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Reaction of *Coffea arabica* seedlings to colonization by wild *Colletotrichum gloeosporioides* and transformed with *gfp.*

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ABSTRACT

Defense reactions of coffee trees in response to invasion by *Colletotrichum gloeosporioides* (Penz.) wild (I2) and transformed with the green fluorescent protein gene *gfp* (I2-T) were studied in this research to identify defense reactions of coffee tree seedlings in response to invasion by *Colletotrichum gloeosporioides* using wild isolates and those transformed with the *gfp* gene by observing the biochemical changes developed by the plants and to check the difference in the pathogen behavior after transformation. The activity of the guaiacol peroxidase (POX) enzyme was evaluated in both types of seedlings obtained, with and without symptoms (MOPCS and MOPSS), respectively. The inoculation of the different isolates (I2 and I2-T) induced the activity of the enzyme, showing higher rates than those observed in the control treatment, demonstrating that there was a possible recognition of the pathogen. Regarding the activity of the enzyme polyphenoloxidase (PPO), similar behavior was observed between the control and the isolates studied at different exposure times. The highest peak in the activity of the enzymes studied occurred at 6 and 24 hours after inoculation with I2. It was observed that the time the plants were exposed to the pathogen increased the levels of total soluble phenols.

Keywords: *Coffea arabica*, Enzymes, Proteins.

Reação de mudas de *Coffea arabica* à colonização de *Colletotrichum gloeosporioides* selvagens e transformados com *gfp.*

RESUMO

Reações de defesa de cafeeiro em resposta à invasão por *Colletotrichum gloeosporioides* (Penz.) selvagens (I2) e transformado com o gene da proteína verde fluorescente *gfp* (I2-T) foram estudadas nesta pesquisa, com objetivo de identificar reações de defesa de mudas de cafeeiro em resposta à invasão por *Colletotrichum gloeosporioides* utilizando-se isolados selvagens e transformados com o gene *gfp* através da observação das alterações bioquímicas desenvolvidas pelas plantas e verificação da diferença no comportamento do patógeno após transformação. Para a atividade da enzima peroxidase de guaiacol (POX) em ambos os materiais estudados, mudas obtidas de plantas com e sem sintomas (MOPCS e MOPSS), respectivamente. A inoculação dos diferentes isolados (I2 e I2-T) induziu a atividade da enzima, apresentando índices superiores ao observado no tratamento controle, demonstrando que houve um possível reconhecimento do patógeno. Em relação à atividade da enzima polifenoloxidades (PPO), observou-se comportamento semelhante entre a testemunha e os isolados estudados, nos diferentes tempos de exposição. O maior pico da atividade das enzimas estudadas ocorreu às 6 e 24 horas após inoculação com I2. Observou-se que o tempo de exposição das plantas ao patógeno aumentou os teores de fenóis solúveis totais.

Palavras-chave: *Coffea arabica,* Enzimas, Proteínas.

1. Introduction

Plants are irreplaceable food resources, synthesizing their food and storing the excess as a reserve (Bar On et al., 2018). On the other hand, they are exposed daily to environmental conditions, biological agents, and endogenous factors, which provide the plant with immediate defense mechanisms (Fernandes et al., 2009). When challenged by phytopathogens such as fungi, bacteria, nematodes, and viruses, plants recognize the infection and induce defense mechanisms against the pathogen, for example, through the production of enzymes, specific or non-specific toxins, hormones, and effectors, which act on the plant metabolism and cellular functions (Dallagnol et al., 2021).

A plant defense mechanism concerning the action of the pathogen can be characterized by the constitution of physical barriers, preventing its penetration, or by using chemical mechanisms, such as substances that act on the development or survival of the pathogen (Stangarlin et al., 2011). The variation in the activity of oxidative enzymes, such as peroxidases in plant tissues, has been related to the host capacity for resistance or susceptibility (Dallagnol et al., 2021).

The production of reactive oxygen species (ROS), mainly in the form of superoxide and hydrogen peroxide (H_2O_2), acts on the expression of defense genes, intensifying the host resistance to the pathogen (Evans et al., 2009).

