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Alterations in antioxidant metabolism in coffee leaves infected by *Cercospora coffeicola*

Alterações no metabolismo antioxidante de folhas de cafeeiro infectadas por *Cercospora coffeicola*

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ABSTRACT

*Brown eye spot (BE) caused by **Cercospora coffeicola** is the main disease of coffee crop. A variation in symptoms of BE has been reported in the field, raising suspicion of occurrence of new species. However, information about coffee-**C. coffeicola** interaction is still limited. This research aimed to determine the difference between antioxidant metabolism of coffee plants cultivar Mundo Novo inoculated with a strain isolated from a common BE lesion (CML 2984) and a strain isolated from a black BE lesion (CML 2985). The enzyme activity of peroxidase (POX), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and phenylalanine ammonia lyase (PAL) were determined. Activities of POX, APX, and PAL increased in plants inoculated with both strains compared to non-inoculated plants at 12 and 24 hours post inoculation (hpi). CAT activity increased in inoculated plants with black BE strain at 24 hpi and both strains at 48 hpi. The SOD activity only increased in inoculated plants with both strains at 48 hpi. These results show that an elevated antioxidant response was observed when the plants were challenged with both strains of **C. coffeicola**. Both strains produced lesions of the common type, suggesting that other factors lead to the development of black BE lesion type under field conditions and further investigation is needed.*

Key words: ***Coffea arabica** L., "common" brown eye spot, "black" brown eye spot, reactive oxygen species.*

RESUMO

*A cercosporiose, causada pelo fungo Cercospora coffeicola, é uma das principais doenças que afeta o cafeeiro. Uma variação nos sintomas de cercosporiose foi encontrada no campo, levantando suspeitas da ocorrência de uma nova espécie. Considerando que informações sobre a interação cafeeiro-C. coffeicola ainda são limitadas, objetivou-se, neste trabalho, determinar diferenças no metabolismo oxidativo de plantas de cafeeiro, cultivar 'Mundo Novo', inoculadas com isolado proveniente de lesões de cercosporiose do tipo olho pardo (CML 2984) e isolado proveniente de lesões de cercosporiose do tipo negra (CML 2985). Foram determinadas a atividade das enzimas peroxidase (POX), catalase (CAT), superóxido dismutase (SOD), ascorbato peroxidase (APX) e fenilalanina amônia liase (PAL). As atividades das enzimas POX, APX e PAL aumentaram nas plantas inoculadas com ambos os isolados, quando comparadas com as plantas não inoculadas às 12 e 24 horas após inoculação (hai). A atividade da CAT aumentou nas plantas inoculadas com o isolado que causa sintomas de cercosporiose do tipo negra às 24 hai e, para ambos os isolados, às 48 hai. Plantas inoculadas com ambos os isolados demonstraram aumento na atividade da SOD somente às 48 hai. Este estudo mostrou que uma resposta antioxidante elevada foi observada quando as plantas foram inoculadas com os dois isolados de **C. coffeicola**. Ambos os isolados produziram lesões de cercosporiose do tipo comum, sugerindo que outros fatores que causam lesões de cercosporiose do tipo negra ainda necessitam ser investigados.*

Palavras chave: ***Coffea arabica** L., cercosporiose do tipo olho pardo, cercosporiose do tipo negra, espécies reativas de oxigênio.*

INTRODUCTION

Brown eye spot (BE) caused by the fungus ***Cercospora coffeicola*** Berkeley & Cooke, is one of the main diseases of coffee trees (***Coffea arabica*** L.), causing reduced yield and quality of the beverage (LIMA et al., 2012). Observed symptoms can vary under field conditions, resulting in several speculations and

