Antifungal activity against *Alternaria solani* and control of early blight in tomato by essential oil of citronella[1]

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ABSTRACT

An alternative to the agrochemicals is the use of essential oils that can act in plant defense against phytopathogens. The objective of work was to evaluate the antifungal activity, the early blight control, and the enzymatic defense in tomato treated with citronella essential oil. Mycelial disks of the pathogen were added in Petri dishes, with treatments 0, 500, 1000, 1500, 2000 and 2500 μL L^{-1} of essential oil and a control treatment with fungicide, thus evaluated mycelial growth and sporulation. The treatments were applied in the second pair of leaves of plants (treated) and after 72 hours the pathogen was inoculated on the second pair (treated) and also on the third pair leaves (untreated). The severity was expressed through the area under the disease progress curve (AUDPC). The enzymatic activity of peroxidase, polyphenoloxidase, and phenylalanine ammonia-lyase were evaluated. The essential oil reduced the mycelial growth and sporulation of the pathogen. The AUDPC was reduced up to 38.14% in the treated leaves and 51.32% in the untreated, and increases in the activities of enzymes were found. The essential oil of citronella could be an alternative in the control of tomato early blight by antimicrobial activity and/or resistance induction local and systemically.

Keyword: *Cymbopogon nardus*; enzymatic activity; medicinal plant; resistance induction.

INTRODUCTION

The tomato (*Solanum lycopersicum* L.) is one of the most prone crops to the occurrence of diseases, like early blight caused by *Alternaria solani* (Inoue-Nagata *et al.*, 2016), and therefore, very dependent of the use of pesticides to control them.

Substances from plant extracts and essential oils are control options that aim to reduce and/or mitigate the use of pesticides, since the intensive use of these chemicals may generate environmental problems. The adversities of these products include contamination of food, soil, and water; intoxication of farmers; selection of resistant phytopathogens; and eradication of beneficial soil microorganisms (Maia *et al.*, 2015).

Crude extract and essential oils from medicinal plants are sources of biologically active compounds that have the potential to control phytopathogens through direct fungitoxic action and also by inducing host resistance (Stangarlin *et al.*, 2011).

Citronella [*Cymbopogon nardus* (L.) Rendle] essential oil possesses repellent activity and bacterial and fungicidal

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action. The production of its secondary metabolites may vary with the ecological and genetic relationships of the plant. The compounds present in this oil, such as the citronellal and geraniol monoterpenes, act in defense of the plant and can inhibit the growth of fungi (Castro *et al.*, 2007).

The effectiveness of citronella essential oil to minimize fungi effects has proven on some pathosystems, such as *Botrytis cinerea* in strawberry (Lorenzetti *et al.*, 2011), *Sphaceloma ampelinum* in grapes (Fialho & Papa, 2015), *Colletotrichum graminicola*, causing anthracnose in sorghum (Sarmento-Brum *et al.*, 2013), and *Cercospora coffeicola* in coffee tree (Pereira *et al.*, 2011).

The aim of this work was to evaluate the antifungal activity, the early blight control, and the activation of defense enzymes peroxidase, polyphenoloxidase, and phenylalanine ammonia-lyase in tomato leaves treated with citronella essential oil.

MATERIAL AND METHODS

Isolation of pathogen and treatments

The fungus *A. solani* was isolated from symptomatic tomato fruits by the indirect method. The fragments of tomato were deposited in a Petri dish with V8-agar culture medium, and kept at 25 °C and dark in a BOD incubator (Bio-Oxygen Demand). The isolate was preserved by the Castellani method (sterile distilled water) described by Gonçalves *et al.* (2016).

The essential oil of citronella (*C. nardus*) was obtained from a manipulation pharmacy by the ViaFarma company and used at six concentrations (0; 500; 1000; 1500; 2000 and 2500 μL L^{-1}). The additional treatment with the fungicide was Amistar Top® (Syngenta company), used commercially (azoxystrobin + difenoconazole, 200 + 125 g L^{-1}, respectively).

In vitro experiments

The *in vitro* experiments were conducted in a laboratory with a completely randomized design with five replications, each petri dish corresponding to one repetition. Mycelial growth and sporulation tests were conducted to evaluate the antifungal activity on the pathogen using the V8-agar juice culture medium (Pulz & Massola Junior, 2009). Treatments were added to the still-flowing culture medium, and Tween 20 detergent was added at a 1:1 (v/v) ratio to homogenize the culture medium with the citronella essential oil.

The fungicide concentration was used according to the manufacturer's recommendation, corresponding to 40 mL 100 L^{-1} of water. After solidification of the culture medium in each Petri dish (90 mm in diameter), a 6 mm mycelial disc of the 14-day-old colony was put in the center of the plate and incubated at 25 °C in dark.

The antifungal activity was assessed by daily measurements of colony diameter (mm) on two perpendicular axes, starting 24 hours after the installation of the experiment until a treatment reached the entire surface of the Petri dish, thus calculating the mycelial growth of all treatments. Daily measurements performed the relationship between pathogen mycelial growth and evaluation days until all colonies covered the whole surface of the culture medium or did not show any evolution of mycelial growth over the days.

At the end of the mycelial growth evaluation, the sporulation of the fungus was assessed. Thus, 10 mL of deionized water was added to each Petri dish, and after scraping the colony with a glass slide, the suspension was filtered through gauze, and the number of spores per mL was determined in a Neubauer chamber. Sporulation was calculated based on the mycelial area of each treatment.