The *Colletotrichum* genus complex in coffee is associated with blister spots and the drying out of plant tips, and anthracnose symptoms are notorious on leaves and fruit (Munaut et al., 1998), especially in conditions of high humidity and mild temperatures of around 22 ºC. This infection is present in various pathosystems (pathogen and host relationship), which has sparked interest in research into the penetration process and determining the pathogen resistance. Thus, understanding the molecular mechanisms underlying coffee tree *Colletotrichum gloeosporioides* interactions is essential for developing control measures and breeding programs for disease resistance (Paradela Filho et al., 2001).

Recent research has progressed into pathosystems involving biotrophic and necrotrophic fungal species, but understanding the mechanisms mediating plant infection by hemibiotrophs is still very limited.

The use of transgenic isolates is considered an alternative that has provided a better understanding of the interaction of phytopathogenic fungi with their host, methods of penetration, colonization, and dissemination of different pathogens, as well as obtaining resistance to pathogens (Dong and Ronald, 2019). The fluorescent proteins used in forming a transgenic gene can be easily cloned into different vectors and presented in various cell types, thus being considered an important tool that has revolutionized cell biology, especially in the study of living cells (Chalfie et al., 1994).

This study aimed to identify the defense reactions of coffee seedlings in response to invasion by *C. gloeosporioides* using wild isolates and those transformed with the *gfp* gene by observing the biochemical changes developed by these plants.

2. Material and Methods

The experiment was conducted in a randomized block design (RBD) with four replications, each consisting of two micropropagated seedlings. The seedlings were obtained from coffee seeds of the cultivar Catuaí Vermelho from plants with and without symptoms of blister spot, which were taken to the Tissue Culture Laboratory at the Federal University of Lavras, where they were micropropagated in BDA culture medium, then transferred to 500 ml cups and placed in the greenhouse until the end of the trial. The seedlings were inoculated with a suspension of conidia collected in the experimental field of the Federal University of Lavras and stored in the Diagnosis and Control Laboratory of the same university at a concentration of $2x10^6$ conidia.ml^{-1}, with the isolate of *C. gloeosporioides* genetically transformed using the transformation technique via the pCT74 vector (pSC001), containing the sgfp, with the PtOx promoter gene of the fungus *Aspergillus nidulans*, containing a resistance gene (HPR) to the antibiotic hygromycin B. The plasmid was provided by researcher Dr. Theo van der Lee (Plant Research International, Netherlands), according to Armesto et al., 2012 expressing the fluorescent protein GFP (I2-T) and one (1) wild isolate obtained from the mycoteca of the Diagnosis Laboratory of the Federal University of Lavras (non-transformed I2). They were kept in a 2% MEA (Malt Extract and Agar) culture medium for the duration of the study (approximately 48 months).

Inoculation was done by spraying the conidia suspension using a 500 mL manual atomizer onto the previously wounded coffee seedlings. Three days before inoculation, these plants were subjected to humid chamber conditions using plastic bags and kept in a greenhouse at 25 ºC. The wounds were made using entomological needles positioned at 0.5 mm equidistant, totaling an area of 1.3 cm in diameter, to ensure the best penetration of the pathogen into the leaf tissue. After inoculation, the seedlings were kept in the greenhouse.

The entire seedling was collected for the analysis, except for the roots, using two seedlings/replication/time of exposure to the pathogen (6, 12, 24, and 48 hours after inoculation). During collection, the material was protected with aluminum foil, placed in Styrofoam containing liquid nitrogen, and then taken to the Plant

Disease Diagnosis and Control laboratory at the Federal University of Lavras (UFLA), where it was stored in a freezer at -80 ºC for seven days until the extracts were prepared for enzymatic analysis.

