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Essential oil vapours suppress the development of anthracnose and enhance defence related and antioxidant enzyme activities in avocado fruit

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ABSTRACT

Anthracnose caused by *Colletotrichum gloeosporioides* is a major postharvest disease in avocados that causes significant losses during transportation and storage. Complete inhibition of the radial mycelia growth of *C. gloeosporioides in vitro* was observed with citronella or peppermint oils at 8 μL plate^{-1} and thyme oil at 5 μL plate^{-1}. Thyme oil at 66.7 μL L^{-1} significantly reduced anthracnose from 100% (untreated control) to 8.3% after 4 days, and to 13.9% after 6 days in artificially wounded and inoculated 'Fuerte' and 'Hass' fruit with *C. gloeosporioides*. GC/MS analysis revealed thymol (53.19% RA), menthol (41.62% RA) and citronellal (23.54% RA) as the dominant compounds in thyme, peppermint and citronella oils respectively. The activities of defence enzymes including chitinase, 1, 3-β-glucanase, phenylalanine ammonia-lyase and peroxidase were enhanced by thyme oil (66.7 μL L^{-1}) treatment and the level of total phenolics in thyme oil treated fruit was higher than that in untreated (control) fruit. In addition, the thyme oil (66.7 μL L^{-1}) treatment enhanced the antioxidant enzymes such as superoxide dismutase and catalase. These observations suggest that the effects of thyme oil on anthracnose in the avocado fruit are due to the elicitation of biochemical defence responses in the fruit and inducing the activities of antioxidant enzymes. Thus postharvest thyme oil treatment has positive effects on reducing anthracnose in avocados.

1. Introduction

Anthracnose caused by *Colletotrichum gloeosporioides Penz. & Sacc. In Penz.* (Sanders and Korsten, 2003) is a common postharvest disease that affects the shelf-life, fruit quality and marketability of avocado (*Persea americana*). Synthetic fungicide prochloraz is commonly used for control of postharvest decay due to quiescent infection in South Africa (Nell et al., 2003). Due to global concern over the often indiscriminate use of pesticides and their hazardous side effects on environment and human health, more stringent product registration requirements have been developed. Furthermore, due to the emergence of fungicide resistant strains, postharvest fungicide application is often not considered a long term solution for the industry (Ippolito and Nigro, 2000). Prochloraz solution for postharvest dip treatment is prepared in large tanks (~1500 L volume) and maintained for several days before the residue is disposed. Toxic waste disposal is a costly exercise and hazardous waste poses serious environmental problems. Due to the increasing consumer demand for organically produced fruit, fruit industries are in search of natural environmental friendly alternative fungicides to control postharvest diseases.

Antifungal activity of essential oil is well documented and proven to inhibit the fungal growth of *C. gloeosporioides in vitro* or *in vivo* in avocados (Regnier et al., 2010). Essential oils and their components are gaining increasing interest due to their volatility, relatively safe status, and wide acceptance by consumers, and their eco-friendly and biodegradable properties (Tzortzakis and Economakis, 2007). Regnier et al. (2010) recommended a combination of essential oils (*Lippia scaberrima* Sond., rich in R-(−)-carvone, (d)-limonene and 1,8-cineole) with a commercial coating (Avoshine®) as an acceptable postharvest treatment to the organic market to control anthracnose in avocados. However, the organic niche markets especially in the developed countries prefer fruit that is free from fruit coatings. On the other hand, the application of essential oil in vapour phase is preferred to liquid phase application due to its volatility, which leads to more activity, a need for lower concentrations and absence of effect on the sensory properties of foodstuffs (Laird and Phillips, 2011).

There is little information available regarding the effect of essential oils on induced defence related enzymes (chitinase,

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1, 3-β-glucanase, phenylalanine ammonia-lyase (PAL) activity, peroxidase (POD), antifungal compound (phenols) and their influence on reactive oxygen species (ROS) metabolism (superoxide, hydrogen peroxide [H_2O_2] and hydroxyl radical) (Wang et al., 2008; Jin et al., 2012a) and the enzymes catalyzing the ROS scavenging activity. Superoxide dismutaste (SOD) catalyses the dismutation of O_2^- to H_2O_2, catalase (CAT) dismutates H_2O_2 to oxygen and water, and peroxidase (POD) decomposes H_2O_2 by oxidation of phenolic compounds (Wang et al., 2008; Jin et al., 2012a). These enzymes are considered to be the main antioxidant enzymatic systems for protecting cells against oxidative damage. Therefore, in order to develop a suitable application of essential oil treatment in vapour phase, the effects of essential oil in vapour phase on decay control, defence and the array of antioxidant enzymes need to be investigated. The objectives of our investigation were to evaluate the effects of selected essential oils on (1) the control of *C. gloeosporioides* in avocado fruit (cvs. Hass and Fuerte) by identifying the antifungal activities of *in vitro* and *in vivo*, and (2) to determine the induction of defence related enzymes chitinase, 1, 3-β-glucanase, PAL, POD, and antifungal phenol compounds, and antioxidant enzymes catalase and superoxide dismutase.

2. Materials and methods

2.1. Essential oils

The three essential oils citronella (*Cymbopogen nardus*), peppermint (*Mentha piperita* L.), and thyme (*Thymus vulgaris* L.), were obtained from Burgess and Finch (Vital Health Foods S.A. Distributor, Kuils River, South Africa); Dis-Chem (Pty) Ltd. Randburg, South Africa and stored at 4 °C.

