BIOLOGICAL CONTROL OF *Hemileia vastatrix* Berk. & Broome WITH *Bacillus subtilis* Cohn AND BIOCHEMICAL CHANGES IN THE COFFEE

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ABSTRACT: *Hemileia vastatrix* stands out as one of the main phytosanitary problems of the coffee culture. The objective of this study was to evaluate the efficiency of the biological control of *Hemileia vastatrix* and detect some biochemical changes in plants of cultivars Icatu and Mundo Novo treated with foliar spray of *Bacillus subtilis* and azoxystrobin under field conditions. After the applications, leaves were collected for visual assessment of *H. vastatrix* severity in accordance with diagrammatic scales. For the biochemical evaluations related to plants defense, leaf tissue samples were collected for the analysis of proline, total phenols, total protein and lipid peroxidation. *B. subtilis* acted effectively in control of *H. vastatrix* in both cultivars, but with less efficiency than the azoxystrobin. Plants treated with *B. subtilis* presented greater proline, total phenols and total proteins in their leaves on the application of azoxystrobin. The cultivar Mundo Novo was more responsive to treatments that Icatu.

Index terms: *Coffea arabica*, induced resistance, rhizobacterium.

CONTROLE BIOLÓGICO DA FERRUGEM COM *Bacillus subtilis* E ALTERAÇÕES BIOQUÍMICAS NO CAFEEIRO

RESUMO: *A ferrugem destaca-se como um dos principais problemas fitossanitários da cultura do café. O objetivo deste trabalho foi avaliar a eficiência do controle biológico da ferrugem e detectar alterações bioquímicas nas plantas das cultivares Icatu e Mundo Novo em função da pulverização foliar com Bacillus subtilis e azoxistrobina em condições de campo. Após as aplicações foram coletadas folhas para avaliação visual da severidade da ferrugem de acordo com escalas diagramáticas. Para a avaliação bioquímica relacionada com a defesa nas plantas, foram coletadas amostras de tecido foliar para realização de análises de prolina, fenóis totais, proteínas totais e peroxidação de lipídeos. B. subtilis atuou de forma eficaz no controle da ferrugem nas duas cultivares testadas, porém com desempenho inferior a azoxistrobina. O tratamento com B. subtilis proporcionou maiores teores de prolina, fenóis totais e proteínas totais nas folhas em relação à aplicação de azoxistrobina, com a cultivar Mundo Novo sendo mais responsiva aos tratamentos que a Icatu.*

Termos de indexação: *Coffea arabica, indução de resistência, rizobactéria.*

1 INTRODUCTION

The fungus *Hemileia vastatrix* Berk. & Broome (coffee rust) stands out as the main cause of the coffee disease due to major damages. It causes a foliar disease that initially cause chlorotic spots on the underside of the leaf and in time will spread throughout the limbo, forming larger patches of yellow-orange coloration, leading to death of the affected tissue (MAIA et al., 2009).

Chemical control is the most used way to control the disease through the use of protective and systemic fungicides. Fungicides chemical group of strobilurins, which belongs azoxystrobin, have a biochemical mode of action to inhibition of mitochondrial respiration. This is by blocking the electron transfer in the mitochondrion site, interfering with breathing pathogen (PARREIRA; NEVES; ZAMBOLIM, 2009).

Genetic improvement has already released in the market cultivars resistant to *H. vastatrix*, which have high productivity and quality of grain, and this would lead to decreased application of pesticides (CARVALHO et al., 2012).

The cultivar Icatu has more rustic features, high size, canopy with larger diameter, vigorous growth, average maturity late and moderate resistance to *H. vastatrix* and drought, as well as being widely used as a way of expressing drink. Already the Mundo Novo presents high size, high vigor and longevity, with average maturity and susceptibility to coffee rust, besides presenting great quality drink (GUERREIRO FILHO; FAZUOLI; AGUIAR, 2006). Both cultivars have excellent adaptation in the producing regions of Brazil coffee (CARVALHO et al., 2006).

Biological control has been gaining ground in order to minimize the use of fungicides, reduce environmental impacts and costs in production. It is a form of control that aims to reduce the inoculum and the activities of pathogens through natural agents that activate the plant defense system. This defense is based primarily on the production and/or accumulation of some molecular compounds that the plant develops when under stress (COSTA; ZAMBOLIM; RODRIGUES, 2007).

