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A. INVESTIGACIONES

Cymbopogon citratus essential oil bioactivity and the induction of enzymes related to the pathogenesis of *Alternaria solani* on tomato plants

Bioactividad del aceite esencial Cymbopogon citratus *y la inducción de enzimas relacionadas con la patogénesis de* Alternaria solani *en plantas de tomate*

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ABSTRACT

The objective of this work was to evaluate *in vitro* and *in vivo* the bioactivity of the essential oil of *Cymbopogon citratus* on the mycelial growth and sporulation of *Alternaria solani*, and also some biochemical and structural mechanisms of resistance induction on tomato plants. The plants were treated with essential oils at 0, 250, 500, 750, 1000 and 1500 µL L^{-1} 72 hrs before the fungi inoculation. Foliar discs were collected at 0, 12, 24 and 48 h after the inoculation to verify the activity of peroxidase, polyphenol oxidase and β–1,3 glucanase. Spore germination, appressorium formation, and the mechanisms of structural resistance were evaluated 48 h after the inoculation. The *in vitro* responses showed direct toxic activities unlike the ones observed in vivo, where the essential oils had no fungitoxic effects on the fungi spores. *In vitro*, the peroxidase and polyphenol oxidase enhanced both local and systemic activities, unlike the β–1, 3-glucanase which enhanced only local activities at the highest concentrations. The essential oils of *C. citratus* have, therefore, potential to induce resistance in tomato plants.

Key words: induced resistance, pathogenesis-related proteins, *Solanum lycopersicon*.

RESUMEN

El objetivo de este trabajo fue evaluar la bioactividad in vitro *e* in vivo *del aceite esencial de* Cymbopogon citratus *sobre el crecimiento micelial y la esporulación de* Alternaria solani*, y también algunos mecanismos bioquímicos y estructurales de la inducción de resistencia en plantas de tomate. Las plantas fueron tratadas con aceite esencial a 0, 250, 500, 750, 1000 y 1500 µL L^{-1} 72 horas antes de inocular los hongos. Se recogieron discos foliares a 0, 12, 24 y 48 h después de la inoculación para verificar la actividad de la peroxidasa, polifenol oxidasa y β–1, 3 glucanasa. Se evaluó la germinación de esporas, formación de apresorios, y los mecanismos de resistencia estructural 48 horas después de la inoculación. La respuesta* in vitro *mostró actividad tóxica directa, a diferencia de lo observado* in vivo*, donde los aceites esenciales no tuvieron efectos fungitóxicos sobre las esporas de hongos.* In vitro*, la peroxidasa y polifenol oxidasa mejoro su actividad, tanto local como sistémica, a diferencia de la β–1, 3-glucanasa que aumentó solo su actividad local en las concentraciones más altas. Los aceites esenciales de* C. citratus *tienen, por lo tanto, el potencial para inducir resistencia en plantas de tomate.*

Palabras clave: *resistencia inducida, proteínas relacionadas con la patogénesis,* Solanum lycopersicum.

Introduction

Resistance induction may be an efficient, practical, and alternative method of controlling plant diseases, being used as a toll for studying biochemical and physiological mechanisms of resistance and susceptibility of plants against pathogens (Walters and Fountaine, 2009). The biochemical mechanisms expressed by enzymes related to pathogenesis permit the self-protection of plants from diseases incited by numerous pathogenic organisms (Schwan-Estrada & Stangarlin, 2005).

These mechanisms work in a dynamic and coordinate way to structural mechanisms as the

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papillae and halo, for example. The responses from resistance induction using the crude plant extracts or the essential oils have all the benefits of natural products. This alternative to the conventional treatments has the objective of reducing the food, soil and water contamination, farmers and handy workers intoxication, and pathogen resistance, which affects the population of numerous soil-borne organisms (Prithiviraj *et al*., 1997).

