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Bioactivity of essential oils in the control of *Alternaria alternata* in dragon fruit (*Hylocereus undatus* Haw.)

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ABSTRACT

This study isolated and identified the fungus that causes postharvest disease in dragon fruit (*Hylocereus undatus* Haw.). The *in vitro* and *in vivo* antifungal activity of some essential oils were evaluated against the fungus. Morphophysiological and molecular identification confirmed the fungus was *Alternaria alternata*. The essential oils of *Cinnamomum zeylanicum*, *Cymbopogon flexuosus*, *Eucalyptus globulus*, *Eugenia caryophyllus*, and *Rosmarinus officinalis* were evaluated by the microdilution broth technique, disc diffusion, scanning electron microscopy, and fluorescence microscopy. Evaluation of the composition of the essential oils by gas chromatography/mass spectrometry revealed substantial amounts of eugenol as a major constituent of *E. caryophyllus* and *C. zeylanicum* (90.50% and 80.70%, respectively). The other essential oils of *R. officinalis* contained α-pinene (24.5%) and camphor (22.0%) as major components. *E. globulus* contained 1,8-cineole (78.9%). *C. flexuosus* contained neral (35.1%) and geranial (42.6%). *C. zeylanicum* and *E. caryophyllus* were the most active against the isolated fungi at minimum inhibitory concentrations of 250 and 500 μg/ml, respectively, causing morphological changes in the hyphae. The *in vivo* assay indicated that the fruits that were treated with *E. caryophyllus* at concentrations of 500 and 1000 μg/ml exhibited a 31% reduction of mycelial growth compared with the control. These results suggest that the essential oils of *C. zeylanicum* and *E. caryophyllus* are active against *A. alternata* both *in vitro* and *in vivo*, which may be promising for control of the microorganism.

1. Introduction

Hylocereus undatus (Haw.), known as dragon fruit, belongs to the Cactaceae family that originated in the Americas (Ortiz-Hernández et al., 1999; Brunini and Cardoso, 2011). The fruits of the cactaceae family in tropical and subtropical regions has a high nutritional content (Jaafar et al., 2009; Zhuang et al., 2012), an exotic form, and an attractive color, thus generating great interest by the food industry (Le Bellec and Vaillant, 2011). However, its postharvest period is relatively short because it deteriorates rapidly under environmental conditions through exposure, storage conditions, and the physiology of the fruit.

Diseases that are caused by microorganisms lead to large postharvest losses. Fruit can be attacked by microorganisms, such as fungi, during both the production and postharvest stages, resulting in black spots, necrosis, rot, and deterioration, making the fruit unsuitable for consumption and resulting in disposal (Ortiz-Hernández and Carrilo-Salazar, 2012).

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The *Alternaria* genus is a spoilage microorganism. *Alternaria* spp. is a latent fungus that develops during the cold storage of fruit. It becomes visible during the commercialization period (Troncoso-Rojas and Tiznado-Hernández, 2014) and is associated with the deterioration of fruit (Ostry, 2008). More than 100 plant species have been reported to be affected by *Alternaria* spp. (Armitage et al., 2015).

To effectively treat fruit and control microorganisms, natural compounds are receiving increasing attention (Moghaddam et al., 2015). Essential oils (EOs) are natural volatile substances with a complex of compounds (Li et al., 2015). Such compounds are synthesized in various parts of the plant, such as the leaves, flowers, seeds, fruits, and roots (Bakkali et al., 2008; Texeira et al., 2013). They are widely used in various fields, including the cosmetics, pharmaceutical, and food industries (Harkat-Madouri et al., 2015). The constituents of these compounds have been shown to have antibacterial, virucidal, fungicidal, antiparasitic, insecticidal, and medicinal activity that is considered protective for plants (Bakkali et al., 2008).

Although studies have reported the presence of *Alternaria* spp. in various fruits, little information is available on the presence of *Alternaria* spp. in dragon fruit and its relationship with postharvest disease. The objective of the present study was to isolate and identify the fungus that is responsible for the contamination of *H. undatus* (Haw.) during refrigerated storage for 25–30 days and evaluate the *in vitro* and *in vivo* antifungal activity of EOs (*Cinnamomum zeylanicum*, *Cymbopogon flexuosus*, *Eucalyptus globulus*, *Eugenia caryophyllus*, and *Rosmarinus officinalis*) against *A. alternata*.

2. Material and methods

2.1. Pathogen isolation

Hylocereus undatus (Haw.) fruits were collected in Marialva, Paraná (coordinates: 23°46′35.51″S, 51°79′71.10″W), selected, washed, and sanitized with 1% sodium hypochlorite. The microorganism was isolated from the fruits by incubation for 25–30 days at 8 °C until rot had clearly appeared in the epidermis, with typical symptoms of the *Alternaria* spp. fungus. Tissue of the epidermis (1 cm × 1 cm) that contained the microorganism was inoculated in potato dextrose agar (PDA) and incubated at 28 °C for 7 days to allow identification according to Carvalho et al. (2011), with slight modifications.