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The *Bacillus subtilis* Cohn, is a biocontrol agent widely used for acting antagonist manner, releasing toxic substances that act and compete with pathogens. This species has been assessed as an antagonist of several pathogenic fungi under controlled conditions (FIGUEIREDO et al., 2010; KUPPER; BELLOTTE; GOES, 2009). In addition, this microorganism promotes plant growth, proving to be very versatile and efficient in disease control and crop development (GRIGOLETTI JÚNIOR; SANTOS; AUER, 2004). This is due to production growth regulators and plant phytohormones in the rhizosphere, as abscisic acid and indole acetic acid (ARAÚJO; HENNING; HUNGRIA, 2005), as well as solubilization of nutrients (MACHADO et al., 2012).

The objective of this study was to evaluate the efficiency of the biological control of *H. vastatrix* and detect some biochemical changes in plants of cultivars Icatu and Mundo Novo treated with foliar spray of *B. subtilis* and azoxystrobin under field conditions.

2 MATERIAL AND METHODS

The experiment was carried out in the experimental area of Universidade do Oeste Paulista of Presidente Prudente, SP, Brazil, 22° 07' 04" S and 51° 22' 57" W, altitude 472 m, from October 2013 to June 2014. On site is made coffee cultivation for nine years, being the area with the cultivars Mundo Novo and Icatu chosen for this study presents infestation history of *H. vastatrix*. The experimental design was randomized blocks with distribution in factorial arrangement of two cultivars, three treatments (bacteria, fungicide and control) and four replicates, totaling 24 plots. Each replicate was represented by four coffee plants properly identified with the name of the cultivar and treatment.

The control strategy was used to apply a biologically input the base of bacteria of the species *B. subtilis* in liquid suspension, with a concentration of 1,0 x 10^9 cells/ml and a conventional chemical fungicide of the strobilurin group and the active ingredient azoxystrobin (commercial product *AMISTAR*® WG). The control treatment received no spraying.

Four monthly foliar sprays with products using backpack sprayer, constant pressure and manual drive have been performed. Each spray was used dosage of 5 L of water with 50 mL of bacteria and fungicide to 5 L of water to 1,25 g of the product, the first spray being conducted in October 2013.

The severity of *H. vastatrix* in the coffee was evaluated after the four applications. The evaluations were conducted in eight reproductive branches per plant, four branches on each side of the row, in the middle third of the plants, and each branch was rated the third or fourth pair of leaves.

The quantification of the severity of *H. vastatrix* was made according to the diagrammatic scale proposed by Cunha et al. (2001), establishing scores from 0 to 5, with 0 (no disease), 1 (<3% severity), 2 (3 to 6% severity), 3 (6 to 12% severity), 4 (from 12 to 25% severity) and 5 (25 to 50% severity).

The collection of leaves for biochemical assessment was done concurrently with the collection for severity assessment, 10 days after the last spraying. The proline concentration in leaves analysis (µg mg^{-1}) was performed according to the method described by Bates, Waldren and Teare (1973), in which 400 mg of fresh material was taken from the middle third of the plants of each treatment. The material was macerated in 10 mL of 3% sulfosalicylic acid. Filtering material and 2 mL of the supernatant obtained was performed was removed and placed in a test tube with 2 mL of ninhydrin acid and 2 mL of glacial acetic acid. The samples were kept in boiling water bath for 1 hour and after this period were cooled on ice. Then, the reading was performed at the spectrophotometer at 520 nm in triplicate.

The determination of total phenols content (µg mL^{-1}) present in the leaves was carried out using the Folin-Ciocalteu method, according to the methodology proposed by Singleton, Orthofer and Lamuela-Raventós (1974), using gallic acid as standard, solution of methanol and sodium carbonate, in triplicate.

For analysis of total protein, leaf samples were weighed (1g) and macerated with liquid nitrogen. Then put the extraction buffer and centrifuged for 30 minutes (10.000 rpm and 4°C). With drew aliquots of 100 mL of the supernatant, from which was made the determination of protein content (mg mL^{-1}), based on the reactant adsorption Coomassie Brilliant Blue G-250 (BRADFORD, 1976) using bovine serum albumin as standard.

