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In vitro and *in vivo* activity of essential oils extracted from *Eucalyptus staigeriana*, *Eucalyptus globulus* and *Cinnamomum camphora* against *Alternaria solani* Sorauer causing early blight in tomato

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ABSTRACT

Use of plant compounds has been reported to reduce severity of diseases in many crops. In an attempt to establish new control practices against plant pathogens with low mammalian toxicity and low persistence in the environment, the objective of this work was to study the activity of essential oils extracted from *Eucalyptus staigeriana* F. Muell. ex Bailey, *Eucalyptus globulus* L. and *Cinnamomum camphora* Ness and Eberm var. *linaloolifera* Fujita against *Alternaria solani*, causing early blight on tomato, under *in vitro* and *in vivo* conditions. In order to test *in vitro* antifungal activity, potato dextrose agar (PDA) medium was used with essential oils diluted at 0.1–10.0 μL mL^{-1} concentrations. Inhibition of disease severity on tomato was evaluated under greenhouse conditions and essential oils concentrations used were 2.0 and 10.0 μL mL^{-1}. Results showed that all essential oils were capable to control early blight disease, for both *in vitro* and *in vivo* assays. Tomato plants treated with essential oils presented an area under the disease progress curve (AUDPC) ranging from 126.71 to 166.43 and fungicide azoxystrobin ranging from 154.97 to 160.89, differing from control condition which demonstrated an AUDPC of 279.14. Therefore, essential oils could be an effective alternative to control tomato early blight disease.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is the second most cultivated vegetable, after potato crop, and has a major role in global economy due to production volume and value (Heuvelink, 1996; FAO, 2013). However, more than two hundred diseases caused by biotic and abiotic agents had been reported worldwide, contributing to a significant decrease in tomato productivity (Jones et al., 1991). Early blight caused by *Alternaria solani* Sorauer is an important and frequent fungal disease of tomato crop around the world. Disease develops in areas of high humidity and temperatures, occurring on leaves, stems, petioles and fruits. The fungus prefers mature tissues and is more frequent at the fruiting stage, causing high economic losses (Foolad et al., 2000; Chaerani and Voorrips, 2006).

Although genetic resistance is the most effective control method, currently there are no viable tomato varieties with acceptable levels of genetic resistance against early blight. Control method most used and more effective for this disease is application of synthetic fungicides. However, they are generally used in excessive doses, causing environment contamination, health problems to workers and consumers, and favor the development of pathogen resistant strains (Chaerani and Voorrips, 2006; Feng and Zheng, 2007; Grigolli et al., 2011). Therefore, alternative products should be developed to reduce the dependence on synthetic agrochemicals, and natural products from plant extracts and essential oils can provide that. Plant extracts and essential oils are favorable compounds for the control of phytopathogens, since they have proven antifungal activity and can also induce pathogenesis-related enzymes, phytoalexins and leaf lignification (Fawzi et al., 2009). Essential oils are complex mixtures of volatile organic substances, consisting of oxygenated compounds and hydrocarbons, such as sesquiterpenes and monoterpenes. These compounds are responsible for the antimicrobial activity of essential oils and have their mechanism associated to their lipophilic nature, which interacts with microbial membranes leading to leakage of cell compounds and causes energy losses of microbial cells (Feng and Zheng, 2007; Nerio et al., 2010; Tian et al., 2012).

Many investigations have been conducted on the use of essential oils as alternative products to control phytopathogens under *in vitro*

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conditions and just very few studies have been conducted in *in vivo* conditions (Soylu et al., 2010). Essential oils of *Eucalyptus globulus* L., *Eucalyptus staigeriana* F. Muell. ex Bailey and *Cinnamomun camphora* Ness and Eberm var. *linaloolifera* Fujita have been shown to exhibit antifungal action against many different fungi already proven in literature (Hili et al., 1997; Batish et al., 2008; Gilles et al., 2010), but its action against *A. solani* was not investigated yet. In this work, the ability of three essential oils extracted from *E. staigeriana*, *E. globulus* and *C. camphora* var. *linaloolifera* to inhibit the development of *A. solani* in laboratory conditions and in tomato plants grown under greenhouse conditions was evaluated.

2. Materials and methods

2.1. Plant material, essential oils extraction and chemical identification

Leaves of *Eucalyptus staigeriana*, *Eucalyptus globulus* and *Cinnamomum camphora* var. *linaloolifera* were harvested from plants located at the Institute of Biotechnology, University of Caxias do Sul, Rio Grande do Sul State, Brazil. Voucher specimens were deposited at the Herbarium of the University of Caxias do Sul (HUCS), by: HUCS37973, HUCS37972 and HUCS37976, respectively. Essential oils were extracted by hydrodistillation method from dried plant leaves for 1 h in a Clevenger-type apparatus according to Agostini et al. (2009). For chemical identification, a gas chromatograph HP 6890 coupled with a mass selective detector Hewlett Packard MSD5973 (GC–MS), equipped with HP Chemstation software and Wiley 275 spectra data was used to perform the analyses. Analyses were conducted using a fused silica capillary column HP-Innowax (30 m × 0.25 mm i.d., 0.25 μm film thickness, Hewlett Packard, Palo Alto, USA) with following conditions: column temperature, 40 °C (8 min) to 180 °C at 3 °C/min, 180–230 °C at 20 °C/min, 230 °C (20 min); interface 280 °C; split ratio 1:100; carrier gas He (56 KPa); flow rate: 1.0 mL/min; ionization energy 70 eV; mass range 40–350. Volume injected was 0.4 μL (diluted in hexane 1:10). Analytical gas chromatography was carried out in a Hewlett Packard 6890 gas chromatograph with a flame ionization detector (FID) equipped with a HP Chemstation software. A HP-Innowax bonded phase capillary column (30 m × 0.32 mm i.d., 0.50 μm film thickness, Hewlett Packard, Palo Alto, USA) was used with following conditions: column temperature, 40 °C (8 min) to 180 °C at 3 °C/min, 180–230 °C at 20 °C/min, 230 °C (20 min); injector temperature 250 °C, detector temperature 250 °C; split ratio 1:50; carrier gas H_2 (34 KPa). Injection volume was 1 μL (diluted in hexane 1:10). Identification of individual components was based on comparison of their GC retention times (R.T.) on polar columns and comparison with mass spectra of components by GC–MS. Components were identified by a combination of mass spectrum of the Wiley library and by comparison with data from literature (Adams, 1995).

