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Antifungal activity of essential oils associated with carboxymethylcellulose against *Colletotrichum acutatum* in strawberries

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ABSTRACT

The antifungal activity of the essential oils (EOs) of *Eucalyptus staigeriana, Lippia sidoides* and *Pimenta pseudocaryophyllus* was evaluated *in vitro*, by direct contact and by exposure to volatiles, against *Colletotrichum acutatum*, an important pathogen of strawberry. The chemical composition of the EO with the highest activity and its effects on the morphology of the pathogen were verified. The *in vivo* antifungal activity of this EO associated with carboxymethylcellulose (CMC) coating, in preventive and curative applications, was also evaluated. *L. sidoides* EO presented the highest antifungal activity *in vitro*, being more efficient by direct contact than by volatilization. This EO has a predominance of the compound thymol and was able to cause dehydration and rupture of the pathogen hyphae. *In vivo*, strawberries treated with CMC associated with *L. sidoides* EO presented a reduction in disease severity, when treated in a curative way. Thus, the association of *L. sidoides* EO with CMC can be a potential alternative for the control of this disease.

1. Introduction

Strawberry, a fruit appreciated because of its pleasant flavor and aroma, represents a valuable source of bioactive compounds. Nevertheless, these properties can be undermined since this fruit is susceptible to mechanical damages, desiccation, physiological disorders and, especially, the degradation by fungal diseases, limiting its commercialization and consumption (Ugolini et al., 2014; Mohammadi et al., 2015).

Anthracnose fruit rot, an important disease in strawberries, can be caused by several fungal species of the genus *Colletotrichum* (Freeman, 2008), with *C. acutatum* being one of the most prejudicial in the economic standpoint (Zhang et al., 2016). The application of synthetic fungicides, which represents 15% of the total operational costs, and the use of less susceptible cultivars, are important tools for the control of this disease (Legard et al., 2005; Forcelini et al., 2017). Nevertheless, the use of these fungicides can bring potential risks to consumers and the environment and contribute to the selection of resistant pathogens. Thus, there is a strong need of alternative strategies for the control of this disease and the extension of strawberry postharvest shelf life (Aguado et al., 2012; Romanazzi et al., 2016).

In this sense, the essential oils (EOs) are alternatives to extend fruit shelf life, due to their antimicrobial activity and low risk for the development of pathogen resistance, because of their complex composition and their different mechanisms of action (Rehman et al., 2016). The use of EOs in fruits represents a challenge, since their components can interact with fruit constituents, besides the occurrence of losses of their active compounds by rapid volatilization or the action of other factors, such as light, which reduces or impairs its application (Turek and Stintzing, 2013).

The incorporation of EOs in edible coating, for the protection of their compounds and controlled release, has been indicated as an efficient strategy. The carboxymethylcellulose (CMC), one of the most common cellulose derivatives (Embuscado and Huber, 2009), can be used in association with the EO, acting as a selective barrier for the transfer of gas and moist, in the reduction of microbial growth and in

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the decrease of the intense aroma of the EOs, thus preserving its quality with shelf life extension (Vieira et al., 2016). CMC can be originated from abundant and low-cost sources, such as sugarcane bagasse, one of the major by-products of sugar cane industry. Some studies have shown the association of EOs with edible coating, like chitosan, as an alternative for increasing the shelf life of strawberries (Perdones et al., 2016; Badawy et al., 2017). However, few studies have been done for this purpose combining EOs with edible coatings less expensive and from abundant and affordable sources, such as CMC is.

Some studies have revealed the antifungal power of EOs on *C. acutatum* (Elshafie et al., 2016a,b), However, studies regarding the antifungal activity of the EOs extracted from leaves of the species *Eucalyptus staigeriana*, *Lippia sidoides* and *Pimenta pseudocaryophyllus* on fruit-deteriorating fungi in the postharvest period are scarce, despite the proven antimicrobial action of these oils (Yokomizo and Nakaoka-Sakita, 2014; Herculano et al., 2015). Therefore, this study evaluated the *in vitro* antifungal activity of these EOs on *C. acutatum* by different methods (direct contact and exposure to volatiles), determining the EO with the highest activity. The chemical composition and the effects on pathogen morphology were also observed for the EO with the highest activity. Still, the effect of the association of the EO with CMC, considering the preventive and curative application, was evaluated *in vivo*.

2. Materials and methods

2.1. Plant material and extraction of the essential oils

The EOs were extracted from leaves of *E. staigeriana* (Itatinga - SP, Brazil), *L. sidoides* (Campinas - SP, Brazil) and *P. pseudocaryophyllus* (Cananéia - SP, Brazil), by hydrodistillation, for 4 h, in a Clevenger equipment at up to 100 °C. The EO was dehydrated in anhydrous sodium sulfate and stored at −5 °C. The yield of each EO was calculated by the formula *R* (%) = [(*EO volume* x *density*) /*dry mass of the leaves*] x 100 (Girard et al., 2017).