The lipid peroxidation was measured by determining the concentration of malondialdehyde (MDA), according to the methodology of Heath and Packer (1968), 100 mg of leaf tissue without the midrib were processed. The assay was performed in triplicate and the values expressed in nanomol of MDA by fresh weight of grass (nmol g MF^{-1}).

The data were submitted to analysis of variance by F test by SISVAR statistical program, and when significant, the averages were compared by Tukey test at 5% probability.

3 RESULTS AND DISCUSSION

According to Table 1, the results of the analysis of variance F were significant treatment and cultivar in *H. vastatrix*. Regarding the biochemical evaluations - proline, total phenols, total protein and MDA, all results were significant, including the interaction between treatment and cultivar.

The results demonstrate that both the biological and chemical control was effective to reduce the *H. vastatrix* in the coffee (Table 2). The cultivar Icatu showed greater resistance to the pathogen due to lower incidence of *H. vastatrix*. In a study by Montes, Paulo and Fischer (2012), to evaluate the *H. vastatrix* in the coffee, disease severity was lower in Icatu and Obatã cultivars. The cultivar Icatu is a hybrid obtained from *Coffea arabica* L. and *Coffea canephora* Pierre ex A.Froehner, crossing, having inherited from *C. canephora* greater resistance to *H. vastatrix* (BOTTCHER et al., 2012). Martins, Mendez and Alvarenga (2004), comparing agro-ecosystems of Minas Gerais, Brazil, planted with resistant cultivars and susceptible to *H. vastatrix*, observed that the cultivar Icatu not reached the level of damage of the disease, unlike other susceptible cultivars. According to Botelho et al. (2007), the population of Icatu cultivars has a germplasm of great importance in breeding programs for resistance to *H. vastatrix*.

The treatments performed in both cultivars presented significant amounts of *H. vastatrix* control and *B. subtilis* had underperformed the control provided by the fungicide (Table 2). Treatment with fungicide showed 53% control in the cultivar Icatu and 45% in the Mundo Novo, while the bacteria provided with a control of 24% and 17%, respectively. This result confirms what was reported by Dorighello (2013), that by using isolates of *B. subtilis* QST 713 and AP-3 in the control of *Phakopsora pachyrhizie* in the soybean, noted a significant reduction in germination uredospores, and in field trials treatments with fungicides alone or associated with *B. subtilis* have excelled in reducing the severity of disease and defoliation.

Angonese et al. (2009) showed in a study on the fungistatic effect of *Bacillus* spp., which isolates this bacterium or molecules released by them have potential to be used in the integrated management of plant diseases, associated for example with the use of fungicides, allowing the rotation of the use of products with different modes of action to prevent pathogen resistance, ensuring greater security for the production system. This becomes possible due to the mode of action antagonist of this microorganism and the characteristic to stay alive for a long time on the plant, interfering with the attachment of the fungus (LANNA FILHO; FERRO; PINHO, 2010), releasing toxic substances, such as iturin, that act and compete with pathogens (ARAÚJO; HENNING; HUNGRIA, 2005; FIGUEIREDO et al., 2010).

Tofoli et al. (2002), in a job with azoxystrobin in controlling of the *H. vastatrix* in the coffee under induced wet conditions, showed the potential of the chemical group of strobilurin fungicides for the disease control under adverse conditions, in addition to promoting lower levels of defoliation, an important factor in plant maintenance and productivity of future harvests. This chemical group of fungicides include azoxystrobin inhibits mitochondrial respiration by blocking the transfer of electrons in the mitochondria site, interfering with breathing pathogen (PARREIRA; NEVES; ZAMBOLIM, 2009). Studying the control of the *Puccinia triticina* in the wheat with fungicides, Navarini and Balardin (2012) concluded that the effective control of this disease is due to the action of strobilurins.

With respect to biochemical changes in the coffee depending on the treatment, it was found that the leaf proline contents were larger in the Mundo Novo cultivar compared to Icatu. Treatment with *B. subtilis* resulted in a 84% increase in proline content in the cultivar Mundo Novo. On the other hand, for the cultivar Icatu, treatment with *B. subtilis* and fungicide did not differ but resulted in higher proline contents than the control (Table 3).

