (Table 2).

Table 2 - Identification of *Bacillus* spp. most promising for coffee bacterial blight management.

Isolate	Identity	Access	Similarity
B04	*Bacillus megaterium*	KT986110.1	90.11%
B05	*Bacillus methylotrophicus*	MF449164.1	92.32%
B18	*Bacillus subtilis*	KM983016.1	91.81%
B22	*Bacillus thuringiensis serovar israelensis*	CP013278.1	81.40%
B31	*Bacillus subtilis subsp. inaquosorum*	CP029465.1	100.00%
B33	*Bacillus subtilis*	KX692273.1	99.53%
B53	*Bacillus safensis*	KX242459.1	93.33%
B105	*Bacillus amyloliquefaciens*	JN558839.1	85.71%
B202	*Bacillus subtilis*	HM588163.1	74.78%
B264	*Bacillus cereus*	CP016360.1	88.89%
B208	*Bacillus megaterium*	CP003020.1	87.10%
B266	*Bacillus amyloliquefaciens*	JN558839.1	85.71%
B294	*Bacillus cereus*	KU884469.1	74.56%

The different isolates of *Bacillus* spp. produced different metabolites. Isolates B04, B05, B22, B31, B53, B202, B208, B264, B266 and B294 showed ammonia production. Isolates B04, B33 and B294 showed hydrocyanic acid production. Finally, isolates B04, B05, B18, B22, B33, B53, B105, B202, B208, B264 and B294 showed siderophores production.

The antagonism of *Bacillus* spp. against phytopathogens such as *P. syringae* pv. *garcae* may be related to competition for space and nutrients, antibiosis and/or synthesis of antimicrobial substances such as ammonia, hydrocyanic acid and siderophores (Leelasuphakul et al., 2008). According to Knaak et al. (2010), antibiosis is a metabolite-producing process that can lead to cell lysis or dissolution. Bacteria of the genus *Bacillus* spp. produce ammonia, a volatile compound associated with phytopathogen control. Isolates that produce ammonia can expand their inhibition halo, reaching a larger inhibition area around colonies that produce this compound (Lanna et al., 2010). Siderophores are Fe(III) binders largely produced by facultative aerobic and anaerobic species, where they scavenge and transport Fe(III) as gram-positive and gram-negative bacteria capable of removing iron from phytopathogens, disrupting the pathogen cycle (Benite et al., 2002). Phytopathogenic bacteria use low iron levels as a signal to induce virulence genes in specific systems of iron uptake, storage and homeostasis regulation (Cornelis & Andrews, 2010). Fe^{3+} is used as a prosthetic group or protein cofactor involved in amino acid synthesis, nitrogen fixation, citric acid cycle, redox stress resistance, oxygen transport, electron transport in cell respiration and light uptake, which is an important component for the metabolic functioning of bacteria (Braun & Hantke, 2011). Phytopathogenic bacteria find iron complexed with organic acids or host proteins in reduced concentration as a host strategy to prevent the infection process (Hood & Skaar, 2012). Hydrocyanic acid, in turn, is a potent enzyme inhibitor involved in the respiration of microorganisms, and is synthesized by some bacteria (Ramette et al., 2003). Santiago et al. (2015) observed that hydrocyanic acid production is one of the mechanisms of action involved in the suppression of eucalyptus bacterial wilt by rhizobacterial isolates.

The most promising isolates obtained under greenhouse conditions in the present study have different mechanisms of action to control *P. syringae* pv. *garcae*. Isolate B05 produces ammonia and siderophores; B18, siderophores; B22, ammonia and siderophores; B33, hydrocyanic acid and siderophores; B53, ammonia and siderophores; and B202, ammonia and siderophores.

Ammonia, siderophores, and hydrocyanic acid syntheses by bacterial isolates have been used as criteria for choosing potential plant disease biocontrol agents and/or plant growth promoters (Mota et al., 2017; Radhakrishnan et al., 2017; Santiago et al., 2015; Ramette et al., 2003).

It is likely that several substances besides those studied here are responsible for controlling coffee bacterial blight. Given the promising results obtained in the present study, a detailed analysis of the chemical compounds involved becomes necessary. In addition, experiments under field conditions should be conducted to confirm the potential of these isolates for the management of coffee bacterial blight.

Conclusion

Isolates B05, B18, B22, B33, B53 and B202 are promising biological control agents against coffee bacterial blight.

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