The leaf tissues were macerated in liquid nitrogen until a fine powder was obtained. Subsequently, 2 g of the fine powder was placed in a Falcon tube with 50 mM sodium acetate buffer pH 5.2 (10.0 mL of buffer for each gram of sample) and homogenized for 10 seconds while shaking. After this process, the suspension was centrifuged at 12,000 rpm for 10 minutes (4 °C). The supernatant was removed, placed in plastic microtubes, used as an enzyme source, and stored at -80 ºC for later analysis. The activity of guaiacol peroxidase (POX) was determined by adding 10 µL of the enzyme source, as described above, 20 µL of sodium acetate buffer (50 mM pH 5.2), and adjusted to 200 µL of a solution containing 473 µL of sodium acetate buffer (50 mM pH 5.2), 500 µL of guaiacol (20 mM), and 500 µL of hydrogen peroxide (60 mM). After incubation at 30 °C for 10 minutes, the absorbance was measured in a spectrophotometer at 480 nm.

Polyphenol oxidase (PPO) activity was determined by adding 50 µL of the enzyme extract, adjusted to 200 µL of a solution containing 473 µL of sodium phosphate buffer (50 mM pH 5.2), and 527 µL of catechol (50 mM). After incubation at 30 °C for 10 minutes, the absorbance was measured in a spectrophotometer at 410 nm. All enzyme assays were conducted in triplicates.

The leaf tissues collected 48 hours after inoculation were ground in liquid nitrogen. The samples were then freeze dried for 24 hours. An aliquot of 30 mg of the freeze dried material was transferred to a 2 mL microtube, homogenized with 1.5 mL of 80% methanol, and kept shaking for 15 hours in a rotary shaker, protected from light at room temperature. The suspension was centrifuged at 12,000 rpm for five minutes. The supernatant (methanolic extract) was transferred to a new microtube, which was used to determine total soluble phenols, while the solid residue was used to determine soluble lignin. In microplates, aliquots of 15 µL of methanolic extract were mixed with 15 µL of 80% methanol and 30 µL of 0.25 N Folin Ciocalteau reagent for five minutes, homogenized with 30 µL of 1 M Na_2CO_3 for ten minutes and diluted with 110 µL of distilled water at room temperature for one hour.

Based on a study by Düsman et al. in 2017, the absorbance values of this reaction were determined at 725 nm in a compatible EIA spectrophotometer and calculated based on the chlorogenic acid curve. The total phenolic compounds were expressed as microgram equivalents of chlorogenic acid per milligram of dry mass.

The experiment was conducted in a 2 x 3 x 4 factorial scheme (two types of micropropagated seedlings from plants with and without blister spot symptoms, two isolates of *C. gloeosporioides* transformed and non-transformed and non-inoculated plants sprayed with water, and collection at 6, 12, 24, and 48 hours after inoculation). The means, when significant by the F test, were compared by the Tukey test ($P \leq 0.05$) using the Sisvar® 5.1 program Ferreira (2011).

3. Results and Discussion

There was a significant interaction between the types of seedlings and treatment and between treatment and the time the plants were exposed (hours after inoculation) to *C. gloeosporioides* for the activity of the POX enzyme. In both types of seedlings studied (seedlings obtained from plants with symptoms MOPWS and seedlings obtained from plants without symptoms MOPWT), the inoculation of the different isolates of *C. gloeosporioides* (I2 and I2-T) induced the activity of the POX enzyme, showing higher activity than that observed in the control treatment (inoculation with water) (Table 1), showing that there was a possible recognition of the pathogen when observing the increase in the activity of this enzyme.

The maximum peak in the guiacol peroxidase activity was 6 hours after inoculation with isolate I2-T and 48 hours after inoculation with isolate I2. However, it can be seen that there was a greater increase in enzyme activity in the first few hours after inoculation with I2 compared to I2-T, which can be attributed to later germ tube formation and infection processes compared to I2 (Figure 1). This was confirmed by Shiraishi et al. (1995), working with pathogenic *Erysiphe graminis* f.sp. *hordei* (race 1) and non-pathogenic *Erysiphe pisi*, who emphasized the importance of the primary germ tube for the infection process and the influence on POX levels through the attempt to penetrate these tubes into host tissues.