In vivo experiments

The *in vivo* experiment was conducted in a greenhouse and the experimental design was randomized blocks, with the same treatments of *in vitro* assay and using four replications, where each plant was an experimental unity.

Soil fertilization was performed according to chemical analysis and the needs of the crop. Tomato seeds "Caqui" cultivar "Odete" were sown in a 200 cell expanded polystyrene tray containing commercial substrate (composed of pine bark, sand, organic compost and vermiculite). The seedlings were transplanted at 30 days after sowing and supplemented with fertigation.

Thirty days after seedling transplantation, the second pair of leaves at the bottom of each plant was treated with fungicide and essential oil, with localized spraying, at the concentrations described above. To obtain a homogeneous mixture between the essential oil and water, Tween 20 was used in a 1:1 (v/v) ratio. After 72 hours of the treatments, the second pair of treated leaves and the third pair (untreated) leaves were inoculated with $1x10^4$ mL^{-1} spores *A. solani*. The spore suspension was prepared by adding 10 mL of deionized water in a Petri dish containing the 30-day growth pathogen in the V8-agar juice culture medium. The spore suspension was sprayed on tomato leaves and plants were kept in humidity chamber for 12 hours.

Severity assessments of early blight occurred every two days, beginning seven days after pathogen inoculation. Tomato leaves were photographed and analysed using the Quant software (Vale *et al.*, 2003), which calculated the severity of the disease. The area under the disease progression curve (AUDPC) was calculated by the trapezoidal integration method (Shaner & Finney, 1977), based on the average disease severity per plant, the number of evaluations, and the interval between two applications using of the formula:

$$AUDPC = \sum_{i=0}^{n} \left(\frac{Y_{i+n1} + Y_i}{2} \right) \left(X_{i+1} - Xi \right)$$

where, n = number of observations; Yi = disease severity in the i^{th} evaluation; Xi = time in days in the i^{th} evaluation.

Enzymatic activity

Tomato seedlings were cultivated to evaluate the enzymatic activity, as a previous experiment, using the concentration of 2000 µL L^{-1} of essential oil, which was one of the concentrations that showed the smallest area under the disease progress curve (AUDPC). After 30 days of transplanting the seedlings, 1-cm-diameter discs of the plant tissue were collected in the treated leaves (second pair of leaves) and untreated (third pair of leaves), in the interval of 0 h (time of treatment), 24 h, 48 h, 72 h (time of inoculation), 96 h, 120 h, and 144 hours after the treatments. The samples were also performed on control plants, with no treatment and just inoculated with the pathogen.

A randomized block design was used with five replicates in a 7 × 3 factorial arrangement (seven collection times: 0, 24, 48, 72, 96, 120, and 144 hours, and three leaf conditions: second pair, third pair, and leaves of plants that were only inoculated). Each sample collected was placed in aluminium foil envelopes and frozen at -20 °C for further biochemical analysis.

The leaf discs were mechanically homogenized in 4 mL of 0.01 M sodium phosphate buffer (pH 6.0) in a porcelain mortar. The homogenate was centrifuged at 20,000*g* for 25 min at 4 ºC, and the supernatant obtained was considered as an enzymatic extract.

The enzymatic activities of peroxidase (POD) (Hammerschmidt *et al.*, 1982); polyphenoloxidase (PPO) (Duangmal & Apenten, 1999) and phenylalanine ammonia-lyase (PAL) (Umesha, 2006) were analysed. Protein content was determined by the method of Bradford (1976).

Statistical analysis

The data obtained were subjected to analysis of variance and, when significant, submitted to regression analysis with a 5% probability of error for essential oil concentrations. The enzyme data were analysed by Tukey test at 5% using Sisvar statistical software (Ferreira, 2011). The fungicide was compared with the other treatments by the Dunnett test, with 5% probability level of error, by Genes statistical software (Cruz, 2006). The data from the enzyme activity experiment were compared by the Tukey test at 5% significance level. The data were analysed using the statistical software Sisvar (Ferreira, 2011).

RESULTS AND DISCUSSION

In vitro experiments

The mycelial growth of *A. solani* submitted to citronella essential oil concentrations showed significant differences resulting in a quadratic decrease, presenting 100% inhibition with the calculated concentration of 2417 µL L^{-1}, although the concentrations 2000 and 2500 µL L^{-1} showed total inhibition of pathogen mycelial growth. The concentration of 1500 µL L^{-1} showed 48.77% of pathogen inhibition; 1000 µL L^{-1} corresponded to 27.68%, and the concentration of 500 µL L^{-1} presented 11.42% inhibition of *A. solani*, as shown in Figure 1.

The correlation of higher pathogen inhibitions with higher citronella essential oil concentrations may be linked to the number of oil substances available in each treatment. According to Fiori *et al.* (2000), the antifungal activity of essential oils may be related to their hydrophobic property, that is, the oil substances, in contact with the fungus, promote the alternation of plasma membrane permeability, causing structural disturbances and exposure of the cellular content of the pathogen.

The antifungal effect of citronella essential oil is proven by several authors in different pathosystems, according to the present study. The concentration of 1000 µL L^{-1} showed 79% inhibition of *Botrytis cinerea* mycelial growth in strawberry (Lorenzetti *et al.*, 2011). The development of *A. solani* was also inhibited by approximately 30% when subjected to a concentration of 1000 µL L^{-1} citronella essential oil (Lucas, 2012).