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hypotheses such as the possibility of genetic variation in the pathogen and different environmental and nutritional effects (phosphorus deficiency, nitrogen and potassium) (NELSON, 2008; MATIELLO & ALMEIDA, 2013). Typical symptoms on coffee leaves due to infection by ***C. coffeicola*** are small necrotic spots consisting of a light-colored center surrounded by a purple brown ring with yellow edges, giving rise to the name common BE. The atypical symptom is characterized by black BE lesions which are larger than normal and resembled leaf spot (MATIELLO & ALMEIDA, 2013). It is known that ***Cercospora*** produces a toxin called cercosporin which causes damage to the cellular membrane of the host in the presence of sunlight, resulting in lipid peroxidation, loss of electrolytes and formation of reactive oxygen species (ROS) (SHARMA et al., 2012; DAUB et al., 2013). In order to minimize damage caused by this oxidative stress, plants developed mechanisms of enzymatic and non-enzymatic defence (SCANDALIOS, 2005). Peroxidase (POX), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione-S-transferase (GST), glutathione redutase (GR), phenylaline ammonia lyase (PAL) and glutathione peroxidase (GPX) are among the enzymes used in enzymatic defence (SHARMA et al., 2012).

Previously conducted research reported changes in the defense system antioxidant of the plants in response to induced stress by biotic and abiotic agents (DEBONA et al., 2012; NASCIMENTO et al., 2014; DOMICIANO et al., 2015). It is unknown whether the coffee plant responds in a different way to ***C. coffeicola*** strains causing the two distinct BE symptoms (common BE and black BE). Considering the significant differences in the host biochemical response, it can be useful as an indirect indicator of the existence of genetic variability among the strains that cause distinct symptoms. Considering this, the aim of this research was to investigate whether the alterations in the oxidant metabolism of coffee leaves is similarly induced by the two ***C. coffeicola*** strains from the typical common BE and the atypical black BE symptoms. Effects of different strains on coffee can be measured and used as an indicator for what strain is affecting the coffee, eg, a biomarker. This is essential to research, especially those related to epidemiology and management strategies including resistance and chemical control.

MATERIALS AND METHODS

Plant material and growth conditions

Six-month-old ***Coffea arabica*** seedlings from cultivar 'Mundo Novo 376/4', susceptible to ***C. coffeicola***, were used in all experiments. Plants were grown under controlled temperature (21±2°C) and 12 hours photoperiod.

Fungal strains and inoculum preparation

Two ***C. coffeicola*** strains were used in this research. Strain CML 2984 and CML 2985 were selected from a collection of strains previously isolated from coffee leaves collected from various locations in Brazil. Strain CML 2984 was isolated from a common BE lesion in a field located in Bonfinopolis, Minas Gerais, Brazil and will be referred to as 'common BE' from now on; strain CML 2985 was isolated from a black type BE lesion collected from a coffee field in Tres Pontas, Minas Gerais, Brazil.

Induction of *in vitro* sporulation of ***C. coffeicola*** was performed by following the method developed by SOUZA et al. (2011). Briefly, six disks (5mm in diameter) were removed from the border of the medium containing the mycelial growth and transferred to a 25mL flask containing 10mL of V8 medium (10% tomato juice V8). They were kept in a shaker at 25°C with 110rpm for four days. Fungal growth of each flask was poured into Petri dishes containing 1.5% water agar medium. These plates were kept open in an incubator at 40 cm from the light bulbs with 12 hours photoperiod and 25°C. This dehydration process is used for the induction of sporulation in ***C. coffeicola*** (SOUZA et al., 2011). After five days, an aliquot of 10mL of distilled water was added to each Petri dish and the colony was scraped with a glass rod, and the spore suspension was filtered through cheese cloth. The concentration of conidial suspension used was adjusted to $8.25x10^4$ conidia mL^{-1} for both strains. Temperature and relative humidity were daily assessed using the datalogger (HT-500, Instrutherm, Sao Paulo, Brazil).