Praxedes, Ferreira and Gomes Filho (2009), in studies with bean under salt stress, found that stressed plants had higher proline accumulation in their leaves, suggesting a protective mechanism. The accumulation of proline in plants occurs in response to saline, water stress, UV radiation, heavy metals, pathogenic and oxidative stress (LIANG et al., 2013).

TABLE 1 - F values analysis of variance.

F.V.	*H. vatatrix*	Proline	Total phenols	Total protein	MDA
Treatment	52,05**	167,46**	929,71**	669,63**	20,62**
Cultivar	8,78**	787,42**	785,02**	800,23**	30,36**
Treat.XCult.	0,21	154,91**	543,79**	864,96**	41,43**
C.V.	12,36	6,80	1,25	8,06	6,41

* 5% probability ** 1% probability.

TABLE 2 - Effect of the application *B. subtilis* and azoxystrobin in controlling the *H. vastatrix* in two coffee cultivars. Values established by grading scale.

Treatments	Icatu	Mundo Novo
Control	2,95 a	3,20 a
Fungicide	1,40 c	1,77 c
B. subtilis	2,22 b	2,66 b
Mean	2,19 B	2,54 A

Values followed by the same letter, in the columns and the mean line, have no significant value by Tukey test at the 5% level of probability.

TABLE 3 - Forms of control and cultivars in coffee infested with *H. vastatrix* for proline content ($\mu g\ mg^{-1}$) in the leaves.

Treatments	Icatu	Mundo Novo
Control	6,72 b	16,67 b
Fungicide	10,89 a	14,49 c
B. subtilis	9,57 a	30,73 a
Mean	9,06 B	20,63 A

Values followed by the same letter, in the columns and the mean line, have no significant value by Tukey test at the 5% level of probability.

Generally, the function of the proline is associated with osmotic adjustment and its action in regulating water stress. However, proline also serves to balance the cellular oxidative stress in the chelation of metals and removal of reactive oxygen species (ROS) (MATYSIK; ALIA BHALU; MOHANTY, 2002; SZABADOS; SAVOURE, 2010). Thus, understanding the mechanisms by which proline increases the response to biotic and abiotic stresses is of great importance for agricultural research and genetical enhancement.

However, the Mundo Novo cultivar, despite having a higher concentration of proline in the leaves, not found to be more resistant to *H. vastatrix* (Table 2), indicating by this that other mechanisms may be related to this resistance. Rampazzo (2013) noted that the proline accumulation was affected by inoculation with rhizobacteria in sugarcane, and the inoculated plants and under water stress increased 2,2 times the concentration of proline in the leaves when compared to inoculated without presence of stress. This information can be related to the data from this study, in which to cultivate the Mundo Novo, where there was a higher incidence of the *H. vastatrix* and consequently greater stress, showing higher concentration of proline in treatment with *B. subtilis*.

Regarding the total phenol content in leaves, the highest values were found in the cultivar Mundo Novo. However, treatments with bacteria and fungicides did not differ, but both cultivars responded to treatments applied in increasing phenolic content with the control (Table 4).

TABLE 4 - Forms of control and cultivars in coffee infested with *H. vastatrix* for total phenols content (µg mL^{-1}) in the leaves.

Treatments	Icatu	Mundo Novo
Control	6,60 b	9,24 b
Fungicide	9,38 a	9,53 a
B. subtilis	9,53 a	9,71 a
Mean	8,50 B	9,49 A

Values followed by the same letter, in the columns and the mean line, have no significant value by Tukey test at the 5% level of probability.

In a study of resistant soybean cultivars and susceptible to bacterial blight (*Pseudomonas savastanoi* pv glycinea), Sbalcheiro (2010) held the application of *Bacillus* spp. and found increase in phenol content in plants. Mello, Frighetto and Valarini (2009) observed an increase in the total phenol content in soybean leaves exposed to *P. pachyrhizie* in response to treatment with an isolated endophytic bacteria. Phenolic compounds are secondary metabolites produced by plants that are not directly related to development of the same, but with resistance to pests and diseases. In this defense mechanism of plants deviated carbohydrates of the major metabolic pathway for the secondary producing the phenols, which are protective substances, fungitoxic, antibacterial and antiviral, and inhibit the germination of fungal spores, such as the isoflavones (SALGADO, 2004).