The essential oils or the crude extracts from the medicinal plants have potential to control the plant pathogens either by direct fungitoxic effects or resistance induction because the presence of elicitor compounds (Schwan-Estrada & Stangarlin, 2005). The fungitoxic effects of the crude extracts and essential oils of *Achillea millefolium*, *Cymbopogon citratus*, *Eucalyptus citriodora* and *Ageratum conyzoides* on *Didymella bryoniae* was verified *in vitro* by evaluating the germination of spores and the mycelial growth. Scanning electron microscopy (SEM) observations revealed significant alterations in the growth pattern of *D. bryoniae* hyphae using the essential oil of *A. millefolium* (Fiori *et al.*, 2000). Anaruma *et al.* (2010) tested 28 essential oils, found the activity of 15 of them against *Colletotrichum gloeosporioides*, agent of anthracnose on yellow passion fruit (*Passiflora edulis*). Of the oils tested *C. citratus* to a performance similar to fungicides. Essential oils of plants of the family Lamiaeceae, *Origanum syriacum*, *Lavandula stoechas* and *Rosmarinus officinalis* L., demonstrated effect in controlling *Botrytis cinerea* in tomato, oil *O. syriacum* being the most efficient (Soylu *et al.*, 2010).

Numerous researchers have demonstrated that plant extracts induce the mechanisms of resistance. For example, Asha and Kannabiran (2001) detected the protection of hot pepper seeds against *Colletotrichum capsici* during 35 days using extract of *Datura metel* at 10%, verified significant increases in the activity of peroxidase and polyphenol oxidase. In rice, *R. solani* and *Xanthomonas oryzae* pv. *oryzae* were significantly reduced by *D. metel* under glasshouse conditions (Kagale *et al.*, 2004). Reduction on fungus development was also verified *in vitro*. The resistance induction was detected by significant increases in the activities of peroxidase, chitinase, β–1, 3-glucanase and PAL. Chakraborty *et al.* (2005), observed increases in β–1, 3-glucanase and chitinase activities of tea plants (*Camellia sinensis*) using extracts of neem (*Azadiractha indica*) and vinca (*Catharanthus roseus*) which were associated to decreases in the severity of *Curvularia pallescens*. The plant extract of *A. indica* was more efficient than the extract of *C. roseus* to reduce the disease severity, but both plant extracts were efficient to induce β–1, 3-glucanase, PAL and chitinase. These authors associated the disease reduction to the enzyme induction. Itako *et al.* (2008) also evaluated the fungitoxic effects of crude extracts (EBAs) from *Achillea millefolium, Artemisia camphorata, C. citratus* and *Rosmarinus officinalis* against *A. solani*, and their protective effects on tomato plants cultivated under greenhouse conditions. These authors observed that the crude extract did not inhibit the mycelial growth, but the sporulation and the conidia germination as the EBAs of *A. camphorata, C. citratus* and *R. officinalis* were reduced at concentrations above 20%. In terms of plant protection by these extracts, there was significant reduction in the number of lesions in the leaves above because of these systemic effects. However, these authors did not evaluate the enzymes involved in the systemic protection.

Thus, the objective of this work was to verify *in vitro* and *in vivo* the antifungal potential of the essential oils from *C. citratus* on the *Alternaria solani*, and the induction of the biochemical mechanisms of resistance through the activities of peroxidase, polyphenol oxidase and β–1, 3-glucanase.

Material and Methods

The experiment was conducted in the Laboratory of Agriculture Biotechnology at the Universidade Estadual de Maringá, Maringá, Northwestern Paraná State, Brazil. Isolates of *A. solani* was supplied by the Universidade Estadual do Oeste do Paraná, at Marechal Cândido Rondon, Western Paraná, Brazil. The fungus was stored in the PDA medium (potato dextrose agar) under dark conditions at 25 ± 2 ºC. In February, fresh and healthy leaves of *Cymbopogon citratus* were collected at the Botanical Garden of Medicinal Plants in the Universidade Estadual de Maringá between 12:00 and 02:00 PM. The essential oils were distilled using hot water steam, packed into dark-glass bottles, and stored into refrigerator.

In vitro and *in vivo* studies

In vitro, the sterilization of the essential oils was done with 0.45 µm Millipore membranes. Thereafter, aliquots of 0, 5, 10, 20, 40 and 50 µL of

these essential oils were added into autoclaved and liquefied PDA medium and poured into sterilized Petri dishes. After the solidification, 15-day-old mycelium discs of *A. solani* measuring 8 mm in diameter were transferred to the centre of every Petri dish which was wrapped in plastic film and incubated at 25 ºC ± 2ºC under darkness. The mycelial growth was evaluated by measuring two opposite diameters of every colony at 24-hrs intervals, and these measurements were finished when the control had the fungus colony established on two-thirds of the growing media surface. The percentage of inhibition was calculated according to Bastos (1997). Finally, 10 mL of distilled water was added to every Petri dish from where the mycelium was scraped for counting the number of conidia. The mixture was filtered and the conidia were counted in the Neubauer chamber assembled under an optical microscope.