Several essential oils can inhibit the mycelial growth of other fungi. To *Sphaceloma ampelinum*, citronella oil at 3000 µL L^{-1} inhibited 81% of pathogen growth, and at 10000 µL L^{-1} there was 100% inhibition, showing the antifungal potential of the essential oil (Fialho & Papa, 2015).

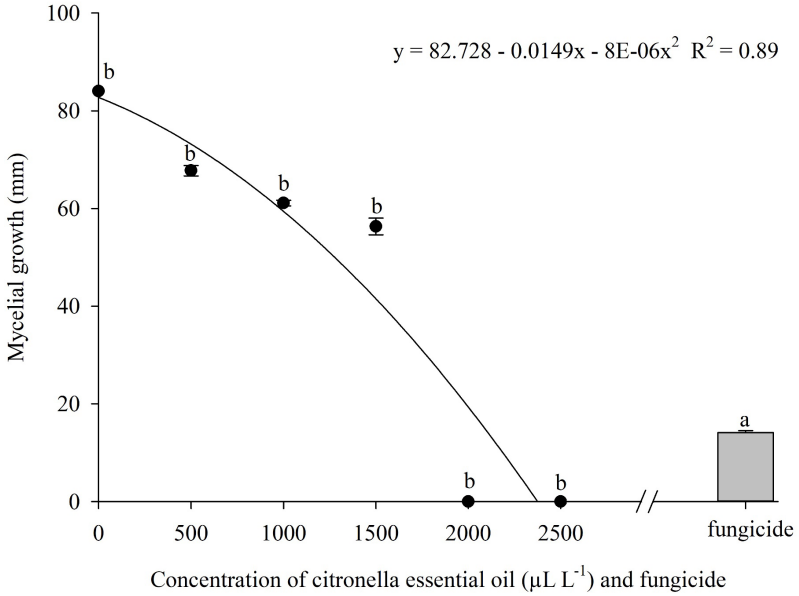

Figure 1: Mycelial growth (mm) of *Alternaria solani* in the presence of citronella essential oil (µL L^{-1}) and fungicide on the tenth day of evaluation. Note: different letters indicate a significant difference by Dunnett test. Bars indicate the standard error.

Mycelial growth was evaluated daily, and *A. solani* submitted to 0 µL L^{-1} concentration reached the entire surface of the Petri dish on the 11th day of evaluation. Higher concentrations were stunted by three days for the concentration of 500 µL L^{-1} and four days for the concentrations of 1000 µL L^{-1} and 1500 µL L^{-1}, which reached the entire surface of the plate on the 15th day. The pathogen with concentrations of 2000 µL L^{-1} and 2500 µL L^{-1} did not show mycelium growth at any moment of the experiment. The fungicide provided *A. solani* growth, but from day 11, it became stable (Figure 2).

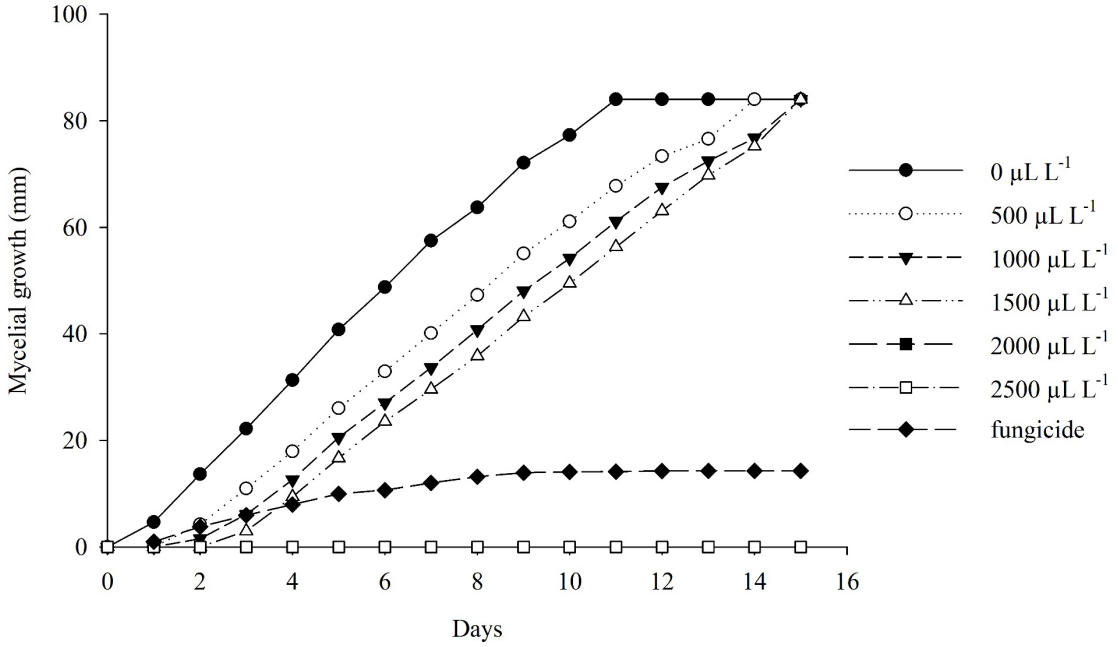

Figure 2: Mycelial growth (mm) of *Alternaria solani*, according to the days of evaluation, subjected to concentration of citronella essential oil (µL L^{-1}) and fungicide.

