Alterations in antioxidant metabolism in coffee leaves infected by *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.

**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
The coffee plant responds in a different way to C. coffeicola (DOMICIANO et al., 2015). It is unknown whether the 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; SHARMA et al., 2012). Peroxidase (POX), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione-S-transferase (GST), glutathione reductase (GR), phenylalanine ammonia lyase (PAL) and glutathione peroxidase (GPX) are among the enzymes used in enzymatic defence (SCANDALIOS, 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 reductase (GR), phenylalanine 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 (N2) 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 N2 and 1% (w/v) polyvinylpyrrolidone (PVP) to obtain a fine powder. The powder was homogenized in 1.5mL of sodium phosphate buffer 50mM (pH 6.5) containing...
1 mM 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 determined following the method of KAR & MISHRA (1976). A mixture of 80 µL potassium phosphate buffer 100 mM (pH 7.0), 40 µL of pyrogallol 50 mM and 40 µL of hydrogen peroxide 125 mM was added to 40 µL of extract. Absorbance was measured at 420 nm 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 mg⁻¹ of protein. Enzyme activity of PAL was analyzed by using the modified method of GUO et al (2007) where 5 µL of the extract is added to a mixture containing 145 µL of Tris-HCl buffer 50 mM (pH 8.8) and 50 µL of 50 mM L-phenylalanine. Absorbance of the trans-cinnamic acid derivatives was measured in a Gen5™ spectrophotometer at 290 nm and the trans-cinnamic acid derivatives was measured through the method of 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), 0.2 g of leaf fragments were macerated as described above. The powder obtained was homogenized in 1.5 mL of potassium phosphate buffer 400 mM (pH 7.8) containing EDTA 10 mM, ascorbic acid 200 mM 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 200 mM (pH 7.0), distilled water and hydrogen peroxide 250 mM. The reaction occurred at 25°C under lighting lamps of 15 W. After 10 min 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 560 nm 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 200 mM (pH 7.0), hydrogen peroxide 2 mM and ascorbate acid 10 mM. The reaction was initiated by adding 10 µL of leaf extract and the activity was measured through ascorbate oxidation at 290 nm, during 3 minutes at 25°C. 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).

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 common 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
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-
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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$_2$O$_2$ 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$_2$O$_2$ from plant cells. In the first reaction of catalyzation, APX uses two molecules of ascorbate to reduce H$_2$O$_2$ 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$_2$O$_2$ 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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REFERENCES


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**MATERIAL AND METHODS**

Characterization of the hypocotyls were performed via quantitative reverse transcriptase polymerase chain reaction (q-RT-PCR). For this purpose, the leaves of the coffee plants were infected with *Cercospora coffeicola* and subjected to different levels of light intensity: 0, 10, 20, 30, and 40 μmol m⁻² s⁻¹. The expression of the gene encoding peroxidase (PER) was assessed by q-RT-PCR.

**RESULTS AND DISCUSSION**

The expression of the PER gene was significantly increased in the leaves of coffee plants infected with *Cercospora coffeicola*, especially under low light conditions. These results suggest that the hypersensitivity response induced by the pathogen is associated with the activation of the PER gene, which is involved in the antioxidant defense system of plants.

**CONCLUSIONS**

The results of this study contribute to the understanding of the role of antioxidant metabolism in the response of coffee plants to *Cercospora coffeicola* infection. Further studies are needed to elucidate the mechanisms underlying the activation of the PER gene and the antioxidant response in coffee plants under different light conditions.

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**REFERENCES**