Peroxidases are fundamental in plant defense against pathogens and are induced by the plant when infected by fungi, bacteria, viruses, and viroids, as stated in the literature by Loon et al. (1998). The role of these enzymes in plant defense is to strengthen the cell wall with the formation of lignin, increase the production of reactive oxygen species, i.e., cell protection against oxidative reactions, and increase the production of phytoalexins (Kristensen et al. 1999).

Based on research by Anterola and Lews (2002), the activity of the enzyme peroxidases is often increased in response to attack by phytopathogens.

Table 1. Guiacol peroxidase (POX) activity in seedlings obtained from plants with and without symptoms of blister spot and inoculated with *C. gloeosporioides* non-transformed (I2) and transformed with the green fluorescent protein gene *gfp* (I2-T).

Treatment	MOPWT	MOPWS
I2	3.65 Aa	3.48 Aa
I2-T	3.46 Aa	2.25 Bb
Control	0.84 Ba	0.67 Ca
CV (%)	37.58	

Means followed by the same letter, lowercase in the column and uppercase in the line, do not differ by the Tukey test (P≤0.05).

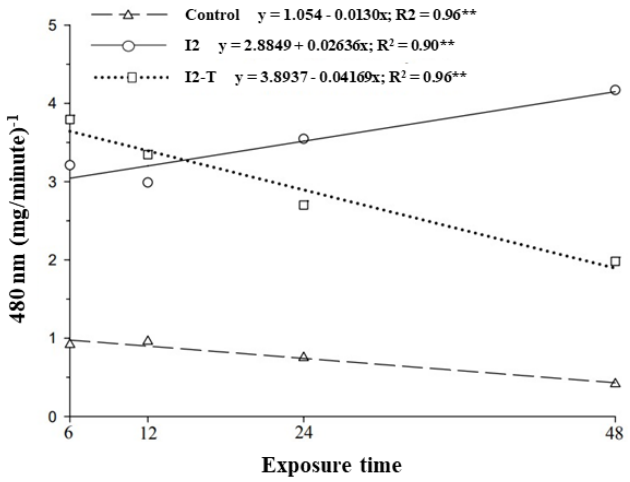

Figure 1. Guaiacol peroxidase activity in coffee leaves after inoculation with non-transformed (I2) and transformed (I2-T) *C. gloeosporioides* according to the exposure time to the pathogen.

Following the same reasoning as the authors above, Pimenta et al. (2004) point out that environmental factors such as insect attacks, microorganism infections, physiological alterations, and mechanical damage cause rapid deterioration of plant tissue because once the cell membrane is broken, there is greater contact between the enzymes and the chemical compounds present in the tissue, both intra and extra cellular, thus causing chemical reactions that modify their original composition.

In this context, the POX enzyme activity was due to the damage caused to the membrane by the infection of the pathogen in the plant tissue (leaves) 6 hours after inoculation. Concerning the activity of the enzyme polyphenol oxidase (PPO), significant differences were observed for treatment and type of seedlings. When the plants were exposed to the pathogen, both I2-T and I2 did not influence the amount of PPO in either MOPWS or MOPWT and did not differ statistically from the control.

In MOPWS, higher PPO enzyme activity peaks were observed compared to MOPWT (Table 2). As with POX, it is believed that the PPO activity observed was due to the damage caused to the membrane by the fungus attacking the host (polyphenoloxidase peak). In addition, it can be said that the infection caused by the fungus can lead to an oxidative explosion and the accumulation of these phenolic compounds in both compatible and incompatible relationships, as observed in this study, since in plants with the "susceptibility" factor (MOPWS) the peaks of this enzyme were higher than in plants without this factor (MOPWT).

The transformed and non-transformed isolates behaved differently from the control, showing that recognition of the pathogen interfered with the increase in this enzyme activity. Similar results were found by Ribeiro Júnior et al. (2006), where in the *Verticilium dahliae* Cocoa tree pathosystem, the application of ASM (0.2 g/L) followed by inoculation of the pathogen induced an increase in PPO activity when compared to the control. According to Vieira et al. (2011), high peaks of this enzyme were identified when working with coffee plants artificially inoculated with *C. gloeosporioides*.