2.2. GC–MS analysis of essential oils

The GC/MS analysis was carried out on an Agilent 7890A gas chromatograph equipped with split/split-less inlet in combination with an Agilent 5973N MSD. The HP-5MS column (30 m × 0.25 mm id × 0.25 μm) was used for the separation (Agilent part number 19091S-433) and helium was used as a carrier gas at a constant pressure of 65 kPa (9.43 psi). The essential oil (20%) was injected (1 μL) into the column with a split ratio of 25:1 and injector temperature was 250 °C. The temperature programme was 60–240 °C at 3 °C min^{-1} and total run time of 60 min for the separation of components. The mass spectra were taken at 70 eV, under positive electron impact ionization, with a mass range from 50 to 550 amu, solvent delay of 2 min and transfer line 300 °C. The technique used the retention locking (RTL) method using a standard compound n-pentadecane (Adams, 1998). Compound identification was confirmed by comparison of the mass spectra with NIST08 (National Institute of Standards and Technology 08) and also comparison with published data.

2.3. Pathogen

The avocado postharvest pathogen *C. gloeosporioides* was isolated from symptomatic infected fruit. The culture was maintained on Potato Dextrose Agar (PDA) at 25 °C. Fourteen days old culture was used for the preparation of spore suspension. The spores were separated from the media by flooding with sterile distilled water and gently rubbing with a sterile glass rod. The mycelial fragment was removed from suspension by filter through three layers of muslin cloth. The spore suspension was adjusted to 1×10^5 spore mL^{-1} with haemocytometer using sterile distilled water.

2.4. Antifungal activity of essential oils on mycelial growth of the pathogen

The essential oils (citronella, peppermint, and thyme) were tested for antifungal activity *in vitro* by adopting a disc volatilization method (Dafarera et al., 2000; Tzortzakis and Economakis, 2007) as briefly. The Petri plates (90 mm diameter) containing 15 mL of PDA medium were inoculated with 6 mm plugs from the margin of 7 days old culture. The sterilized Whatman filter paper disc (6 mm diameter) was placed in the middle of the lid and different concentration of oils from 1 μL $plate^{-1}$ to 8 μL $plate^{-1}$ was added to filter paper discs and a blank served as the control (Arrebola et al., 2010). The Petri dishes were sealed with parafilm and incubated for 7 days at 25 °C. The radial mycelia growth of *C. gloeosporioides* was measured with a Vernier calliper (Digimatic; Mitutoyo Co., Japan) in mm and expressed as percentage inhibition of radial mycelial growth. The assays were repeated twice with ten replicates.

2.5. Inoculation and measurements of disease progress

Freshly harvested, unblemished 'Fuerte' and 'Hass' avocado fruit were selected from Bassan Fruit Packers (Tzaneen, Limpopo Province, South Africa). Fruit at correct stage of maturity were selected according to finger feel firmness score 2 (1 = hard, 2 = slightly soft, just starting to ripen, 3 = very soft), surface sterilized by dipping in 70% ethanol for 1 min and air-dried. Thereafter, fruit were uniformly wounded (2 mm deep and 6 mm wide) with a sterilized cork-borer and inoculated with 20 μL of a spore suspension of *C. gloeosporioides* (10^5 spores mL^{-1}) at the equatorial region and left to air-dry. After inoculation, fruit were placed in 27.5 cm × 18.5 cm × 9 cm glass boxes. The essential oils were introduced into the glass boxes (90% RH) containing the inoculated fruit by placing the specific essential oil at a specific concentration in a Petri plate lids inside the glass boxes. The inoculated fruit were exposed to thyme oil (16.7 μL L^{-1}, 33.3 μL L^{-1} and 66.7 μL L^{-1}) or peppermint and citronella oils (26.7 μL L^{-1}, 53.33 μL L^{-1} and 106 μL L^{-1}). The commercial fungicide prochloraz treatment (0.05% for 5 min dip) was included for comparison. After introducing the essential oil to the inoculated fruit, glass boxes were sealed with lids and the experimental set up was placed at 20 °C. The experiment was repeated twice with 12 replicate fruit per specific essential oil at each concentration. Disease incidence and lesion diameter (mm) were recorded on days 4 and 6 after inoculation. The disease incidence was determined according to Xing et al. (2010) using the following equation

$$\text{Disease incidence}\,(\%) = \frac{\text{Number of infected wounds}}{\text{Total no of inoculated fruit}} \times 100$$

2.6. Measurements of active defence response-related enzymes, antioxidant enzymes activities and total phenolic content in avocado fruit

Fruit exposed to 66.7 μL L^{-1} thyme oil and 106 μL L^{-1} peppermint or citronella oil vapour treatment were subjected for PAL, β-1,3-glucanase, chitinase, POD, SOD, CAT and total phenolic content determination. Fruit tissue samples (1 g) from 15 fruit were collected from 2 mm away from the wound inoculated region. The samples were homogenized with specific buffers and centrifuged at 15,000 × *g* for 30 min at 4 °C and supernatants were used to determine the enzyme activities. Sodium phosphate buffer (100 mM, pH 7) was used for POD and CAT. Sodium phosphate buffer (100 mM, pH 7.8) was used for SOD. For chitinase and β-1,3-glucanase, the samples were extracted by 50 mM sodium acetate buffer (pH 5.0). Borate buffer (100 mM, pH 8.8) containing 5 mM β-mercaptoethanol and 2 mM EDTA was used for the PAL.