Determination of the activities of enzymes involved in the oxidant metabolism

Samples of the first and second pair of leaves from the apex to the base (total of 6 leaves through repetition of each treatment) were collected at 12, 24, 36 and 48 hours post inoculation (hpi). Samples were individually stored in aluminum foil, immediately flash frozen in liquid nitrogen (N_2) and stored at -80°C until further analysis. To determine the activities of POX (EC 1.11.1.7) and PAL (EC 4.3.1.5), 0.2g of leaf tissue were macerated in a mortar and pestle containing liquid N_2 and 1% (w/vol) polyvinylpyrrolidone (PVP) to obtain a fine powder. The powder was homogenized in 1.5mL of sodium phosphate buffer 50mM (pH 6.5) containing

1mM phenylmethyl sulfonic fluoride (PMSF) and centrifuged at 13,000×g for 25 min at 4°C. Supernatant was used to determine enzyme activity.

POX activity was determinated following the method of KAR & MISHRA (1976). A mixture of 80µL potassium phosphate buffer 100mM (pH 7.0), 40µL of pyrogallol 50mM and 40µL of hydrogen peroxide 125mM was added to 40µL of extract. Absorbance was measured at 420nm in a Gen5™ spectrophotometer (BioTek® Instruments, Winooski, USA). The coefficient of molar extinction of 2.47 mM^{-1} cm^{-1} was used to calculate POX activity (CHANCE & MAEHLEY, 1955), which was expressed in mM of purpurogallin produced by min^{-1} mg^{-1} of protein. Enzyme activity of PAL was analyzed by using the modified method of GUO et al (2007) where 5µL of the extract is added to a mixture containing 145µL of Tris-HCl buffer 50mM (pH 8.8) and 50µL of 50mM *L*-phenylalanine. Absorbance of the trans-cinnamic acid derivatives was measured in a Gen5™ spectrophotometer at 290nm and the coefficient of molar extinction of 10^4m M^{-1} cm^{-1} (ZUCKER, 1965) was used to calculate PAL activity (in µM min^{-1} mg^{-1} of protein).

To obtain the extract for enzymatic determination of CAT (EC 1.11.1.6), SOD (EC 1.15.1.1) and APX (E 1.11.1.11), 0.2g of leaf fragments were macerated as described above. The powder obtained was homogenized in 1.5mL of potassium phosphate buffer 400mM (pH 7.8) containing EDTA 10mM, ascorbic acid 200mM and PVP 1% (wt vol^{-1}). The homogenate was centrifuged as described previously. CAT activity was determined through the method of CAKMAK & MARSCHNER (1991) where the reaction mixture was composed of potassium phosphate buffer 200mM (pH 7.0), distilled water and hydrogen peroxide 250mM. The reaction was initiated after the addition of 10µL of the crude enzyme extract, and the activity was determined by the rate of H_2O_2 decomposition at 240nm for 3 minutes at 25ºC. Coefficient of molar extinction of 36 M^{-1} cm^{-1} (ANDERSON et al., 1995) was used to calculate CAT activity (mM min^{-1} mg^{-1} of protein). SOD activity was determined by adding 30µL of leaf extract in 170µL of reaction mixture composed of potassium phophate buffer 100mM (pH 7.8), methionine 70mM, *p*-nitrotetrazole blue (NBT) 1mM, EDTA 10µm and riboflavin 0.2mM. The reaction occurred at 25°C under lighting lamps of 15W. After 10min of light exposure, the light was interrupted and the production of formazan blue due to the photo reduction of NTB was monitored by the increase in absorbance at 560nm in the a Gen5™ spectrophotometer. A single unit of SOD was defined as the amount of enzyme necessary to inhibit NBT photoreduction by 50%. The APX activity was determined through the method of NAKANO & ASADA (1981). The reaction mixture was composed of potassium phosphate buffer 200mM (pH 7.0), hydrogen peroxide 2 mM and ascorbate acid 10mM. The reaction was initiated by adding 10µL of leaf extract and the activity was measured through ascorbate oxidation at 290nm, during 3 minutes at 25ºC. The coefficient of molar extinction of 2.8 mM^{-1} cm^{-1} (Nakano & Asada, 1981) was used to calculate the APX activity (mM min^{-1} mg^{-1} of protein). The concentration of proteins in each sample was determined according to the colorimetric method described by BRADFORD (1976). All assays were replicated in triplicate.