2.2. Isolation and Molecular identification of C. acutatum

C. acutatum was obtained by direct isolation of fungal structures present on strawberries, from a conventional grower in Jarinu (SP, Brazil), with typical symptoms of anthracnose fruit rot. Genomic DNA of the isolate was extracted using the Kit FastDNA®MP Biomedicals, following the procedures recommended by the manufacturer. Polymerase chain reaction (PCR) was used to confirm the identity of the species complex using the primers ITS4-Universal (5′-TCCTCCGCTTA TTGATATGC-3′) and Cacut-Int2 (5′-GGGGAAGCCTCTCGCGG-3′). Water was used as negative control and a previously identified isolate of the species complex *C. acutatum* was used as positive control.

2.3. Determination of EO antifungal activity in vitro

2.3.1. Method by contact

EO antifungal activity was initially evaluated by measuring *C. acutatum* growth inhibition by the direct contact of the fungus with potato dextrose agar (PDA) culture medium either containing the individual EO or with its binary and ternary mixtures, at concentrations of 31; 62.5; 125; 250 and 500 µl/L (Plaza et al., 2004). The mixtures M1 (*L. sidoides* + *E. staigeriana)*; M2 (*P. pseudocaryophyllus* + *E. staigeriana);* M3 (*L. sidoides* + *P. pseudocaryophyllus)* and M4 (*L. sidoides* + *P. pseudocaryophyllus* + *E. staigeriana)* were tested to evaluate if the combination of EOs could present a higher activity on the control of the pathogen than when evaluated individually. For the homogenization of the EOs and the mixtures to the PDA medium, the emulsifier soy lecithin (0.2% w/v in ethanol) was used. A control treatment, containing only the emulsifier and the culture medium, was also installed.

After solidification of the PDA medium, *C. acutatum* was transferred to the central point of the Petri dish, from an inoculum suspension containing 10^5 spores mL^{-1}. Plates were maintained in growth chambers at 25 °C with photoperiod of 12 h, and mycelial growth measurements of each colony were taken every two days, in two perpendicular directions (diameter in cm). Mycelial growth inhibition at the different concentrations of the individual EOs and of the mixtures was measured by the formula *PI* (%) = (*Growth of the Control - Growth of the Treatment/ Control Growth*) x 100 (Plaza et al., 2004). The Minimum Inhibitory Concentration (MIC), when present, was considered as the lowest concentration of the treatment, among the concentrations evaluated, capable of completely inhibiting the development of *C. acutatum* visible at naked eye.

2.3.2. Method by exposure to volatiles

The treatment that presented the highest antifungal activity *in vitro* by the method of contact was also evaluated by the method of exposure to volatiles, according to (Yun et al., 2013). The inhibition of fungal growth was observed at concentrations of the EOs and their mixtures (0; 31; 62.5; 125; 250 and 500 µl/L), which were emulsified in tween-80 (at the proportion 2:1 v/v) and applied on a circle of filter paper (20 mm^2), fixed in the center of the inner part of a Petri dish that contained solidified PDA. Pathogen inoculation, incubation, mycelial growth measurement and determination of the MIC, were the same as described in the method by contact (item 2.3.1).

In the methods *in vitro*, the experimental design was randomized in factorial scheme, 8 × 5 with eight treatments (Control; *L. sidoides*; *E. staigeriana; P. pseudocaryophyllus* and the binary and ternary mixtures), and five concentrations for the contact method, and in a 2 × 5 factorial scheme, with two treatments (Control; *L. sidoides*) and five concentrations, for the method of exposure to the volatiles. Both experiments had five repetitions per treatment and were performed three times.Data from repeated experiments were combined after tests of homogeneity indicated that variances were homogeneous.

2.3.3. Effects of the essential oil on C. acutatum morphology

The damages caused by the EO with the highest antifungal activity on the pathogen morphology was evaluated by Scanning Electron Microscopy (SEM) according to (Yu et al., 2015) with modifications. For this, 150 mL of potato dextrose broth (PDB) with the addition of 1 mL of a suspension of pathogen spores (10^6 spores mL^{-1}) was incubated for two days at 25 °C and under a photoperiod of 12 h. After this period, the volume of EO corresponding to the MIC determined in the experiment *in vitro* by the method of direct contact in PDA was emulsified with soy lecithin and added to the broth. Subsequently, the incubation followed for additional 6 h at the same conditions described. A treatment with potato broth without the addition of the EO was used as control. The samples were processed according to Escanferla et al. (2009) and the observations were performed in a Scanning Electron Microscope LEO 435 (Zeiss, England). This analysis was repeated twice, with three repetitions.

2.4. Evaluation of the chemical composition of the essential oil

The chemical composition was determined only for the EO that presented the best result in the *in vitro* test. The characterization was performed by gas chromatography coupled to mass spectrometry, using the equipment CGMS 2010 (SHIMADZU) and capillary-column gas chromatography diphenyl dimethylpolysiloxane (5% diphenyl and 95% dimethylpolysiloxane). Reactions were performed at 50 °C for 1.5 min, then elevated at 4 °C min^{-1} until 200 °C, followed by 10 °C min^{-1} until 240 °C, remaining at 240 °C for 7 min. The injector temperatures were set at 240 and 220 °C for the ions and interface sources, respectively. Injection was done on “Split” mode: 1 µL of EO was injected and the “Split” ratio was 1:20. Helium gas was used as the drag gas at 1.2 mL min^{-1}. The mass detector was run on scan mode with scanning range of 40–500 m/z. The volatile compounds were identified by the