2.2. Morphological characterization and molecular identification of isolated species based on sequencing of ITS1-5.8S-ITS2 region

Macroscopic characterization was performed with microorganisms that were grown in solid PDA medium and analyzed with regard to the appearance of colonies, shape of the mycelium, color, and growth time. Microscopic characterization was performed using the micro-cultivation technique (Ribeiro and Soares, 2005). The isolates were viewed under an Olympus fluorescent microscope and photographed with an image capture system. The isolates were identified morphophysiologically (Hoog et al., 2000). The results of the macroscopic and microscopic evaluations were analyzed using identification keys.

The genomic DNA of the isolated fungus was prepared and extracted using an extraction buffer (Pamphile and Azevedo, 2002) and stored at −20 °C. The integrity of DNA was verified using 1% agarose gel and photographed. Amplification of the ITS1-5,8S-ITS2 region of rRNA was performed by polymerase chain reaction (PCR) using the following primers: ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCCCGCTTATTGATATGC-3′; White et al., 1990) according to previously described methodology (Rhoden et al., 2012).

The PCR product was purified using shrimp alkaline phosphatase and exonucleose I enzymes. The rRNA sequence was obtained using an ABI-Prism 3500 Genetic Analyzer (Applied Biosystems). The results were analyzed using BioEdit 7.2.5 software. The isolated nucleotide sequences were identified by comparisons with those in the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov) database using the nBLAST sequence filter type tool (type strains). The sequences were aligned using MEGA 6.05 software and the "neighbor-joining" method, with "p-distance" for nucleotides with the "pairwise gap deletion" option and bootstrap with 10,000 replications to construct the phylogenetic tree.

2.3. Essential oil

Essential oils (EOs) of cinnamon (*Cinnamomum zeylanicum*), lemon grass (*Cymbopogon flexuosus*), eucalyptus (*Eucalyptus globulus*), clove (*Eugenia caryophyllus*; Bio Essência, Jaú, São Paulo, Brazil), and rosemary (*Rosmarinus officinalis*; By Samia, São Paulo, Brazil) were obtained in Maringá, Paraná, Brazil.

2.3.1. Characterization of the essential oils

The detailed chemical composition of the EOs was performed using gas chromatography-mass spectrometry (GC–MS) with an automatic injector (FOCUS GC – DSQ II, Thermo Electron Corp). The gas chromatograph-mass spectrometer was equipped with an Agilent DB-5 capillary column (5% phenyl/95% dimethyl siloxane stationary phase, 30 m length, 0.25 mm internal diameter, and 0.1 μm film thickness). Characterization was performed using a column temperature program that began at 70 °C, followed by a temperature increase of 3 °C/min to 230 °C. Helium was used as the carrier gas at a flow rate of 1.0 ml/min (Kim et al., 2015). The total analysis time was 53 min. The temperature of the injector and detector was maintained at 250 °C. A 1 μl volume of the samples was injected for chromatography in split mode (1:10). Each EO was diluted in hexane (high-performance liquid chromatography grade; 0.2 μl of EO to 1000 μl of hexane) to form the stock solution. Prior analyses indicated the necessity of diluting each solution again for injection of the sample in the chromatograph. The stock solution of EOs of *C. flexuosus*, *E. globulus*, *E. caryophyllus*, and *R. officinalis* was diluted in hexane (1/10 [v/v]). *C. zeylanicum* was diluted in hexane (1/20 [v/v]; Falasca et al., 2016; with modifications).

Characterization was performed based on retention time (RT) and compared with the major compounds using Kovats retention rate (Skoog et al., 2006). The compounds of the EOs were identified by analyzing the retention times of the peaks that were obtained for each EO and confirmed via a standard mixture of *n*-alkanes (C_8–C_{20}; Sigma-Aldrich). The compounds of interest were confirmed (Adams, 2007) and are presented as percentages.

2.4. Antifungal activity

The minimum inhibitory concentrations (MICs) of the EOs and eugenol were determined by the microdilution method (Clinical and Laboratory Standards Institute, 2008) with a conidia suspension of 5×10^4 conidia/ml using RPMI 1640 medium and L-glutamine without bicarbonate, buffered with 0.165 M morpholine propane sulfonic acid. Conidia were collected in sterile saline solution after 7 days of incubation. The EOs and isolated compounds were diluted in 1% Tween-80 and tested at concentrations of 62.5–4000 μg/ml. The microplates were incubated at 28 °C for 72 h. The MIC was defined as the lowest concentration of the EO that inhibited the visual growth of the fungus.