Increasing the concentration of citronella essential oil retarded *A. solani* mycelial growth. The concentration of 0 µL L^{-1} presented a growth rate equivalent to 8.18 mm day^{-1}, while to 500 µL L^{-1} was 6.43 mm day^{-1} and to 1000 and 1500 µL L^{-1} was 6 mm day^{-1}. These results are similar to those found by Santos *et al.* (2013), who evaluated *Helminthosporium* sp. The authors found that citronella essential oil reduced the growth rate of the pathogen, with a concentration of 250 µL L^{-1}, presenting a growth rate of 7.08 mm day^{-1} and at 500 µL L^{-1} there was a lower growth, equivalent to 4.11 mm day^{-1}, with the beginning of growth, observed after four days of the implementation of the experiment.

Citronella essential oil may retard and prevent the mycelial growth of *Colletotrichum graminicola*, the causal agent of anthracnose in sorghum, and *Pyricularia grisea* causing brusone in rice (Sarmento-Brum *et al.*, 2013). The authors observed that the growth of pathogens with a concentration of 250 µL L^{-1} started two days after the beginning of the experiment, while at 500 µL L^{-1} provided the pathogen growth only after the sixth day. Higher concentrations as 750, 1000, and 1250 µL L^{-1} inhibited the growth of pathogens entirely.

The sporulation, evaluated at the end of mycelial growth assay, presented a quadratic decrease, and the concentration of 2000 and 2500 µL L^{-1} showed the absence of spores (Figure 3). Based on the quadratic equation, the concentration of 1500 µL L^{-1} presented inhibition of 55.01% compared to the concentration 0 µL L^{-1}; 1000 µL L^{-1} (31.43%) and concentration of 500 µL L^{-1} with 13.10% inhibition of *A. solani* sporulation. The fungicide showed high sporulation compared to the concentration of 0 µL L^{-1}.

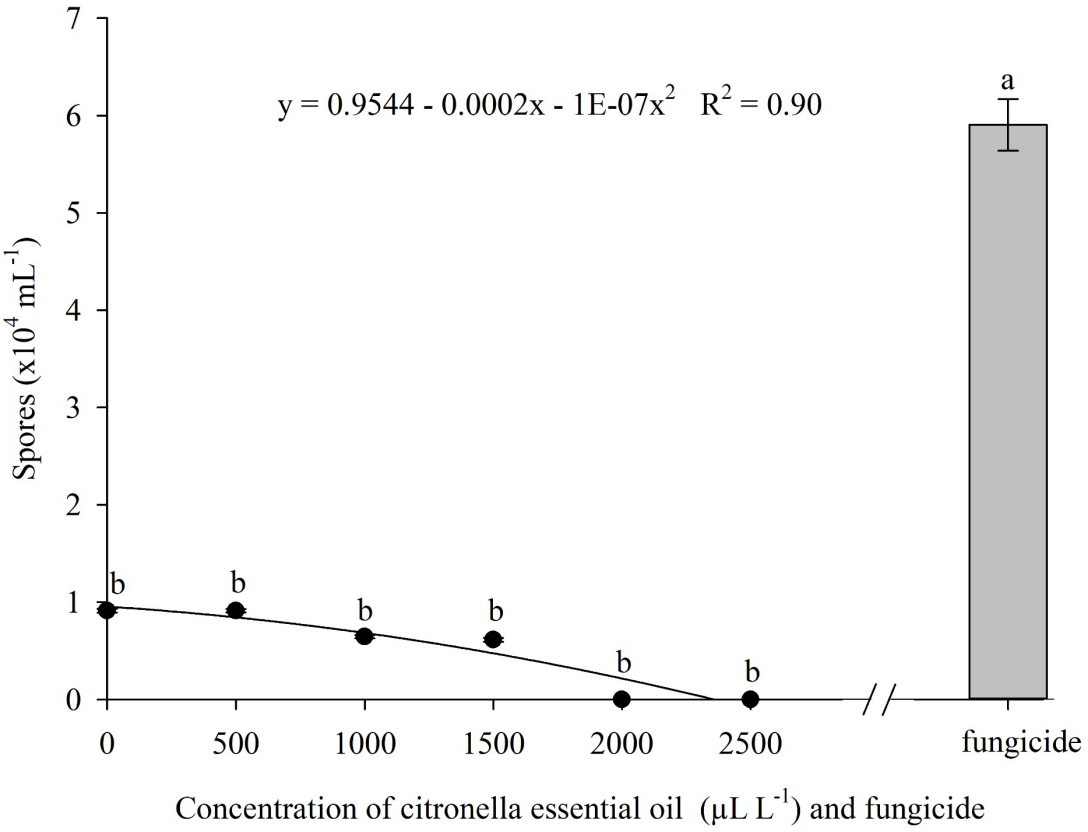

Figure 3: Sporulation of the fungus *Alternaria solani* subjected to concentrations of citronella essential oil (µL L^{-1}) and fungicide. Note: different letters indicate a significant difference by Dunnett test. Bars indicate the standard error.