The experimental design was completely randomized with six treatments and four replications where every Petri dish was the experimental unit. The data were submitted to the analysis of variance and regression using the SAS/Stat and SISVAR (Ferreira, 2011). The goodness of fit was tested at 5% of probability.

In vivo, the leaves of tomato plants cv 'Santa Clara' were treated with the essential oil of *C. citratus* at 0, 250, 750, 1000 and 1500 µL L^{-1} 72 hrs before the inoculation with *A. solani*. Foliar discs with 2 cm in diameter were collected 48 hrs after the inoculation of the leaves by *A. solani*, and they were stored into FAA (formaldehyde: acetic acid: ethanol 50% - 5:5:90 v/v/v). The discs were treated with 95% ethanol at 60 ºC under water bath (Conti *et al.*, 1986) before evaluating the spore germination and the appressorium development. The discs, thereafter, were dyed with cotton blue and then evaluated. The experimental design was completely randomized with six concentration levels of essential oils and five replications with four foliar discs.

Peroxidase, polyphenol oxidase and β–1,3-glucanase activities

The tomato seeds cv 'Santa Clara' were sowed in Styrofoam trays with 128 cells filled with commercial growing medium. Thereafter, 35-day-old seedlings were transplanted into plastic pots of 500 mL. Twenty-five days later, every plant had the second leaf pair treated with essential oils of *C. citratus* at 0, 250, 500, 750, 1000 and 1500 µL L^{-1}. Seventy-two hours later, every second leaf pair treated and the third leaf pair untreated was inoculated with *A. solani* (10^4 conidia mL^{-1}). Thereafter, five foliar discs measuring 8 mm in diameter were collected from these leaves at the periods of 0, 12, 24 and 48 h to detect the presence of local and systemic activities of peroxidase (POD), polyphenol oxidase and β–1, 3-glucanase. These foliar discs were weighed, packed into aluminium foil, identified, and stored at –20 ºC. The foliar discs were mechanically homogenized into 4 mL of acetate buffer at 100 mM (pH 5.0) in porcelain mortar with liquid nitrogen. The homogenized material was centrifuged at 4 ºC under 20,000 g for 25 minutes, and the supernatant was analysed to evaluate the activities of POD, polyphenol oxidase and β–1,3-glucanase.

The POD activity was determined at 30 ºC using the direct spectrophotometric method based on the conversion of guaiacol into tetraguaiacol at 470 nm (Lusso and Pascholati, 1999). In this reaction, the mixture consisted of 0.10 mL of protein extract and 2.9 mL of buffer solution from 250 µL of guaiacol and 306 µL of hydrogen peroxide, 12.5 mL guaiacol at 2.0% in 100 mL 0.01M phosphate buffer (pH 6.0). The reference cuvette had 3 mL from the solution with 250 µL of guaiacol and 306 µL of hydrogen peroxide in 100 mL phosphate buffer 0.01 M (pH 6.0). Absorbance data were read in time intervals of 15 seconds for 3 minutes. The specific activity was reported as Δ_{abs} 470nm min^{-1} μg^{-1} of protein Protein concentration expressed as equivalent µg of bovine serum albumin (BSA) in 1-mL sample (µg protein mL^{-1}) was determined using a standard curve of BSA concentrations from 0 to 20 µg mL^{-1}.

The activity of the polyphenol oxidase was determined using the Duangmal and Apentem method (1999) in which the chemical reaction was performed through the mixture of 900 µL of substratum with 10 µL of enzymatic extract. The substratum was prepared with cathecol at 20 mM dissolved into phosphate buffer at 100 mM (pH 6.0). The temperature of the chemical reaction was 30 ºC, and the direct spectrophotometer readings for 3 minutes were carried out at 420 nm. The results were expressed as Δ_{abs} 470nm min^{-1} μg^{-1} of protein. Protein concentration expressed as equivalent µg BSA in 1-mL sample (µg protein mL^{-1}) was determined by using the standard curve of BSA concentrations ranging from 0 to 20 µg mL^{-1}.

