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Enzymatic Activity in Essential Oil-Treated and Pathogen-Inoculated Corn Plants

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Abstract

Bipolaris maydis and *Exserohilum turcicum* are important fungal pathogens that cause leaf blight in corn whose control have been difficult. Essential oils are a promising and environmentally friendly alternative for disease management, but the mechanisms of action remain poorly studied. Here, we aimed to assess the effect of *B. maydis* and *E. turcicum* as well as the essential oil of *Morinda citrifolia* in the activity of plant defense enzymes in corn plants. Experiments were carried out in a completely randomized design with three replications and six treatments as they follow: (T1): corn plants inoculated with *B. maydis*; (T2): corn plants inoculated with *E. turcicum*; (T3): corn plants treated with essential oil of *M. citrifolia* (0.25%) and inoculated with *B. maydis*; (T4): corn plants treated with essential oil of *M. citrifolia* (0.25%) and inoculated with *E. turcicum*; (T5): corn plants treated with essential oil of *M. citrifolia* (0.25%); and (T6): corn plants non-inoculated and treated with distilled water. Protein content (PC) and activities of the enzymes ascorbate peroxidase, catalase, chitinase (CHI), peroxidase (POX) and superoxide dismutase (SOD) were assessed. PC was significantly decreased, whereas CHI and SOD activity was higher in T1-T5 compared to T6. T4 and T5 significantly increased POX activity relative to T6. Therefore, our findings suggest that the essential oil of *M. citrifolia* may play an active role in disease control by activating defense enzymes in corn plants.

Keywords: *Bipolaris maydis*, *Exserohilum turcicum*, *Morinda citrifolia*, resistance induction

1. Introduction

Corn (*Zea mays* L.) is one of the most important cereals for human and animal consumption and it is grown for grain and forage. Moreover, its demand for both food and biofuel is increasing. Corn ranks first in production, accounting for 1,052 million metric tons in the world. Brazil is the third largest producer with 9% of the world production, after USA (34%) and China (21%) (USDA, 2018).

Several fungal diseases, notably southern leaf blight (SLB) and northern leaf blight (NLB), caused by *Bipolaris maydis* (teleomorph: *Cochliobolus heterostrophus*) and *Exserohilum turcicum* (teleomorph: *Setosphaeria turcica*), respectively, are known to affect corn production worldwide. SLB caused one of the most devastating epidemics in Plant Pathology's history; in 1970, due to the use of cytoplasmic male sterile corn (cms-T), that is highly susceptible to the pathogen's race T, the average loss in yield in the U.S. Corn Belt was 20-30% resulting in a monetary loss of 1 billion dollars (Ullstrup, 1972). Nowadays, SLB is considered to be the most important and wide spread corn leaf disease around the world and it is prevalent in warm humid temperate to tropical regions, where temperatures ranges from 20-30 °C during cropping period (Singh & Srivastava, 2012). NLB is reported to be severe under moderate (15-25 °C) and dew heavy (Levy & Cohen, 1983), with crop losses exceeding 50% if NLB becomes severe prior to flowering (Raymundo & Hooker, 1981; Tefferi et al., 1996). Different strategies, including cultural practices, genetic resistance and fungicide application can be employed in SLB and NLB management. Crop rotation is an important alternative to reduce initial inoculum; however, sometimes it is difficult to find a profitable crop to replace corn. Some resistance genes have been identified and incorporated in commercial hybrids, but genetic variation in pathogen population may become it ineffective (Belcher et al., 2012). Broad-spectrum fungicides, including propiconazole, fluazinam, benzovindiflupyr, chlorotalonil and mancozebe were demonstrated to be effective, but pathogen sensitivity to some of them has been shown to

decrease due to long-term large-scale use of a single fungicide (Chen et al., 2018; Hou et al., 2018). Therefore, it becomes necessary to find novel alternatives for SLB and NLB control in order to decrease the risk of pathogens overcoming resistance genes and fungicide resistance development.