According to Amorim & Pascholati (2018), the production of reproductive structures of a pathogen is directly linked to the environmental conditions in which it is exposed. As the essential oil concentrations increased, therefore, the pathogen was exposed to a higher concentration of substances present in the treatment, which may have disadvantaged the pathogen's spore production. Additionally, could has toxic effects on metabolic pathways related to spore production, by destroying cellular integrity, inhibition of respiration and ion transport processes, and

increasing membrane permeability (Cox *et al.*, 2000).

Lorenzetti *et al.* (2011) observed a 22% reduction in the production of reproductive structures of *B. cinerea* using 1000 µL L^{-1} citronella essential oil. The authors also found inhibition of spore production for the essential oils of cinnamon, lemongrass, cloves, eucalyptus, mint, and palmarosa.

Cymbopogon winterianus oil was efficient in inhibiting the production of *Fusarium solani* spores, according to the increase of dosages (Cruz *et al.*, 2015). The decrease in sporulation provided by citronella essential oil treatments is beneficial for plants, since the number of reproductive structures will be lower, and the occurrence of epidemics will be smaller.

In vivo experiments

The area under the disease progress curve (AUDPC) showed a linear dose-dependent effect for treated leaves (a) and a quadratic effect for untreated leaves (b), as shown in Figure 4.

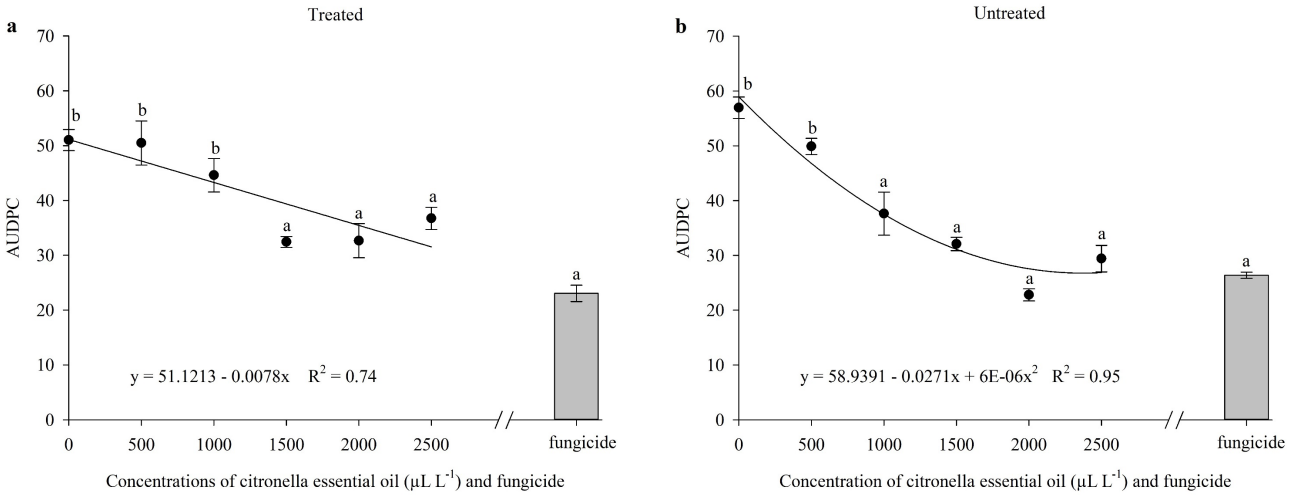

Figure 4: Area under the disease progress curve (AUDPC) in tomato leaves that were inoculated with *Alternaria solani* after being treated with citronella essential oil and fungicide (a) and when they untreated (b). Note: different letters indicate a significant difference by Dunnett test. Bars indicate the standard error.

The concentration of 2500 µL L^{-1} citronella essential oil reduced 38.14% AUDPC for treated tomato leaves, followed by 2000 µL L^{-1} concentration with 30.52% reduction, 1500 µL L^{-1} with 22, 89%, 1000 µL L^{-1} with 15.26% and the 500 µL L^{-1} concentration reduced the AUDPC by 7.63% compared to the 0 µL L^{-1} concentration (values estimated by equation). Thus, citronella essential oil showed local protection for *A. solani*. Concentrations from 1500 µL L^{-1} showed no significant difference with fungicide.

For the untreated tomato leaves the most effective concentration of the experiment was 2500 µL L^{-1} with a 51.32% reduction in AUDPC compared to 0 µL L^{-1} followed by 2000 µL L^{-1} with 51, 24% reduction, 1500 µL L^{-1} with 46.06%, 1000 µL L^{-1} with 35.80% reduction and 500 µL L^{-1} with 20.44% reduction (values estimated by equation). This reduction in disease in untreated leaves is due to the resistance induction potential of citronella essential oil, which increased the activity of defense enzymes. Thus, citronella essential oil has elicitors may trigger defense reactions by mimicking interactions of natural microbe molecular patterns or defense signaling molecules with their respective cognate plant receptors or by interfering with other defense signaling components (Dalio *et al.*, 2020).

The leaves that received or not the standard fungicide treatment showed similar results for AUDPC, since azoxystrobin, the active ingredient of the compound, has mesostemic translocation. This feature enables the compatibility of the product with the leaf surface and can be absorbed by the wax layer (Silva Jr & Behlau, 2018).