The β–1, 3-glucanase was quantified using the colorimetric determination of the glucose released by laminarin after applying the hydrazine from hydroxibenzoic acid (PAHBAH) (Lever, 1972). The solution of 150 µL of the enzymatic extract with 150 µL of laminarin (2.0 mg mL^{-1}) was incubated at 37 ºC for 1 h (Abeles and Foence, 1970). Thereafter, 1.5 mL of PAHBAH was added to the solution which was boiled at 100 ºC for 10 minutes and cooled until 25 ºC when this mixture was used to determine the absorbance at 410 nm in comparison with the buffer of extraction. The absorbance responses were plotted on the standard glucose curve and expressed in µg de glucose μg^{-1} of protein. Protein concentration expressed as equivalent µg BSA in a sample of 1-mL (µg protein mL^{-1}) was determined using the standard curve of BSA concentrations from 0 to 20 µg mL^{-1} (Bradford, 1976).

The experimental design was completely randomized under the factorial arrangement of six concentrations of essential oils at 0, 250, 500, 750, 1000 and 1500 µL L^{-1}, four periods of evaluations at 0, 12, 24 and 48 hrs and four replications. The data were tested for homogeneity of variance and normal errors before the analyses of variance. Thereafter, surface response models ($p \leq 0.05$) were fit to the data using the SAS/Stat.

Results and Discussion

The *in vitro* studies indicated direct fungitoxic effects of the essential oils on the mycelial growth and sporulation of *A. solani*. Every µL of increase in the concentration of essential oils significantly inhibited the mycelial growth by 1.95%. Total inhibition of mycelial growth was observed at 50 µL (Fig. 1A). The sporulation was described by the logarithmic model in which the total inhibition was observed at 40 and 50 µL (Fig. 1B).

These responses are in partial agreement with the report from Abreu (2006) who also verified total inhibition of the mycelial growth using *C. citratus*, but at concentration of 750 µL L^{-1} and the total inhibition of conidia germination at 500 µL L^{-1} and above. Other plant pathogens have also been affected. *Aspergillus flavus*, for example, had the mycelial growth reduced by 64%, *A. fumigatus* by 48%, and *Fusarium moniliforme* by 77% at 500 ppm and concentrations above (Nguefack *et al.*, 2004). Citral, which is the major component in these essential oils, may be responsible for this fungitoxic sensitiveness. Volatile components of essential oil of *C. citratus* provided significant reduction in the development of *Colletotrichum coccodes*, *Botrytis cinerea*, *Cladosporium herbarum*, *Rhizopus stolonifer* and *Aspergillus niger*, between 25 and 500 ppm (Tzortzakis and Economakis, 2007).

The *in vivo* studies, however, did not detect significant differences between the treatments ($F > 0.05$).These treatments, therefore, showed no effect on the spore germination and appressorium formation, and no fungitoxic effects on the conidia were observed under the present concentrations. An explanation for these results could be due the citral volatility described by Schuck *et al.* (2001), as in our experiment, these essential oils were applied 72 hrs before the inoculation of the pathogen.

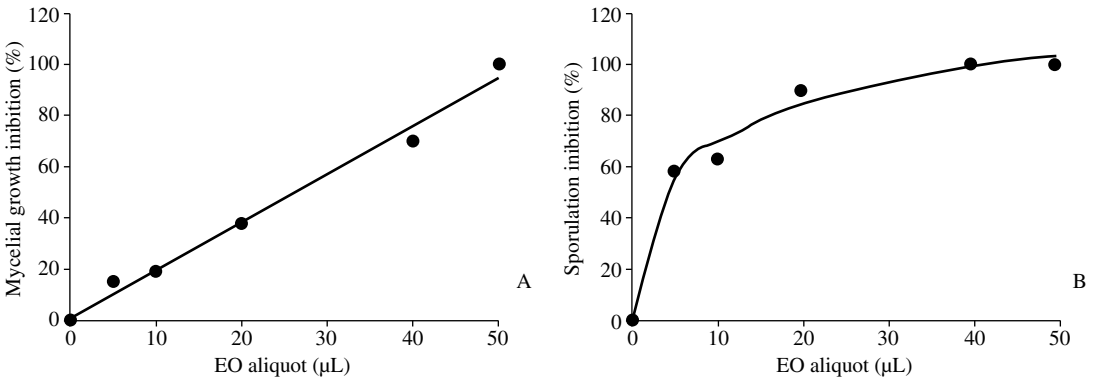

Figure 1. Percentage of inhibition of the mycelial growth (A) and sporulation (B) of *Alternaria solani* treated with essential oils (0, 5, 10, 20, 40 and 50 µl) of *Cymbopogon citratus.*

The capacity of activating biochemical and structural mechanisms of defence in response to pathogenic microorganisms is the main characteristic of resistance inductors unlike their direct antimicrobial activity (Kúc, 2001). This *in vitro* activity is a strong indication of resistance induction because these responses suggested that the potential for inducing resistance was present.

