Essential oil vapors suppress the development of anthracnose and enhance defense 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. The 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 cv. Fuerte and Hass'fruit with *C. gloeosporioides*. The 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.

*Keywords*: Essential oil, postharvest disease, thymol, induced resistance, avocado, antioxidant enzymes

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## 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 (Nel 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, consequently 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, eco-friendly, and biodegradable properties (Tzortzakis, 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 than the liquid phase application; due to its volatility which leads to more activity, requires a low concentration and does not alter 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, 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) (Jin et al., 2012a; Wang et al., 2008) and the enzymes catalysing the ROS scavenging activity. The 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 (Jin et al., 2012a; Wang et al., 2008). 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 effect 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 the (1) control of *C. gloeosporioides* in avocado cultivars (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 compound phenol and (3) Antioxidant enzymes catalase and superoxide dismutase.

## 2. Materials and methods

### *2.1. Essential oils*

The three essential oils such as 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 executed 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 program was 60˚ C to 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 70ev, 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 was done by retention locking (RTL) method using a standard compound n-pentadecane (Adams, 1998). The compound identification was confirmed by comparison of the mass spectra with NIST08 (National Institute of Standards and Technology 08) and also comparison with those published in the literature.

*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 $1 \times 10^5$ spore/mL 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 volatilisation 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 avocado fruits of cvs. Fuerte and Hass 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-slighly soft just started 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 equatorial region and left to air dry. After inoculation, fruit were placed in 27.5 x 18.5 x 9 cm glass boxes. The essential oils were introduced into the glass boxes containing (90% RH) the inoculated fruit by placing the specific essential oil at a specific concentration in a Petri plates lid 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

commercially adopted 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 specific concentration. The 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 cultivars*

Fruit (cv. Fuerte and Hass) 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 buffer 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.

PAL activity was determined according to Assis et al. (2001), with slight modification. The enzyme extract (75 µL) was incubated with 150 µL of borate buffer (50 mM, pH 8.8) containing 20 mM L-phenylalanine for 60 min at 37° C. After incubation time, the reaction was stopped by adding 75 µL of 1 M HCl and the production of cinnamate was measured at 290 nm (Zenyth 200rt Microplate Reader UK-Biochrom Ltd). The specific activity of enzyme was expressed as nanomoles cinnamic acid $h^{-1}$mg of $protein^{-1.}$

β-1, 3 glucanase activity was determined using a method described by Abeles et al. (1971) with a slight modification. The 100 µL of enzyme extract was mixed with 100 µL of 2% (w/v) laminarin (Aldrich, USA) and incubated for 24 h at 40˚ C. After the incubation period, 25 µL 3, 5-dinitrosalicyclic reagent was added for reaction. Then the samples were heated in boiling water for 5 min to stop the reaction. The amount of reducing sugar was determined at 540 nm (Zenyth 200rt Microplate Reader UK-Biochrom Ltd). The enzyme activity was expressed in units with one unit defined as the amount of enzyme necessary to catalyse the formation of one µmol glucose equivalents $h^{-1}$mg of $protein^{-1}$.

Chitinase activity was estimated according to Abeles et al. (1971). The reaction mixture consisted of 600 µL of the enzyme extract and 125 µL of 2% (w/v) dye-labelled chitin azure in 50 mM sodium acetate buffer (pH 5.0) and incubated for 2 h at 40˚ C. After incubation the reaction was terminated by adding 25 µL of 1 M HCl, The supernatant was measured at 550 nm (Zenyth 200rt Microplate Reader UK-Biochrom Ltd). One unit was defined as the amount of enzyme needed to catalyse the formation of one nmol product $h^{-1}$ mg of $protein^{-1}$.

POD activity was determined according to Jiang et al. (2002) method with a slight modification. The 36 µL of enzyme in 144 µL buffered substrate (100 mM sodium phosphate, pH 7 and 20 mM guaiacol) was incubated for 5 min at 30° C. Thereafter, 72 µL of $H_2O_2$ (100 mM) was added and the increase in absorbance at 460 nm for 120 s was measured (Zenyth 200rt Microplate Reader UK-Biochrom Ltd). The specific activity of the enzyme was expressed as Δ *A*460 $min^{-1}$ mg of $protein^{-1}$.

CAT activity was estimated by the method of Beers and Sizer (1952) with a slight modification. The reaction mixture contained 150 µL of sodium phosphate buffer (100 mM, pH 7.0), 50 µL of $H_2O_2$ (100 mM) and 50 µL of enzyme. The $H_2O_2$ decomposition was measured at 240 nm absorbance (Zenyth 200rt Microplate Reader UK-Biochrom Ltd). The enzyme activity was expressed as units per mg protein (one unit: catalase converts one µmol of $H_2O_2$ $min^{-1}$).