Plant essential oils have shown great potential in disease control. *In vitro*, essential oil of *Callistemon citrinus* and *Cymbopogon citratus* were demonstrated to completely inhibit the mycelial growth of *Alternaria padwickii* and *Bipolaris oryzae* and experiments in the field showed that brown spot severity was reduced by 20-80% in rice (Nguefack et al., 2013). Essential oils of cinnamon, citronella, lemongrass, clove, tea tree, thyme, neem and eucalyptus were promising for the control of coffee rust since they inhibited germination of urediniospores of *Hemileia vastatrix*; transmission electron microscopy analysis showed that urediniospores exposed to oils of clove, citronella and thyme promoted cellular disorganization and cytoplasmic vacuolization (Pereira et al., 2012). Total inhibition of urediniospore germination of *Phakopsora pachyrhizi* was also found after treatment with essential oils of *Hyptis marrubioides*, *Aloysia gratissima* and *Cordia verbenacea* and their curative application reduced area under the Asian rust curves by 33-41% (Silva et al., 2014). In maize, essential oil of *Cymbopogon citratus* reduced the progress of the Curvularia leaf spot when applied preventively and it was also showed to inhibit 100% of conidia germination, but not mycelial growth of *Curvularia lunata* (Mourão et al., 2017). In addition, we have demonstrated that essential oil of *Morinda citrifolia* inhibited conidia germination of *E. turcicum* and reduced area under the disease progress curve in preventive applications in corn (Silva et al., 2017).

Despite the reports of their effectiveness against several diseases, mechanisms of the action of essential oils remain elusive. Although a direct effect in pathogen is well documented (Pereira et al., 2012; Nguefack et al., 2013; Silva et al., 2014, 2017; Mourão et al., 2017), an indirect effect, by inducing plant defenses, cannot be ruled out. Therefore, this work aimed to assess the effect of *B. maydis* and *E. turcicum* as well as the essential oil of *M. citrifolia* in the activity of defense enzymes in corn plants.

2. Material and Methods

Experiments were carried out in a completely randomized design with three replicates and six treatments as they follow: (T1): corn plants inoculated with *Bipolaris maydis*; (T2): corn plants inoculated with *Exserohilum turcicum*; (T3): corn plants sprayed with essential oil of *M. citrifolia* (0.25%) and inoculated with *B. maydis*; (T4): corn plants sprayed with essential oil of *M. citrifolia* (0.25%) and inoculated with *E. turcicum*; (T5): corn plants sprayed with essential oil of *M. citrifolia* (0.25%); (T6): corn plants sprayed with distilled water (control). One pot with three plants was considered as experimental unit. *B. maydis* and *E. turcicum* were chosen because their importance in limiting corn yield in the region of Gurupi (TO, Brazil). *M. citrifolia* was selected because we demonstrated previously its potential to control *E. turcicum* (Silva et al., 2017).

Five corn seeds (hybrid 30F53YH) were sown in 2 L pots, filled with Red-Yellow Latosol (Oxisol) and cattle manure (2:1) as substrate. After emergence, each pot was thinned to three seedlings and watered daily with the aid of a watering can.

Essential oil of *M. citrifolia* was obtained from ripe fruits collected in the region of Gurupi as described elsewhere (Silva et al., 2017). Fruits were washed in running water, cut in small cubes and submitted to the extraction of essential oil by the hydrodistillation method. In a round-bottom flask 200 g of noni ripe fruits were added. Following this, the flask was attached to Clevenger distiller for a two-hour period. After the extraction, the essential oil was collected in the supernatant form, placed in amber bottle, identified, and stored at 4 °C. A stock solution of essential oil at 1% was prepared using a 1% Tween 80 solution as a dispersing agent. From the stock solution, a dilution was performed in order to obtain a concentration of 0.25% of essential oil of *M. citrifolia*.