The preventive application of essential oils onto the leaves of tomato increased the local (treated leaves) and the systemic (untreated) activities of POD and polyphenol oxidase unlike the β^{-1}, 3-glucanase which presented only local increases (Fig. 2, 3 and 4).

Significant interaction between time and concentration on the activity of the peroxidase was

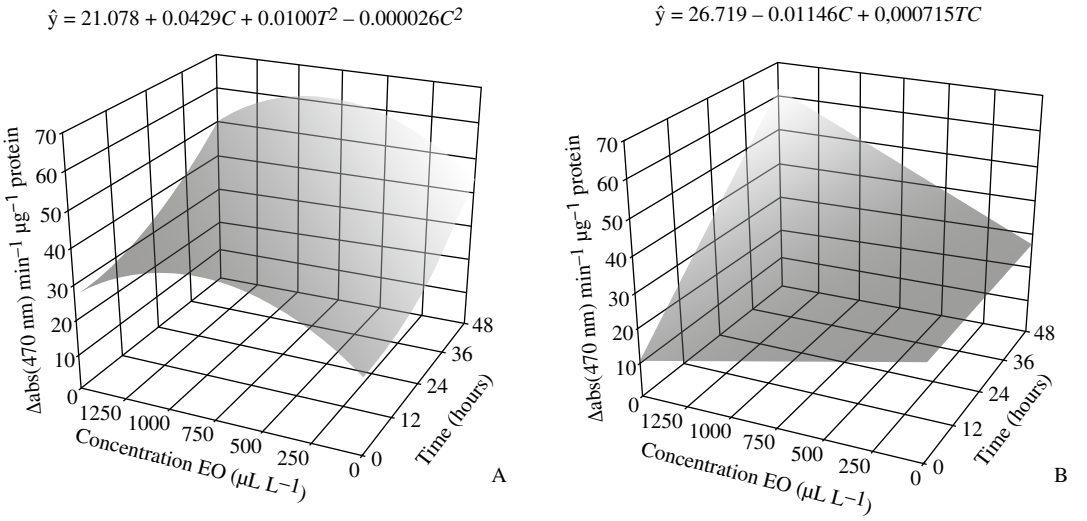

Figure 2. Peroxidase activities at 0, 12, 24 and 48 hrs after inoculating *Alternaria solani* 72 hrs after the leaf treatments with the essential oil (0, 250, 500, 750, 1000 and 1500 µL L^{-1}) of *Cymbopogon citratus* (A) and the control (B).

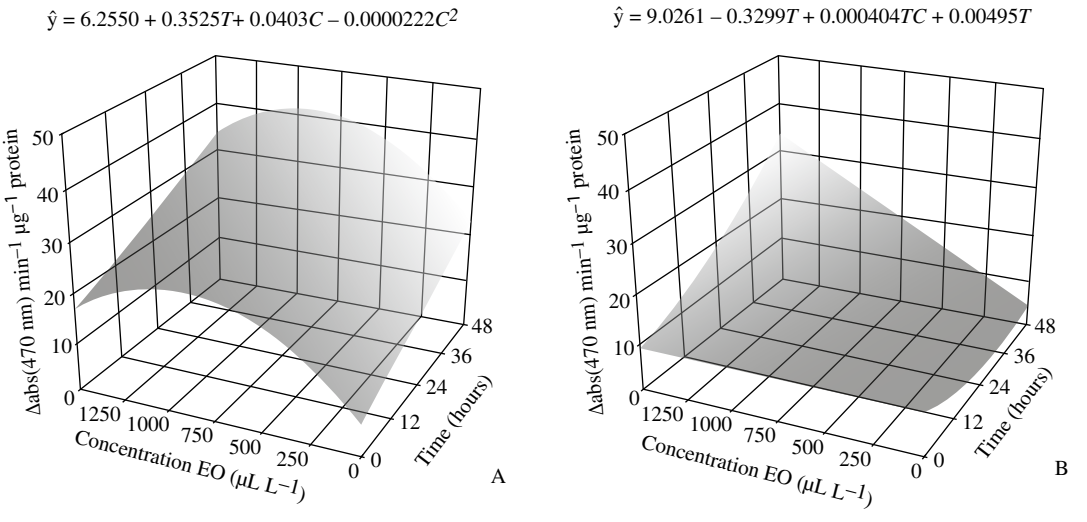

Figure 3. Polyphenol oxidase activities at 0, 12, 24 and 48 hrs after *Alternaria solani* inoculated 72 hrs after leaf treatments with the essential oil (0, 250, 500, 750, 1000 and 1500 µL L^{-1}) of *Cymbopogon citratus* (A) and the control (B).