According to the results observed in the enzyme tests, it can be inferred that, even after undergoing the genetic transformation process, the *C. gloeosporioides* fungus does not lose its ability to manifest itself in the host and be perceived by it so that the results of signal perception are conducted, and the plant defense mechanisms are developed.

Table 2. Polyphenoloxidase activity in coffee leaves from seedlings obtained from plants without (MOPWT) and with (MOPWS) blister spot symptoms after inoculation (HAI) with non-transformed and transformed *C. gloeosporioides*.

Type of seedlings	PPO activity (mg/min)
MOPWT	0.133625 B
MOPWS	0.180750 A
CV (%)	68.20

Means followed by the same letter do not differ by the Tukey test (P≤0.05).

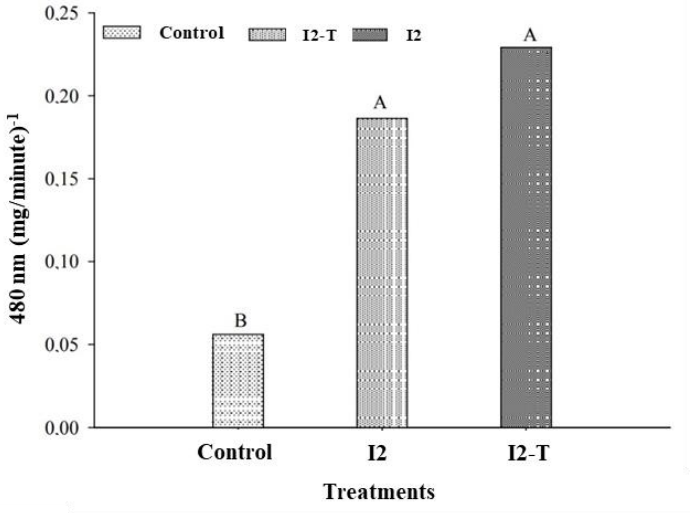

Figure 2. Polyphenol oxidase activity in coffee leaves after inoculation (HAI) with non-transformed and transformed *C. gloeosporioides*.

The time the plants were exposed to the pathogen, both I2-T and I2, did not influence the levels of total phenols in MOPWS and did not differ statistically from the control. The distribution and location of phenols in plants is not known. However, it can be seen that the quantities vary according to the organs, age, stage of plant development, and climatic conditions (Salgado et al., 2008).

Therefore, the lack of these compounds in the MOPWS tissues may be related to the age of the plant, as they were very young with leaves that had not yet fully expanded. Salgado et al. (2008) state that young leaves have lower total phenol levels than older leaves. In the case of MOPWT, for I2-T and I2, higher levels of total soluble phenols than the control at 48 hours after inoculation were observed (Figure 3).

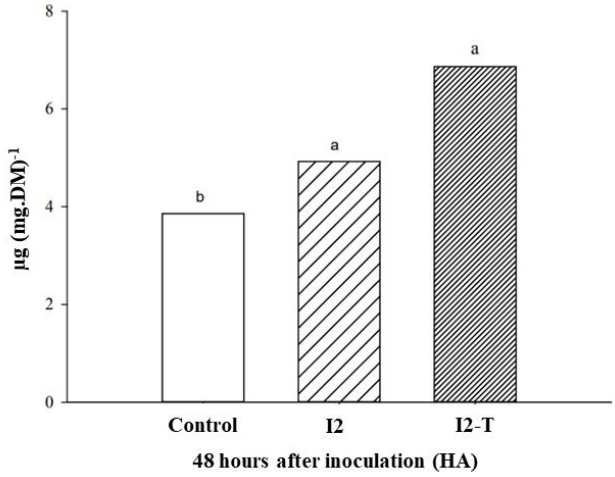

Figure 3. Total soluble phenol content µg (mg DM)$^{-1}$ in coffee leaves from seedlings obtained from plants without (MOPWT) blister spot symptoms 48 hours after inoculation (HAI) with non-transformed and transformed *C. gloeosporioides*.