Lucas (2012) evaluating essential oils at 1000 µL L^{-1} to control early blight in tomato, observed a control of *A. solani* equivalent to 59% with the application of citronella oil before the inoculation of the pathogen. Lemongrass

essential oil, also belonging to the genus *Cymbopogon*, showed 81% control of the pathogen.

Lemongrass (*C. citratus*) essential oil at 5000 µL L^{-1} reduced 25.62% of the soft rot in lettuce plants (Silva *et al.*, 2012). In coffee plants, citronella (*C. nardus*) essential oil at 1000 µL L^{-1} showed a 43.08% reduction in cercosporiosis (Pereira *et al.*, 2011). These results prove the efficiency of *Cymbopogon* oils in disease control.

Enzymatic activity

The enzymatic activity of POD increased for the second and third pairs of tomato leaves as a function of time, with the treatment of citronella essential oil with a concentration of 2000 µL L^{-1} from the 96 hours. The maximum increase in enzyme activity for the control was 144 hours (Figure 5).

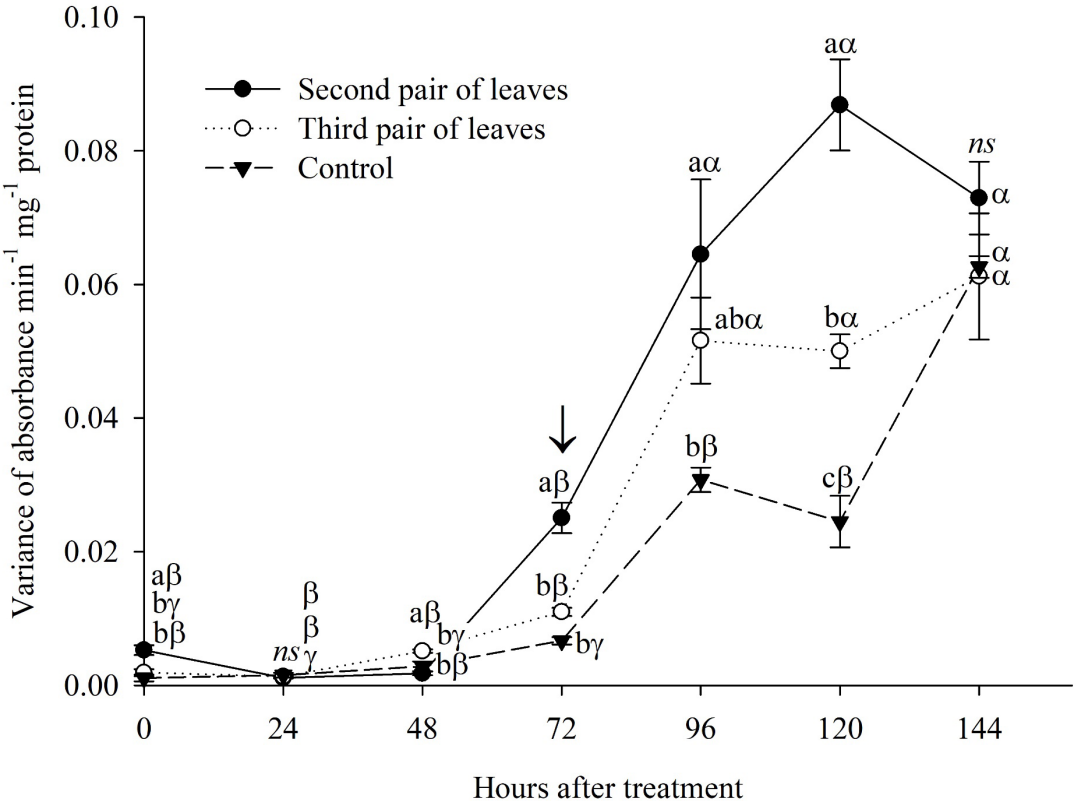

Figure 5: Peroxidase activity in tomato treated with 2000 µL L^{-1} of citronella essential oil. The treatments were performed on the second pairs of leaves, 72 hours before the inoculation of *Alternaria solani* (time 0). Both the second and third pairs of leaves were inoculated with *A. solani*. The control corresponds to the non-treated plants inoculated with the pathogen. The arrow indicates inoculation with *A. solani*. Means followed by the same letter do not differ by Tukey test: Latin letters were used for comparison of treatment within each time; Greek letters were used for comparison between times within a treatment; *ns*: not significant. Bars indicate the standard error.

An increasing tendency for the second leaf pair started at 48 hours after treatment, and with the pathogen inoculation (72 hours), the activity increased. In the course of the evaluations, this increase was achieved for the second and third leaf pairs, different from the control, showing that the increase was not only due to the pathogen but also to an inducing effect that occurred due to the oil treatment.

POD is a membrane protein involved in cell wall lignin synthesis that, together with cellulose, acts as a physical barrier to fungal penetration. Additionally, POD operates in the plant defense process, reinforcing the cell wall from lignin formation, presenting antimicrobial action, and also as a signalling agent (Vance *et al.*, 1980).

Similar results were found by Itako *et al.* (2013) that observed an increase in POD activity in treated and untreated tomato leaves inoculated with *A. solani* when lemongrass (*Cymbopogon citratus*) essential oil was used as an inducing agent. The preventive application of lemongrass

essential oil showed a localized and systemic increase in tomato leaves, according to our study.

