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Antifungal effect of poly(lactic acid) films containing thymol and *R*-(-)-carvone against anthracnose pathogens isolated from avocado and citrus

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ABSTRACT

Biodegradable antifungal films were developed to be used for controlling postharvest anthracnose pathogens. Two antifungal compounds, thymol and *R*-(-)-carvone, were incorporated into poly(lactic acid) (PLA)-based polymer at 10, 15 and 20% (w/w). Antifungal activity of the pure compounds and the antifungal films against *Colletotrichum gloeosporioides* isolated from avocado and citrus was evaluated at 12 and 25 °C using vapor diffusion assays. The results indicated that the colony diameter was affected by the vapor concentration of the antifungal compounds in the headspace. At 12 °C, 20% thymol showed complete growth reduction of avocado isolate, while at 25 °C, 15 and 20% thymol showed complete growth reduction of both avocado and citrus isolates. The PLA films incorporated with 15% *R*-(-)-carvone and 20% thymol were the most effective at 12 °C in suppressing the mycelial growth of the avocado and citrus *C. gloeosporioides* isolates, respectively, whereas the film incorporated with 20% thymol had the highest antifungal activity against both anthracnose isolates at 25 °C. The inhibitory effect of the antifungal films against anthracnose isolates was correlated to the vapor concentration of the antifungal compounds remaining in the headspace of the Petri dish. Antifungal packaging films can potentially be used to control postharvest pathogens of fresh produce.

1. Introduction

Anthracnose, a fungal disease caused by *Colletotrichum gloeosporioides*, is the main postharvest disease of many tropical and subtropical fruit such as mango, papaya, banana, avocado, and citrus. The potential loss of produce due to this pathogen is significant. For example, it was reported that this disease can cause postharvest losses of avocado fruit up to 80% (Bill, Sivakumar, Korsten, & Thompson, 2014; Kuru, Adugna, & Berecha, 2016). The initial infection usually occurs at the preharvest stage when the fungal spores or mycelia infect young fruit through pedicel and peel wounds. Normally, preharvest infection remains latent or hidden until the fruit undergoes ripening (Dinh, Chongwungse, Pongam, & Sangchote, 2003), which offers appropriate conditions and nutrients for fungal growth, and symptoms appear. Although controlled atmosphere or modified atmosphere packaging (MAP) could delay ripening and prolong the shelf-life of many fruit, anthracnose decay was observed at the end of storage after the fruit ripened (Mpho, Sivakumar, Sellamuthu, & Bautista-Baños, 2013; Sellamuthu, Mafune, Sivakumar, & Soundy, 2013a). The combination of antifungal films with MAP could be an effective method to maintain the quality of many tropical and subtropical fruit which are susceptible to fungal decay.

There is increasing concern associated with the use of synthetic fungicides postharvest (Mastromatteo, Conte, & Del Nobile, 2010; Mpho et al., 2013). As a result, natural substances have been investigated for their antifungal activity to be used as alternatives to synthetic chemicals for both preharvest and postharvest pathogen control. Numerous studies reported the antifungal activity of many essential oils from different plant extracts against postharvest pathogens. Combrinck, Regnier, and Kamatou (2011) reported that thymol followed by *R*-(-)-carvone possessed the highest antifungal

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activity against anthracnose isolated from mango and avocado. Thyme oil vapor suppressed mycelial growth of *C. gloeosporioides* isolated from avocado *in vitro* and *in vivo* (Sellamuthu, Sivakumar, Soundy, & Korsten, 2013b). In addition, it was reported that thymol fumigation could control brown rot of apricot (Liu & Chu, 2002) and gray mold of sweet cherries (Chu, Liu, Zhou, & Tsao, 1999). Furthermore, incorporation of thyme oil in an edible coating controlled two postharvest papaya diseases, *C. gloeosporioides* and *Rhizopus stolonifer* (Bosquez-Molina, Ronquillo-de Jesús, Bautista-Baños, Verde-Calvo, & Morales-López, 2010). Regnier, Du Plooy, Combrinck, and Botha (2008) reported that *R*-(-)-carvone, which is the major active component in spearmint oil, has antifungal activity against two mango postharvest spoilage pathogens, *C. gloeosporioides* and *Botryosphaeria parva*. In addition, *R*-(-)-carvone was the most effective volatile component of *Lippia scaberrima* essential oil in inhibiting the mycelial growth of *C. gloeosporioides*, *Lasiodiplodia theobromae*, and an *Alternaria* sp. isolated from avocado fruit (Regnier, Combrinck, Du Plooy, & Botha, 2010) and *Penicillium digitatum* isolated from oranges (Du Plooy, Regnier, & Combrinck, 2009). Essential oils rich in *R*-(-)-carvone, incorporated as fruit coating supplements, have also proved to be effective in controlling fungal diseases of mango (Regnier et al., 2008), avocado (Regnier et al., 2010), and citrus (Du Plooy et al., 2009).