2.2. Alternaria solani isolates

Fungi strains used in this work were isolated from tomatoes and preserved in the fungal collection of the Laboratory of Plant Diseases, University of Caxias do Sul, Brazil, on potato dextrose agar (PDA) medium. Four *A. solani* isolates from tomato crops located within Southern Brazil were tested: A03/09 (Veranópolis – RS), A11/09, A13/09 (both from Vacaria – RS) and A1033 (Caxias do Sul – RS).

2.3. In vitro antifungal activity of essential oils

2.3.1. Pathogen mycelial growth

Microorganism development and inhibition were evaluated under different essential oils concentration using PDA medium according to Feng and Zheng (2007) with minor modifications. Essential oils concentrations used were 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 10.0 μL mL^{-1}, with addition of Tween 20 (1:1), diluted on autoclaved and melted PDA (40 °C) under aseptic conditions. The mixed media were transferred onto 90 mm diameter Petri dishes (20 mL per plate). A mycelium disk of 8 mm diameter was cut from a seven-day-old culture of fungus grown on PDA and placed on the center of each Petri dish. Control received the same quantities of Tween 20 mixed with PDA. The experimental design was completely randomized, with 21 treatments per isolate and 10 replicates per treatment. Incubation was performed in growth chamber at 25 °C temperature and 12-h photoperiod, during 14 days. Diameter of mycelial growth of fungus was recorded on the 3rd and 7th day. Mean growth values were converted into percentage of mycelial inhibition using the formula: Inhibition percentage = [(dc − dt)/dc] × 100, where dc (mm) and dt (mm) represent the mean colony diameters of control and treated groups, respectively. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of oil that inhibited mycelial growth completely. Half maximal inhibitory concentration (IC_{50}) was defined by correlation of the growth percentage at the 7th day of evaluation.

2.3.2. Transfer experiments

Transfer experiments were performed to provide a distinction between essential oils fungistatic and fungicidal effect on target microorganism. Plugs that did not grow were transferred to fresh PDA dishes to assess their viability and growth after seven and fourteen days of incubation at 25 °C. Residual fungal growth was monitored by measurements of orthogonal diameter of fungus development.

2.3.3. Pathogen spore germination assay

A modified protocol based on Feng and Zheng (2007) was developed and spore germination assay was performed in potato dextrose broth (PDB). Essential oils were added to Tween 20 (1:1) at concentrations that showed significant results on mycelial growth assays, being diluted in 5 mL PDB and poured in 10 mL glass tubes. At the same time, 100 μL aliquots of pathogen spore suspension (10^6 spores mL^{-1}) of *A. solani* were added to each tube. Evaluation was made by observation of 100 conidia per replicate in optical microscope to determine the germination rate, after 20 h of incubation at 28 °C on a rotary shaker. Experiments were performed three times.

2.4. In vivo antifungal activity of essential oils

2.4.1. Inoculum preparation

Inoculum preparation was based on a modified protocol developed by Marchi et al. (2006). After 14 days of fungal growth, mycelium was collected by flooding three plates with sterile distilled water and brushing the agar surface with a paint-brush. Washed plates remained open and incubation was performed in growth chamber at 25 °C temperature and 12-h photoperiod, during 72–96 h. After this period, spore suspension was obtained by adding 10 mL of sterile distilled water into the plates and brushing the surface with a paint-brush. Spore density obtained was 10^6 spores mL^{-1} and counted using a hemocytometer.

2.4.2. Pathogenicity test

Experiment was carried out in a greenhouse of the Institute of Biotechnology, University of Caxias do Sul, according to protocol adapted from Tolentino Júnior et al. (2011). Tomato seeds (*S. lycopersicum* cv. UC-82–Isla Sementes) were sown in styrofoam trays with 128 cells, filled with autoclaved commercial substrate for seedlings (Plantmax®), which were kept inside the greenhouse, receiving daily irrigation to keep the substrate moist. Six-weeks-old seedlings were transplanted to 8L pots filled with substrate analyzed by the Laboratory of Chemistry and Soil Fertility, University of Caxias do Sul, and corrections were made for acidity and fertility. Pots were placed directly on the ground, forming twelve lines, each one composed of 10 pots, giving a total of 120 pots. Inoculation of the isolates was performed 25 days after transplantation (dat) by spraying the spore