Leaf contents of total protein in treatments with fungicide and *B. subtilis* in the cultivar Icatu were lower than the control (Table 5). This effect was also verified by Kuhn (2007) in the induction of resistance in beans by treatment with *Bacillus cereus*, where plants had a lower protein content in the leaves.

In contrast, treatment with *B. subtilis* was that provided the greatest protein content in the cultivar Mundo Novo. Silva et al. (2009) found that the inoculation of bean seeds with isolated from *Pseudomonas* DFs842 was responsible for the induction of resistance attributed to the significant increase in concentration of total soluble proteins. The increase in total protein in the treatments of *B. subtilis* is also related to the increase in proline found in this study. The proteins are part of the plasma membrane and out as regulators osmosis, and if there is intense stress proteins break their plants to generate electricity or other amino acids accumulate to defend (ABADE et al., 2014). In the assessment of biochemical descriptors in cotton cultivars focusing on increasing disease resistance, Silva et al. (2009) stated that the total protein content was not a useful parameter for the discrimination of resistant or susceptible cultivars.

No differences were found in the leaf content of malondialdehyde (MDA) between any of the treatments in Mundo Novo, which on average had lower MDA values compared to Icatu. This cultivar, in turn, exhibited higher MDA content when treated with fungicide whereas treatment with *B. subtilis* significantly reduced this level compared to control (Table 6).

The MDA is produced by peroxidation of lipids of the cell membrane when the plant is suffering from some kind of stress (HENDGES et al., 2015). The MDA content is often used as a marker of the degree of oxidative damage (OLIVEIRA; GOMES-FILHO; ENÉAS-FILHO, 2010). According to Langaro et al. (2014), when the plant is treated with herbicide, the protective ability of the carotenoid to act against oxidative stress occurs and decreases the lipid peroxidation. The reduction of the MDA from plants treated with *B. subtilis* shows the ability of this biocontrol agent has to maintain the integrity of cell membranes, which requires further study.

B. subtilis has been shown to be an important option in biological control, especially of foliar diseases. This control can be effected by direct inhibition of the pathogen through the production of inhibitory substances, and also by physiological changes in the metabolism of plants, resulting in activation of the defense mechanism of the same.

The results of this experiment can be inferred that the variety Mundo Novo showed higher production of defense substances in response to the treatment used, especially with *B. subtilis*, demonstrating with it greater potential for plant protection when using induced resistance. Bottcher et al. (2012) also found that this cultivar had better performance in the production of defense substances compared to Icatu.

TABLE 5 - Forms of control and cultivars in coffee infested with *H. vastatrix* for total protein content (mg mL^{-1}) in the leaves.

Treatments	Icatu	Mundo Novo
Control	0,07 a	0,04 c
Fungicide	0,03 b	0,07 b
B. subtilis	0,04 b	0,32 a
Mean	0,05 B	0,14 A

Values followed by the same letter, in the columns and the mean line, have no significant value by Tukey test at the 5% level of probability.

TABLE 6 - Forms of control and cultivars in coffee infested with *H. vastatrix* for content of MDA (nmol g^{-1}) in the leaves.

Treatments	Icatu	Mundo Novo
Control	3,19 b	2,49 a
Fungicide	3,54 a	2,52 a
B. subtilis	2,21 c	2,73 a
Mean	2,98 A	2,58 B

Values followed by the same letter, in the columns and the mean line, have no significant value by Tukey test at the 5% level of probability.

The use of biological control with *B. subtilis* has shown promise for its introduction as a tactic of diseases in coffee, because beyond the control of diseases such as *H. vastatrix*, this microorganism can stimulate the plant to trigger defense mechanisms important for increased resistance. This can mitigate the damaging effects of different pathogens culture during their cycle.

4 CONCLUSIONS

B. subtilis acted effectively in control of *H. vastatrix* in both cultivars, but with less efficiency than the azoxystrobin. Plants treated with *B. subtilis* presented greater proline, total phenols and total proteins in their leaves on the application of azoxystrobin, with the cultivar Mundo Novo being more responsive to treatments that Icatu.

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