Experimental design and data analysis

The experiment was conducted in a replicated (triplicate) randomized block design (RBD) with three treatments: plants sprayed with common BE, black BE, or water (mock inoculation used as control) for the evaluation of enzyme activities. Data for the activity of POX, CAT, SOD, APX and PAL were analyzed using Analysis of variance (ANOVA) and the means compared with the Tukey post hoc test at ($P<0.05$). The analyses were realized using Sisvar software (version 5.3).

RESULTS AND DISCUSSION

In this research, the activity of some important enzymes responsible for removing the reactive oxygen species (ROS) during the infectious process of strains of ***C. coffeicola*** in coffee leaves were determined.

Despite the strains being isolated from fields displaying different symptoms, both induced defense responses from the coffee plants.

The POX activity was significantly increased in the plants inoculated with black BE strain compared to non-inoculated plants at 12 hpi and 24 hpi (Figure 1A). Plants inoculated with the commom BE also showed increased POX activity in relation to the non-inoculated plants at 24 hpi (Figure 1A). There was no difference between the treatments for the POX activities (at 36 hpi and 48 hpi (Figure 1A)) and CAT activities (at 12 hpi (Figure 1B)). Plants inoculated with the black BE strain showed increased CAT activity at 24 hpi and 48 hpi (Figure 1B). Non-inoculated plants showed increased CAT activity at 36 hpi (Figure 1B). Plants inoculated with the common