Development of active packaging including antimicrobial films has gained increasing interest in the past decade. Natural antimicrobial substances have been incorporated into packaging materials and several types of polymers have been tested (Muriel-Galet et al., 2012; Ramos, Jiménez, Peltzer, & Garrigós, 2012). Poly(lactic acid) or PLA, which is a renewable and biodegradable aliphatic polyester has received considerable attention for antimicrobial packaging applications (Del Nobile et al., 2009; Liu et al., 2007; Tawakkal, Cran, Miltz, & Bigger, 2014). Recently, Wu et al. (2014) developed antimicrobially active PLA/poly(ε-caprolactone) (PCL) blend films incorporated with thymol using a solvent-casting method. The films showed antimicrobial activity against *Escherichia coli* and *Listeria monocytogenes*. Erdohan, Çam, and Turhan (2013) reported that olive leaf extract incorporated in PLA films could reduce the growth of *Staphylococcus aureus*. Liu et al. (2007) reported that an extruded PLA and pectin composite loaded with nisin suppressed the growth of *Lactobacillus plantarum*.

According to the literature, most researchers studied the activity of antimicrobial or antifungal materials against food-spoilage microorganisms. Only a few studies have investigated the antifungal activity of natural substances against postharvest diseases, while very few studies have investigated incorporation of these substances into packaging materials using a film extrusion process for postharvest decay control. The primary objectives of this study were to develop biodegradable antifungal films containing various concentrations of two natural, volatile compounds—thymol and *R*-(-)-carvone. The antifungal activities of the films were analyzed against anthracnose pathogens isolated from avocado and citrus at 12 and 25 °C using vapor diffusion assays.

2. Materials and methods

2.1. Materials

PLA 4043D (industrial biaxial grade) was purchased from NatureWork LLC (Minnetonka, MN, USA). The two antifungal, volatile compounds used in this study were $\geq$ 99% FCC grade thymol (solid phase, crystal) and 98% FCC grade *R*-(-)-carvone (oil phase), purchased from Sigma-Aldrich (St Louis, USA) for incorporating into the films and Fisher Scientific (Waltham, USA) for studying the antifungal activity of the compounds using filter paper discs.

2.2. Film preparation

Thymol and *R*-(-)-carvone were incorporated into PLA resins to make the antifungal sheets containing 20, 15 and 10% (w/w) thymol and *R*-(-)-carvone using a single screw extruder (Thermo Scientific™ HAAKE Rheomex for the HAAKE PolyLab OS torque rheometer platform, Karlsruhe, Germany). The PLA sheets were then simultaneously biaxially stretched at 3.5 × 3.5 drawing ratios to convert into antifungal films using film stretcher KARO IV (Bruckner Maschinenbau GmbH, Siegsdorf, Germany).

2.3. Remaining contents

The remaining contents of thymol and *R*-(-)-carvone incorporated into the PLA polymer resins after film processing were determined by thermogravimetric analysis (TGA). The TGA results can give an indirect confirmation of the presence of additives in the polymer matrix after processing (Ramos et al., 2012). TGA data were obtained using a Mettler Toledo, TGA/SDTA 851^e (Columbus, OH, USA). Samples of approximately 10 mg from the sheets and 5 mg from the films were heated at 20 °C min^{-1} from 30 to 900 °C, under a dry N_2 gas flow rate of 60 mL min^{-1}. The data were calculated from the percentage of the additives remaining in the PLA films after processing to mg g^{-1}.

2.4. Pathogens

Subcultures (4–7 d old) of *C. gloeosporioides* isolated from avocado and citrus (obtained from the Department of Plant Pathology and Microbiology at the University of California, Riverside) were used.

2.5. Determination of antifungal activity of thymol and *R*-(-)-carvone

The efficacy of the pure volatile compounds to suppress mycelial growth of each fungal isolate was determined using vapor diffusion assays according to Lopez et al. (2005) with slight modification. The Petri dishes containing 15 mL of potato dextrose agar (PDA) with a headspace volume of 40 cm^3 were inoculated with 5 mm mycelial plugs from the margin of a culture which contained actively growing mycelia. Thymol and *R*-(-)-carvone were diluted using methanol to 5, 10, 15 and 20% (g mL^{-1}), respectively. Then 5 μL of the solutions were added to a 5 mm diameter filter paper disc attached at the center position of the inside cover of a 90 mm diameter Petri dish. The filter discs containing 5 μL of methanol were used as the control. The Petri dishes were sealed with Parafilm and stored at 12 and 25 °C. The vapor concentrations generated by each compound were determined using solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS). The SPME fiber assembly with the phase divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (Supelco, Bellefonte, PA, USA) was introduced into the Petri dish. After 5 min the SPME fiber was removed from the sample Petri dish and immediately inserted into the injection port of a gas chromatograph using a SPME liner, where the thermal desorption occurred at 250 °C. Volatile analysis was performed using an Agilent 6890 gas chromatograph (GC; Agilent, Santa Clara, CA, USA), coupled with a 5975B mass spectrometer detector, and equipped with a HP-5MS column (30 m × 0.25 mm, film thickness 0.25 μm). The SPME fiber was injected into the GC inlet at 250 °C manually. The initial oven temperature was set at 80 °C for 2 min, followed by an increase to 170 °C at a rate of 10 °C min^{-1}, and finally increased to 250 °C at a rate of 60 °C min^{-1} (held for 3 min). The helium flow was set at 1 mL min^{-1}. Standard curves of thymol and *R*-(-)-carvone