Resistance induction in tomato against *Xanthomonas vesicatoria* was also found by Cavalcanti *et al.* (2006) using acibenzolar-S-methyl (ASM), that increased the POD enzymatic activity. Silva *et al.* (2004) also verified that *Bacillus cereus* induced the activity of this enzyme in tomato against *A. solani*, *Corynespora cassiicola*, *Oidium lycopersici*, *Stemphyllium solani,* and *X. vesicatoria*.

For PPO activity in the second and third leaf pairs, there was suppression within 24 hours after treatment with citronella essential oil, and within 48 hours, this suppression remained only for the second leaf pair. However, after 72 hours, coinciding with the moment of pathogen inoculation, there was an increase in PPO activity for the second and third leaf pairs, differing from the control (Figure 6). Increasing enzymatic activity only after pathogen inoculation is beneficial to plants as there will be no unnecessary energy expenditure (Kuhn & Pascholati, 2010).

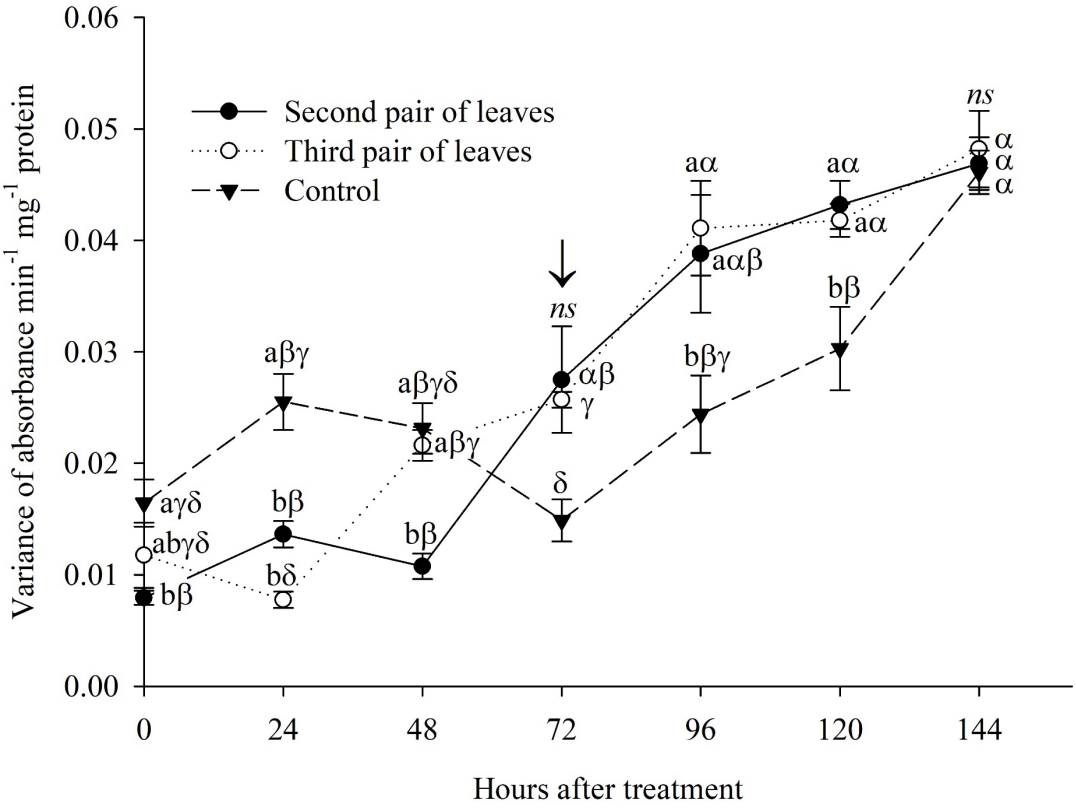

Figure 6: Polyphenoloxidase activity in tomato treated with 2000 µL L^{-1} of citronella essential oil. The treatments were performed on the second pair of leaves, 72 hours before the inoculation of *Alternaria solani* (time 0). Both the second and third pairs of leaves were inoculated with *A. solani*. The control corresponds to the non-treated plants inoculated with the pathogen. The arrow indicates inoculation with *A. solani*. Mean followed by the same letter do not differ by Tukey test: Latin letters were used for comparison of treatment within each time; Greek letters were used for comparison between times within a treatment; ns: not significant. Bars indicate the standard error.

The disruption of a cell caused by the action of insects or pathogens releases the polyphenol oxidases that initiate the oxidation process of phenolic compounds, producing toxic quinones, which present antimicrobial activity (Mohammadi & Kazemi, 2002).

Silva *et al.* (2007) observed a spike in the activity of the PPO enzyme in tomato plants treated with *Lentinula edodes* and *Agaricus blazei* extracts and acibenzolar-S-methyl, and inoculated with *Ralstonia solanacearum* after 72 hours of treatment. According to Portz *et al.* (2011), high enzymatic activity can be observed during the pathogen colonization, due to the defense mechanisms that are activated more intensely after inoculation.

Ramamoorthy *et al.* (2002) also found an increase in PPO activity in tomato plants after seed treatment with *Pseudomonas fluorescens* isolates and inoculated with *Pythium aphanidermatum*.

According to the present study, Itako *et al.* (2013) also observed local (treated leaves) and systemic (untreated leaves) increase in enzymatic activity, characterized by the enzyme PPO, induced by *C. citratus* essential oil (lemongrass) when tomato was inoculated with *A. solani* three days after treatments.