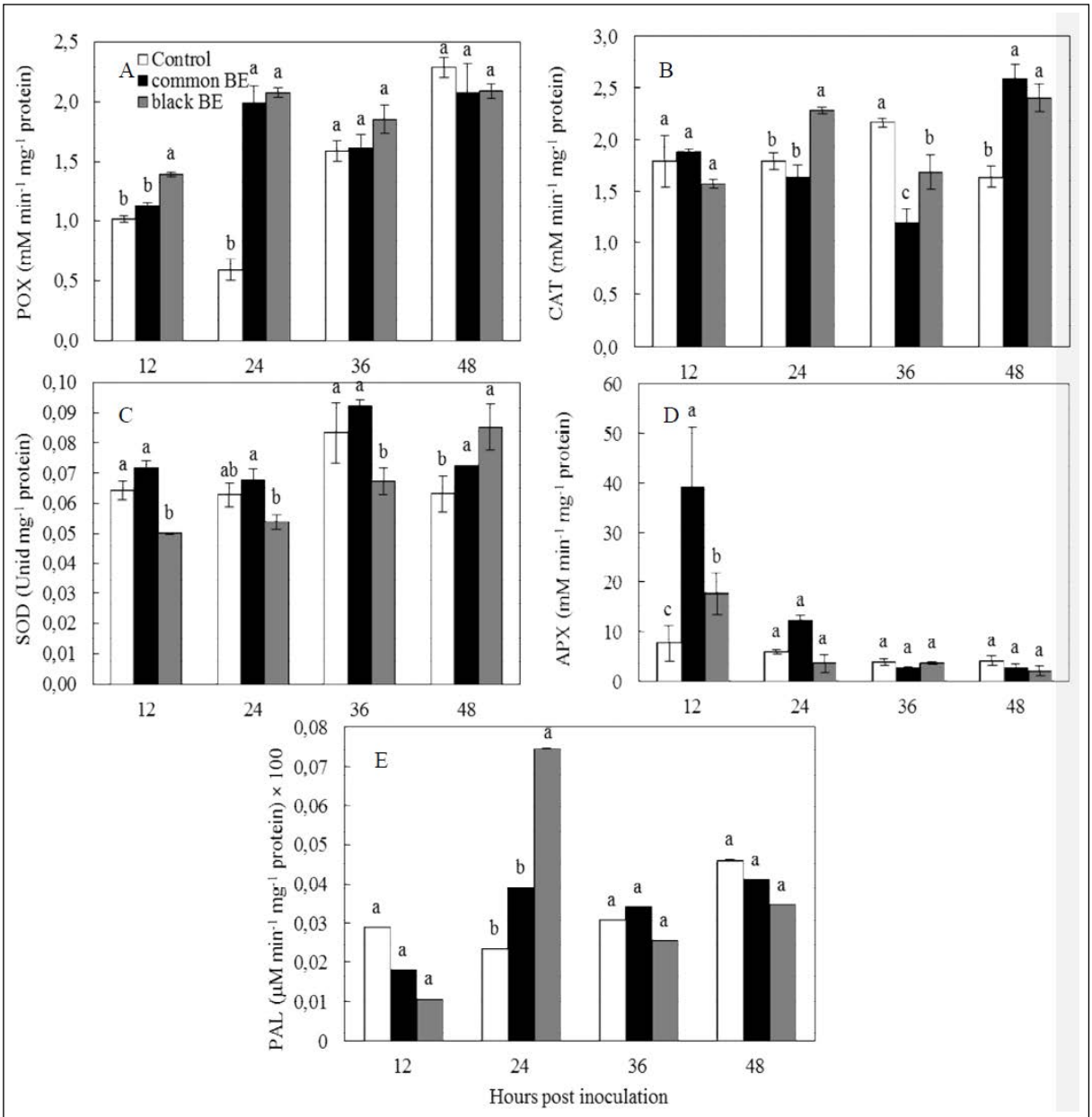

Figure 1 - Activities of peroxidase (POX) (A), catalase (CAT) (B), superoxide dismutase (SOD) (C), ascorbate peroxidase (APX) (D) and phenylalanine ammonia lyase (PAL) (E) in leaves of coffee inoculated with strain isolated from a common brown eye spot (common BE) from lesion and strain isolated from a black brown eye spot (black BE) from lesion and water (mock inoculated used as control). Means followed by the same letter for each sampling time are not significantly different ($P \leq 0.05$) as determined by Tukey's test. Bars represent the standard error of the means.

BE strain also presented increased CAT activity compared with non-inoculated plants at 48 hpi (Figure 1B). Non-inoculated and inoculated plants with the common BE had greater SOD activity at 12 hpi and 36 hpi (Figure 1C). SOD activity was significantly increased in plants inoculated with both strains in comparison to non-inoculated ones at 48 hpi (Figure 1C). Inoculated plants with the common BE strain showed increase in the APX activity in comparison to non-inoculated plants at 12 hpi (Figure 1D). There was no significant difference among the treatments for APX activity at 24, 36 and 48 hpi (Figure 1D). There was no significant difference among the treatments for PAL activity at 12, 36 and 48 hpi (Figure 1E). Plants inoculated with the black BE strain displayed increased PAL activity compared with the non-