Plants from the treatments T1 and T2 were inoculated with a spore suspension of 10^4 conidia mL^{-1} of *B. maydis* and *E. turcicum*, respectively. Conidia were obtained from corn leaves (hybrid 30F53YH) collected in a commercial field in Gurupi and were incubated in a moisture chamber until fungal sporulation. Then, conidia were transferred to the PDA medium in Petri dishes (90 mm) and incubated at 25 °C and photoperiod of 12 h of light until the mycelial growth reached the borders of the plates. Subsequently, the conidia were water-removed with the aid of a soft bristle brush and quantified in a Neubauer chamber and calibrated to the concentration described above. Conidia suspension was applied as a fine mist to the adaxial leaf blades of each plant until runoff using a VL Airbrush atomizer (Paasche Airbrush Co., Chicago). Plants were kept in a moisture chamber in the darkness for 24 h at 25±2 °C. Then, plants were brought to the environment condition (30±2 °C) for further 24 h.

The treatments T3, T4 and T5 were sprayed with 20 mL of 0.25% essential oil solution, whereas the treatment T6 was sprayed just with distilled water and plants were kept at 25±2 °C. Two hours later, the treatments T3 and T4 were inoculated with *B. maydis* and *E. turcicum*, respectively. Thus, plants were kept in a moisture chamber in the darkness for 24 h at 25±2 °C. Subsequently, plants were kept under environmental conditions (30±2 °C).

Twenty-four hours after keeping plants under environmental conditions, corn leaves of the three plants of each replication were collected in order to obtain the crude extract, that was used in the determination of enzymatic activity post-inoculation of the respective treatments.

To obtain the crude extracts, 200 mg of leaf tissue sampled in each treatment were weighed and ground in liquid nitrogen with 50% polyvinylpolypyrrolidone (PVPP). Then, 375 µL of 400 mM potassium phosphate buffer (pH 7.8), 15 µL of 10 mM ethylenediaminetetraacetic acid (EDTA), 75 µL of 200 mM ascorbic acid and 1.035 mL of distilled sterilized water were added. Crude leaf extracts were centrifuged at 13,000 × g for 10 min at 4 °C and supernatants were recovered and stored in freezer at -20 °C for further enzymatic activity determination.

Protein was quantified according to Bradford (1976). In test tubes, 50 µL of corn leaf extract and 1.5 mL of Bradford dye (Quick StartTM Bradford 1x) were shaken on a tube shaker and incubated in the darkness for 5 min. Then, absorbance was read in a spectrophotometer at 595 nm. Protein concentration was determined using a standard curve, prepared with bovine serum albumin (BSA), ranging from 0 to 100 µg mL^{-1}. Results were expressed as µg·mL^{-1} for total protein and as µmol protein·min^{-1} for APX, CAT, CHI, POX and SOD.

Activity of ascorbate peroxidase (APX) was determined at 290 nm and 25 °C by hydrogen peroxide (H_2O_2) degradation (Nakano & Asada, 1981). The reaction mixture consisted in 100 µL of the crude extract, 2.7 mL of 0.5 mM ascorbate buffer and 200 µL of 30 mM H_2O_2.

Activity of peroxidase (POX) was determined at 25 °C through direct spectrophotometric method by the measurement of the conversion of guaiacol into tetraguaiacol at 470 nm (Kar & Mishra, 1976). The reaction mixture had 0.05 mL of the crude extract and 2.55 mL of a solution containing 0.05 mL of 0.2 M guaiacol and 0.5 mL of 0.38 M H_2O_2 and 2.0 mL of 0.02 M sodium acetate buffer (pH 5.0).

Activity of superoxide dismutase (SOD) was determined at 560 nm (Giannopolitis & Ries, 1977; Del Longo et al., 1993). In test tubes, 0.1 mL of the crude extract was combined with 1.0 mL of 50 mM potassium phosphate buffer (pH 7.8), 0.02 mL of 0.1 mM EDTA, 0.4 mL of 14 mM L-methionine and 0.2 mL of 0.1 µM nitro-blue tetrazolium (NBT). The reaction was started by adding 0.02 mL of 2 µM riboflavin. The test tubes were illuminated for five minutes. Two blanks were prepared with the incubation without the crude extract: one was not illuminated and used to zero the spectrophotometer and the other was illuminated together with the samples to determine NBT photoreduction.