Figure 7 shows the activity of phenylalanine ammonia-lyase (PAL) for the second and third pairs of tomato leaves treated with 2000 µL L^{-1} citronella essential oil and control. From the inoculation, the second and third pairs of tomato leaves showed higher PAL activity compared to the control. As function of the time, the increase of enzymatic activity was observed at 120 hours for the second pair of leaves, at 72 and 96 hours for the third pair of leaves, and the times 72 and 96 showed the lowest activities for the control treatment.

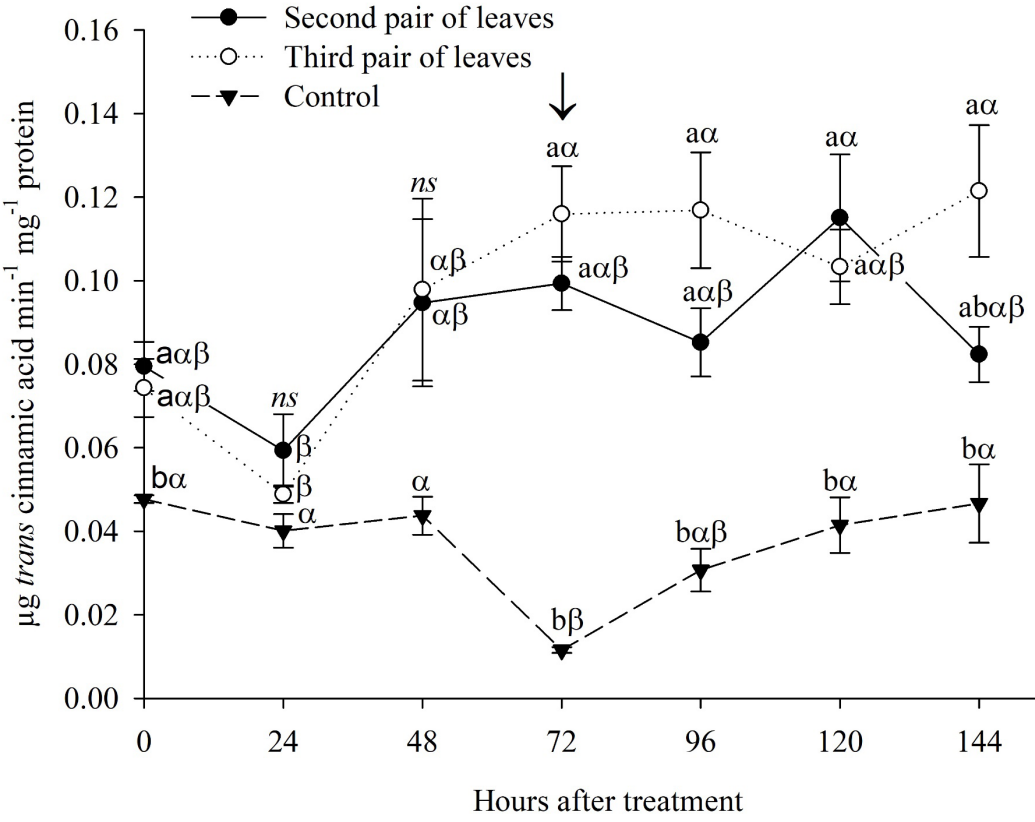

Figure 7: Phenylalanine ammonia-lyase activity in tomato treated with 2000 µL L^{-1} of citronella essential oil. The treatments were performed on the second pairs of leaves, 72 hours before the inoculation of *Alternaria solani* (time 0). Both the second and third pairs of leaves were inoculated with *A. solani*. The control corresponds to the non-treated plants inoculated with the pathogen. The arrow indicates the inoculation with *A. solani*. Mean followed by the same letter do not differ by Tukey test: Latin letters were used for comparison of treatment within each time; Greek letters were used for comparison between times within a treatment; ns: not significant. Bars indicate the standard error.

PAL acts on L-phenylalanine, transforming it into trans-cinnamic acid, the first product formed in the biosynthetic route of phenylpropanoids in higher plants. This acid acts as a precursor to numerous phenylpropanoid compounds that perform various functions in the plant, including the protection against pathogen provided by lignin synthesis (Ritter & Schulz, 2004).

Ramamoorthy *et al.* (2002) observed similar results to the present study when evaluating resistance induction of tomato plants, in which seeds were treated with *P. fluorescens* and challenged with *P. aphanidermatum*. The authors reported that PAL activity remained at high levels throughout during the assay, and for the control treatment PAL activity decreased after the fourth day of evaluation.

PAL activity was verified by Silva *et al.* (2015) with *Cymbopogon flexuosus* essential oil, belonging to the

same genus as citronella, in common bean plants with and without *Colletotrichum lindemuthianum* inoculation. According to the present study, the authors also observed high activity of PAL in all evaluation periods, which may mean that the route of phenylpropanoids has changed, as well as the potentiation of mechanisms such as lignin synthesis.

CONCLUSIONS

Citronella essential oil showed antifungal activity against *A. solani* and early blight control in tomato, which may due either to direct fungitoxic effect on the pathogen, inhibiting mycelial growth and sporulation, or by local and systemic resistance induction mediated by the enzymes peroxidase, polyphenoloxidase, and phenylalanine ammonia-lyase.

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