inoculated and inoculated plants with the common BE strain at 24 hpi (Figure 1E). In literature, there are a few accounts of alterations in the antioxidant metabolism of infected plants by ***Cercospora***, mainly when it refers to strains that cause different symptoms in coffee plants (NASCIMENTO et al., 2014). When comparing the two ***C. coffeicola*** strains (common BE and black BE), the results showed that both strains were able to induce the activity of enzymes in coffee leaves; however, there was variation in the timing of enzymes activation for each strain depending on the enzyme being analyzed. This is probably due to differences in the initial stages of penetration of both strains. The coffee plant recognized the strains in a similar way and was inhibited by H_2O_2 produced by the plant, which can be demonstrated through increased POX and APX activity in the first hours of infection. Similar results were reported by NASCIMENTO et al. (2014), who verified increased POX and PAL enzyme activity in inoculated soybean plants with ***Cercospora sojina***. POX is a pathogenesis related protein and is induced in the host by the presence of pathogens (VAN LOON et al., 2006). Suberization of tissues, catabolism of auxins, strengthening of the cellular wall and responses of defense in plants are responsible for biosynthesis of lignin (HIRAGA et al., 2001 Conversely, PAL is the key enzyme in the phenylpropanoid pathway which is important in coffee plant defence responses. In this study, comparative analyses of PAL activity suggested phenylpropanoids as antioxidant compounds can be extremely important for the antioxidant system of the coffee tree infected with the black BE strain at 24 hpi. Similarly, GHOLIZDEH & KOHNEHROUZ (2010) suggested that PAL could be the main component for the antioxidant system of corn under saline stress. In this study, the observed enzyme activity of POX, APX and PAL suggested that the ***C. coffeicola*** strains likely develop in a similar way on dead plant cells without being inhibited by the accumulation of H_2O_2 to O_2^- produced when the plants are infected by pathogens. This is possibly due to the fact that ***Cercospora*** produces cercosporin toxin that acts in the plasmatic membrane of the host cell, promoting cell death and release of ROS (DAUB et al., 2013). These results suggested that this toxin could have inhibited the defense mechanisms of the plants, thus permitting the fungus to have access to nutrients. The APX enzyme, participant of the ascorbate-glutathione cycle, acts together with POX and CAT, removing H_2O_2 from plant cells. In the first reaction of catalyzation, APX uses two molecules of ascorbate to reduce H_2O_2 with concomitant generation of two molecules of malondialdehyde, the main product in lipid peroxidation of the cellular membranes (SHARMA et al., 2012). The accounts of this study agree with DEBONA et al. (2012), who verified increased APX activity in wheat plants inoculated with ***Pyricularia oryzae***. DOMICIANO et al. (2015) also verified increased activities of these enzymes for the same pathosystem when plants were treated with silicon. In relation to CAT activity, this enzyme was greater in inoculated plants with the black BE strain, compared with non-inoculated plants at 36 hpi. In the same way, DEBONA et al. (2012) verified increased CAT activity in wheat plants inoculated with ***P. oryzae*** compared to non-inoculated plants at 48 hpi. CAT catalyzes the dismutation of two molecules of H_2O_2 into water and oxygen (SHARMA et al., 2012). SOD activity increased in plants inoculated with both strains at 48 hpi, an observation likely to be associated with the beginning of colonization of coffee leaf tissues by the fungal strains. These results are similar to those observed by DEBONA et al. (2012), who reported increased SOD activity in plants inoculated with ***P. oryzae,*** an increase in activity being related to the development of blast symptoms. According to BOLWELL et al. (2002), virulent pathogens can avoid or suppress the recognition of the host, only inducing the initial phase of defence responses. According to LEVINE et al. (1994), the current toxicity of the ROS in the interaction between the pathogen and host is going to depend on the sensitivity of the pathogen to the concentration of these radicals. Some fungi such as ***Botrytis cinerea*** and ***Cercospora*** can benefit from the increase of ROS generated against them in plant defence mechanisms, facilitating host colonization and absorption of nutrients through the exploitation of these host defence mechanisms (GOVRIN & LEVINE, 2000).

In this study, it is speculated that the increased activity of these enzymes could be a strategy by the plant to restrict the colonization of both strains due to the removal of ROS. Despite having observed little differences in the enzymes action in the plants when inoculated with both strains, all the lesions observed in the greenhouse were of the common BE type.

Climate changes that are currently affecting coffee production around the world, mainly in South America (JHA et al., 2014) can have effects on the pathogen, disease development and on coffee production. These drastic changes in the environment could result in ***C. coffeicola*** undergoing selection for new and more aggressive strains. The findings of this research indicated that there are other factors influencing the occurrence of black BE lesions in field conditions.

Overall, studies about the infectious process of strains in different environmental and nutritional conditions, together with the confirmation and accurate identification of these strains causing different symptoms of brown eye spot is necessary through the use of molecular tools.

CONCLUSION

This study clearly demonstrated that both strains were able to induce alterations in the antioxidant metabolism of coffee leaves, suggesting that other factors leading to the black BE lesion type in field conditions are at play. Further investigation is needed to ascertain the cause of these differing symptoms.

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