For catalase (CAT) determination, reaction mixture comprised 0.05 mL of the crude extract and 2.95 mL of 50 mM potassium phosphate buffer (pH 7.8) and 20 mM H_2O_2 (Cakmak & Marschner, 1992). The decrease in absorbance was recorded at 240 nm for five minutes, with readings every 30 s.

Activity of chitinase (CHI) was assessed based on the release of soluble "CM-chitin-RBV" fragments, from remazol-bright-violet-labeled carboxymethylated chitin (CM-chitin-RBV) determined at 550 nm (Wirth & Wolf, 1990). The reaction mixture consisted in 0.2 mL of the crude extract, 0.6 mL of 0.1 M sodium acetate buffer (pH 5.0) and 0.2 mL of "CM-chitin-RBV" (2.0 mg mL^{-1}). Then, samples were incubated at 40 °C for 20 minutes and reaction was stopped by adding 0.2 mL of 1 M HCl, cooled in an ice bath and centrifuged at 10,000 g for five minutes.

Data were submitted to the analysis of variance and means were compared based on Tukeys test ($P \leq 0.05$), using the software Minitab (version 18; Minitab Corporation).

3. Results

Figure 1 shows protein content and activities of the enzymes CHI, CAT, SOD, APX and POX in corn plants that were inoculated with *B. maydis* or *E. turcicum* and sprayed with the essential oil of *M. citrifolia* or distilled water as well as combinations of essential oil of *M. citrifolia* spraying + *B. maydis* or *E. turcicum* inoculation.