The synthesis of these compounds is considered part of an effective response mechanism and may play a role in the active expression of plant resistance to pathogens. Given the above, the results obtained in this study corroborate Nicholson and Hammerschmidt (1992) state that an increase in the synthesis of various phenolic substances with antimicrobial activity has been detected in plants in response to treatment with resistance inducers or the presence of potential pathogens, such as *C. gloeosporioides*, and that the rapid accumulation of these compounds can result in effective confinement of the pathogen at the site of infection.

Further studies should be conducted to elucidate the effect of this pathogen on the activation of defense responses in coffee trees. An alternative could be to increase the time between inoculation and collection of the material and to check the symptoms caused by the isolates.

4. Conclusions

Genetically transformed *Colletotrichum gloeosporioides* do not lose their ability to manifest themselves in the host and be perceived by the plant in seedlings obtained from plants with and without blister spot symptoms, thus activating the plant defense mechanisms. The time the plants were exposed to the pathogen increased the levels of total soluble phenols in seedlings obtained from plants without blister spot symptoms.

Authors' Contribution

During the experiment, Fernanda Gonçalves Martins Maia, Jader Braga Maia, Cecília Armestro, and Maísa de Paula Freitas participated and contributed significantly in all stages of the work, from conducting the fieldwork to writing the manuscript. Pablo da Costa Gontijo helped with statistical analysis and data interpretation.

Bibliographic References

Armesto, C., Martins-Maia, F.G., Abreu, M.S., Figueira, A.R., Silva, B.M., Monteiro, F.P., 2012. Genetic transformation with the *gfp* gene of *Colletotrichum gloeosporioides* isolates from coffee with blister spot. Brazilian Journal of Microbiology, 43(3), 1222-1229. DOI: https://doi.org/10.1590/S1517-83822012000300050

Anterola, A.M., Lewis, N.G., 2002. Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. Phytochemistry, Oxford, 61(3), 221-294. DOI: https://doi.org/10.1016/s0031-9422(02)00211-x

Bar-on, Y.M., Phillips, R., Milo, R., 2018. The biomass distribution on Earth. Proceedings of the National Academy of Sciences of the United States of America, 115(25), 6506-6511. https://www.pnas.org/doi/full/10.1073/pnas.1711842115

Fernandes, C.D., Vieira Junior, J.R., Silva, D.S.G., Reis, N.D., Antunes Junior, H., 2009. Mecanismos de defesa de plantas contra o ataque de agentes fitopatogênicos. Porto Velho, RO: Embrapa Rondônia, 14 p.

Chalfie, M., Euskirchen G., Prasher, D.C., 1994. Proteína Verde Fluorescente como Marcador de Expressão Gênica. Science, 263(5148), 802-805. DOI: https://doi.org/10.1126/science.8303295

Dallagnol, L.J., Pazdiora, P.C., Dorneles, K.R., Morello, T.N., Nicholson P., 2021. Silicon soil amendment as a complement to manage tan spot and fusarium head blight in wheat. Agronomy for Sustainable Development, 41(21), 1-2. https://link.springer.com/article/10.1007/s13593-021-00677-0

Dong, O.X, Ronald, P.C., 2019. Genetic Engineering for Disease Resistance in Plants: Recent Progress and Future Perspectives. Plant Physiology, 180(1), 26-38. DOI: https://doi.org/10.1104/pp.18.01224

Evans, I., Solberg, E., Huber, D.M., 2009. Copper and plant disease. Mineral nutrition and plant disease. Saint Paul, MN. The American Phytopathological Society, 44(5), 177-188. DOI: https://doi.org/10.21273/HORTSCI.44.5.1510a

Ferreira, D.F., 2011. SISVAR: a computer statistical analysis system. Ciência e Agrotecnologia, Lavras, 35(6), 1039-1042. DOI: https://doi.org/10.1590/S1413-70542011000600001