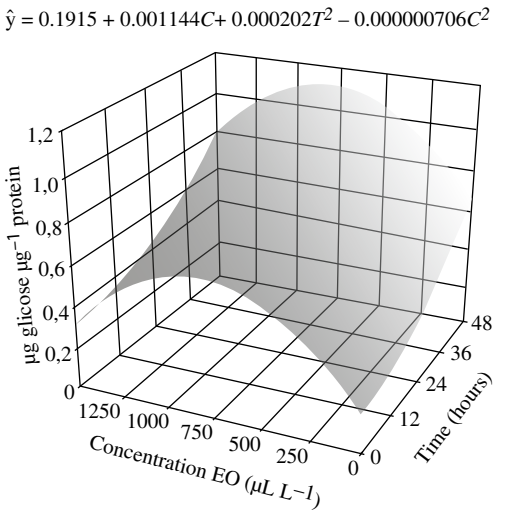

Figure 4. β–1, 3-glucanase activities at 0, 12, 24 and 48 hrs after inoculating *Alternaria solani* 72 hrs after leaf treatments with the essential oil (0, 250, 500, 750, 1000 and 1500 µL L^{-1}) of *Cymbopogon citratus.*

observed and described by surface response analisys. The maximum of 61.82Δ (ABS 470nm) min^{-1} μg^{-1} of protein in the local activity of the peroxidase (Fig. 2A) was observed with 825µL L^{-1}, and 60.55Δ (ABS 470nm) min^{-1} μg^{-1} of protein in the local activity of the peroxidase (Fig. 2B) was observed with 1500 µL L^{-1} for 48 h after the inoculation.

Significant interaction between concentration and time for the local activities of β^{-1}, 3-glucanase was also described by surface response analysis. The maximum of 1.12 µg glucose μg^{-1} of protein in the local activity of peroxidase (Fig. 4) was observed with 810 µL L^{-1} for 48 h. Otherwise, non-significant increases in the enzymatic activities were detected by the analysis of variance.

Enzymes related to the pathogenesis usually have participation in the defence mechanisms during the infection. Peroxidase and polyphenol oxidases are involved in the lignification of cell walls and the β^{-1}, 3-glucanase have direct effects on the glucane present in cell walls of fungi. Indirect effects through elicitor fragments released from plant cell walls start the defence responses (Cutt and Klessig, 1992). Accordingly, the essential oils of *C. citratus* also started the activities of these enzymes in treated and non-treated leaves as reported by Abreu (2006), who verified significant reduction in the level of early blight in tomato plants under glasshouse and field conditions. The treatment with the essential oil of *C. citratus* reduced the disease severity by 90% as they observed with the fungicide application. The production of fruits were similar to the average obtained with fungicide treatment, which was 20% higher than observed in the control. The mechanisms described in the present experiment may explain that level of plant protection.

The induced protection also depends on the time intervals between the treatment and the inoculation due to the specific changes in the plant metabolism. In the present experiment, 72 h may be long enough to activate the mechanisms of induction. Similar responses were observed by Yamunarani *et al.* (2004), who reported efficient control of early blight by the extract of *Quercus infectoria* at 0.5% applied onto tomato plants 72 h before the inoculation with *A. solani.* In terms of resistance induction, there was a significant increase in peroxidase, PAL, β^{-1}, 3-glucanase and chitinase activities. Peroxidase and PAL reached the maximum activity 48 h after the treatment while β^{-1}, 3-glucanase and chitinase reached it after 72 h of treatment. The extract of *Q. infectoria* has potential to induce resistance on the system tomato-*Alternaria solani*.

Conclusion

In vitro, the essential oil of *C. citratus* applied onto tomato plants has direct activity on the development of *Alternaria solani*, and induced the biochemical mechanisms of local and systemic resistance, or both. Essential oils (OE) of *Cymbopogon citratus* do not have toxicity on the tomato plants, but they have fungitoxic effects on *Alternaria solani*. The biochemical mechanisms of plant defence related to the enzymes peroxidase, poliphenoloxidase and β–1, 3-glucanase were activated *in vitro*.

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