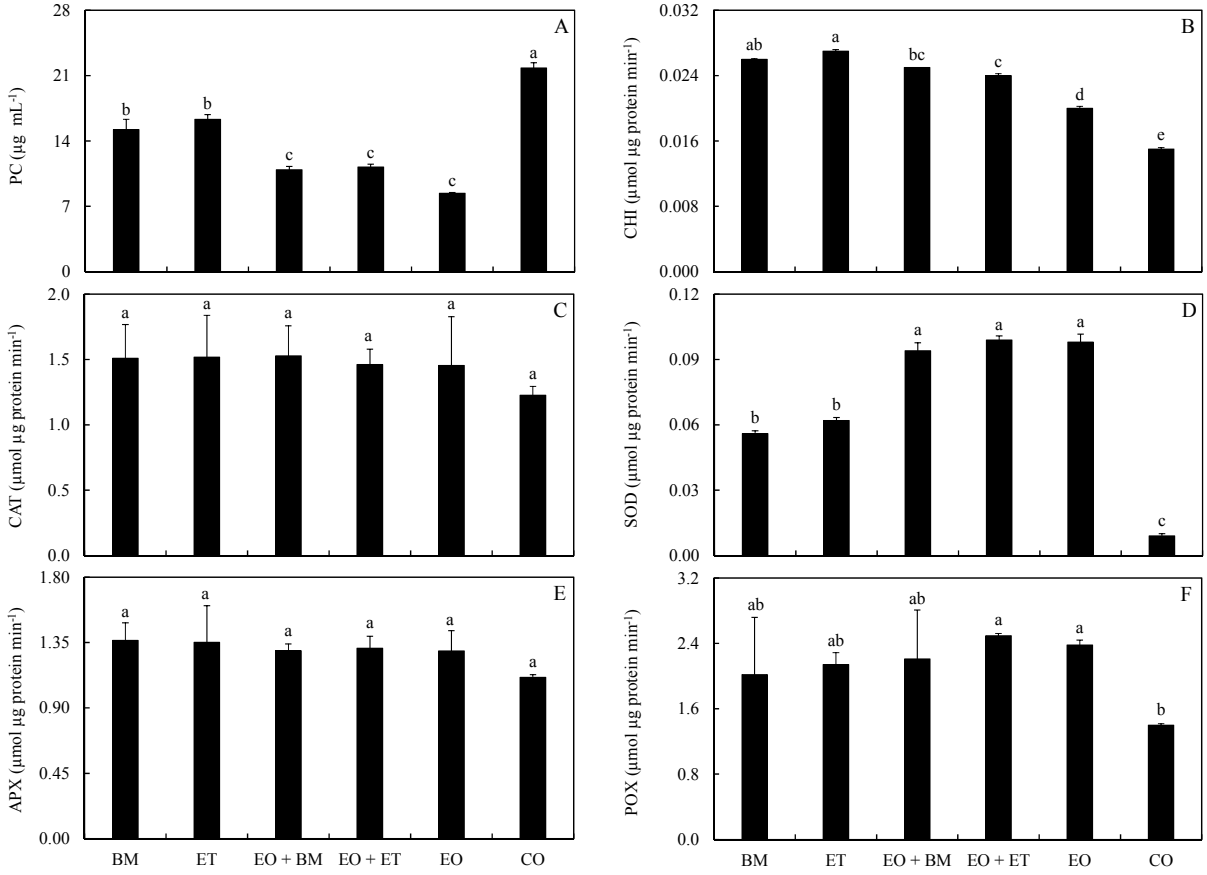

Figure 1. Protein content (PC) (A) and activities of chitinase (CHI) (B), catalase (CAT) (C), superoxide dismutase (SOD) (D), ascorbate peroxidase (APX) (E) and peroxidase (POX) (F), determined in leaves of corn plants that were inoculated with *Bipolaris maydis* (BM), *Exserohilum turcicum* (ET), sprayed with essential oil of *Morinda citrifolia* (0.25%) + inoculated with *B. maydis* (EO + BM), sprayed with essential oil of *M. citrifolia* (0.25%) + inoculated with *E. turcicum* (EO + ET), sprayed with essential oil (EO) only or distilled water (control, CO). Means that are followed by the same letter are not significantly different based on Tukey's test ($P \leq 0.05$)

Protein content was significantly different among treatments and the highest value was observed in the control plants (21.8 µg mL^{-1}) (Figure 1A). Plants that were inoculated with *B. maydis* or *E. turcicum* did not differ between themselves, with protein contents of 15.2 and 16.3 µg mL^{-1}, respectively. Lower protein content were observed in plants that were treated with essential oil of *M. citrifolia* alone or combined with *B. maydis* or *E. turcicum* inoculation, that showed a protein content of 8.4, 10.9 and 11.2 µg mL^{-1}, respectively.

Statistical differences among the treatments were found regarding the CHI activity (Figure 1B). The highest enzyme activity (0.027 µmol µg $protein^{-1}$ min^{-1}) was observed in plants that were inoculated with *E. turcicum*, whereas the lowest activity (0.015 µmol µg $protein^{-1}$ min^{-1}) was verified in the control plants.

Both CAT (Figure 1C) and APX (Figure 1E) activities did not show any significant difference among treatments, indicating that neither the essential oil of *M. citrifolia* nor pathogen inoculation triggered CAT and APX synthesis.

A significant difference was found for SOD activity between control and the other treatments (Figure 1D). The lowest enzyme activity was recorded in the control plants (0.009 µmol µg $protein^{-1}$ min^{-1}). In response to *B. maydis* and *E. turcicum* inoculation, corn plants did not show differences between themselves, but they were different from the other treatments. Plants that were sprayed with the essential oil of *M. citrifolia* and those were sprayed with the essential oil and inoculated with *B. maydis* or *E. turcicum* were not different among themselves (enzyme activities were 0.098, 0.094 and 0.099 µmol µg $protein^{-1}$ min^{-1}, respectively).

There was a significant difference for POX activity between the control and the treatments that received essential oil spraying and those with essential oil spraying and *E. turcicum* inoculation; the latter two treatments were not different from the other treatments. The control plants showed the lowest enzyme activity (1.4 μmol μg protein^{-1} min^{-1}) (Figure 1F).