Kristensen, B.K., Bloch, H., Rasmussen, S.K., 1999. Barley coleoptiles peroxidases: purification, molecular cloning, and induction by pathogens. Plant Physiology, 120(2), 501-512. DOI: https://doi.org/10.1104%2Fpp.120.2.501

Loon, L.C., Van, Bakker, P.A.H.M., Pieterse, C.M.J., 1998. Systemic resistence induced by rhizosphere bactéria. Annual Review of Phytopathology, Palo Alto, 36(1), 453-483. DOI: https://doi.org/10.1146/annurev.phyto.36.1.453

Munaut, F., Hamaide, N., Vander Stappen, J., Maraite, H., 1998. Genetic relationships among isolates of Colletotrichum gloeosporioides from Stylosanthes spp. in Africa and Australia using RAPD and ribosomal DNA markers. Plant Pathology, 47(5), 641–648. DOI: https://doi.org/10.1046/j.1365-3059.1998.00287.x

Nicholson, R.L., Hammerschmid T.R., 1992. Phenolic compounds and their role in disease resistance. Annual Review of Phytopathology, 30(8), 369-389. DOI: https://doi.org/10.1146/annurev.py.30.090192.002101

Paradela Filho, O., Paradela, A. L., 2001. O complexo *Colletotrichum*: cafeeiro. In: ZAMBOLIM, L. (Ed.). Tecnologias de produção de café com qualidade. Viçosa: UFV, Departamento de Fitopatologia, cap. 8. p. 269-275. DOI: https://doi.org/10.1590/S0006-87052005000300013

Pimenta, B.K., Vilela, E.V., Júnior, C.C., 2004. Componentes de parede celular de grãos de frutos de café (*Coffea arabica L.*) submetidos a diferentes tempos à espera da secagem. Acta Scientiarum. Agronomy, 26(2), 203-209. DOI: https://doi.org/10.4025/actasciagron.v26i2.1884

Ribeiro Júnior, P.M., Resende, M.L.V., Pereira, R.B., Cavalcanti, F.B., Amaral, D.R., Pádua, M.A., 2006. Fosfito de potássio na indução de resistência a *Verticillium dahliae Kleb.*, em mudas de cacaueiro (*Theobroma cacao L.*). Ciência Agrotecnologia, 30(4), 629-636. DOI: https://doi.org/10.1590/S1413-70542006000400006

Salgado, P.R., Favarin, J.L., Leandro, R.A., Lima Filho, O.F., 2008. Total phenol concentration in coffee tree leaves during fruit development. Scientia Agricola, 65(4), 354-359. DOI: https://doi.org/10.1590/S0103-90162008000400005

Shiraishi, T., Yamada, T., Nicholson, R.L., Kunoh, H., 1995. Phenylalanine ammonia-lyase in barley: activity enhancement in response to *Erysiphe graminis* f. sp. *hordei* (race 1) a pathogen, and *Erysiphe pisi*, a nonpathogen. Physiological and Molecular Plant Pathology, 46(2), 153-162. DOI: https://doi.org/10.1006/pmpp.1995.1012

Stangarlin, J.R., Kuhn, O.J., Toledo, M.V., Portz, R.L., Schwan-Estrada, K.R.F., Pascholati, S.F., 2011. A defesa vegetal contra fitopatógenos. Scientia Agraria Paranaenis, 10(1), 18-46. https://e-revista.unioeste.br/index.php/scientiaagraria/article/view/5268

Vieira, J.F., Abreu, M.S., Maia, F.G.M., Ogoshi, C., Pierre, R.O., Carvalho, E.A., Silva, B.M., 2011. Tratamento térmico de sementes no manejo da mancha manteigosa causada por *Colletotrichum gloeosporioides* em cafeeiro *Coffea arabica L*. Acta Ambiental Catarinense, 8(1/2), 28-38. https://bell.unochapeco.edu.br/revistas/index.php/acta/article/view/1927