4. Discussion

Despite the importance of SLB and NLB in reducing corn yield in Brazil, their management is still challenging. Resistance and fungicides are available, but variation in races and fungicide sensitivity of the pathogen may become such strategies innefective. Essential oils provides an innovative and environmentally friendly alternative for disease control, but mechanisms of action remain poorly investigated. In this work, we provide novel evidences at the biochemical level of the effect of *B. maydis* and *E. turcicum* as well as the essential oil of *M. citrifolia* in corn leaves and shed light on the potential indirect effect of the essential oil in SLB and NLB control.

Plants that were treated with the essential oil of *M. citrifolia* and inoculated with *B. maydis* and *E. turcicum* displayed lower protein content, which was probably due to the higher consumption of proteins by the corn plants to increase their defense responses against biotic (pathogen infection) or abiotic (presence of the essential oil) stresses. It is well known that stressful conditions increase the demand for proteins involved in biochemical processes, or even the conversion of proteins into specific enzymes in order to equilibrate plant cells since all stresses change gene expression, which are manifested as an induction or repression in the proteins that are produced under normal conditions (Paz et al., 2001).

The highest activity of CHI was recorded in plants that were inoculated with *E. turcicum*, followed by those inoculated with *B. maydis* and by those inoculated with both pathogens and sprayed with the essential oil of *M. citrifolia*. Most likely, the presence of the pathogen may have activated CHI synthesis as a plant defense response. Since chitin is a major component of fungal cell wall, CHI plays an obvious role in plant defense. In addition, CHI releases inducers from the pathogen's cell wall, thus enabling plant to detect it and triggers other lines of defences (Barros et al., 2010). Apart from stopping pathogen's growth, CHI causes the simultaneous release of phytoalexin's elicitors from pathogen mycelia (Maia et al., 2014).

Generation of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide (O_2^-), is a common feature of plans that are attacked by pathogens (Debona et al., 2012). Corn plants that were inoculated with *Stenocarpella macrospora* displayed increased concentrations of H_2O_2 and malondialdehyde (an indicator ofdamage to cell membranse), thereby contributing to the intensification of lipid peroxidation upon damage to cell membranes caused by fungal infection (Bermúdez-Cardona et al., 2015). Given the cytotoxic potential of ROS, their removal by antioxidant enzymes may play a role in disease resistance (Debona et al., 2012). SOD represents the first line of defense against ROS by dismutating O_2^- into H_2O_2 and O_2 (Foyer & Noctor, 2000). In our study, the inoculation with *E. turcicum* and *B. maydis* significantly increased SOD activity, but such increases were boosted by spraying the essential oil of *M. citrifolia*, demonstrating the potential of the essential oil in decreasing oxidative stress induced by fungal infection. Area under of the NLB progress curve was demonstrated to be decreased in preventive spray of the essential oil of *M. citrifolia* (Silva et al., 2017) and findings of the present study indicates that SOD may be involved in corn resistance to NLB mediated by the essential oil. Consistently, the lower development of macrospora leaf spot symptoms in corn leaves was associated with higher acitivity of SOD (Bermúdez-Cardona et al., 2015). By contrast, activities of APX and CAT, enzymes that are involved in H_2O_2 removal (Apel & Hirt, 2004), did not display any change due to pathogen inoculation or essential oil spray.

POX is involved in lignification, being required for the final step of the polymerization of phenolic compounds to lignin (Debona et al., 2012). Polymerization of phenolic compounds, in turn, may affect fungal development through physical blockage, making cell walls more resistant to the mechanical penetration by the fungus, or through reduced nutrient diffusion from the host to the fungus or of the fungal enzymes and toxins to the host (Debona et al., 2012). POX activity was found to be increased in reponse to the spray of the essential oil of *M. citrifolia* as wel as in its combination with inoculation of *E. turcicum*. Therefore, the increase in POX activity as a result of the spray of the essential oil of *M. citrifolia* is thought to be involved in NLB resistance.

In conclusion, besides its well known direct action on pathogens, essential oil of *M. citrifolia* was demonstrated to stimulate activities of CHI, SOD and POX, which may play a role in the essential oil-afforded control of SLB and NLB.

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