SOD activity was assayed according to the Constantine and Stanley (1977) method of with a slight modification. The reaction mixture (200 µL) contained sodium phosphate buffer (100 mM, pH 7.8), methionine (13 mM), 75 µM of nitroblue tetrazolium (NBT), EDTA (10 µM), riboflavin (2 µM), and 100 µL of enzyme extract. Afterwards the mixture was illuminated through fluorescent lamp (60 µmol $m^{-2}s^{-1}$) up to 10 min and then the absorbance read at 560 nm. For the blank, identical solutions were kept under the dark. The enzyme activity was expressed as Unit mg of $protein^{-1}$. One unit was defined as the amount of enzyme that caused 50% inhibition of NBT. The determination of defence related and antioxidant enzymes activities were repeated twice in order to confirm the observations.

The protein content of enzyme extracts was estimated by dye-binding method of Bradford (1976) with bovine serum albumin (BSA) as a standard. All the enzyme assays were carried out for each treatment in triplicates per samples.

*2.7. Analysis of total phenolic content*

The phenolic content was determined by a slight modification of Singleton et al. (1999) method. The sample was extracted with acetone: water (1:1). The 9 µL of sample extract and 109 µL of Folin-Ciocalteu reagent were added to the well and allowed for 3 min. Afterwards 180 µL of sodium carbonate (7.5%) solution was added to each well and incubated at 50° C for 5 min and the absorbance was measured at 760 nm (Zenyth 200rt Microplate Reader UK-Biochrom Ltd). Gallic acid was used as standard and results expressed as gallic acid equivalent per gram of fruit.

*2.8. Statistical Analysis*

A complete randomised design was adopted in this study. The data obtained were subjected to analysis of variance (ANOVA) using SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL, USA). The mean values were compared using Tukey's HSD test at $P < 0.05$

## 3. Results

*3.1. Effect of essential oils on radial mycelia growth of C. gloeosporioides*

The different concentrations of peppermint, citronella and thyme oil were used to determine the most effective concentration to control *C. gloeosporioides*. The essential oils; thyme (5 µL Petri plate$^{-1}$), peppermint (8 µL Petri plate$^{-1}$) and citronella (8 µL Petri plate$^{-1}$) oils completely inhibited the radial mycelia growth of *C. gloesporioides in vitro* (Table 1).

*3.2. Composition of essential oils*

The components present in the three essential oils were analyzed by GC/MS. Thyme oil showed 26 components of which thymol (53.19% RA) and cymol (20.68% RA) were observed as major components (Table. 2) Peppermint oil had 20 components with menthol (41.62% RA) and menthone (20.83% RA) being the predominating components. Citronella oil contained three main components R (+) citronellal (23.54% RA), lemonol (19.03% RA), and B-citronellol (12.18% RA).

*3.3. Effect of essential oils on anthracnose incidence and severity in inoculated avocado cultivars*

The Fig. 1 A and B illustrates the effect of the three essential oils; thyme (16.7 µL L$^{-1,}$ 33.3 µL L$^{-1}$ and 66.7 µL L$^{-1}$), peppermint and citronella (26.7 µL L$^{-1}$, 53.33 µL L$^{-1}$ and 106 µL L$^{-1}$) on anthracnose incidence and severity in inoculated fruit. Although thyme (5 µL Petri plate$^{-1}$), peppermint (8 µL Petri plate$^{-1}$) and citronella (8 µL Petri plate$^{-1}$) showed complete inhibition of radial mycelia growth of *C. gloesporioides in vitro,* these specific concentrations used in *in vivo* study were calculated according to the volume of the 27.5 x 18.5 x 9 cm glass box where the inoculated fruits were exposed to thyme oil volatiles. Increasing the concentration of essential oils significantly ($P < 0.05$)

reduced the anthracnose incidence in both types of avocado cultivars. The incidence of anthracnose and severity (lesion diameter) were significantly ($P < 0.05$) higher in untreated control fruits (cv. Fuerte and Hass). The incidence and severity of anthracnose was observed to increase with the incubation time. Although peppermint and citronella oils significantly ($P < 0.05$) reduced the anthracnose incidence and severity at higher concentrations (106 μL $L^{-1}$), the effect of thyme oil was higher at 66.7 μL $L^{-1}$ for the control of anthracnose in inoculated fruits (cv. Fuerte and Hass). Moreover, the effective control on anthracnose incidence shown by thyme oil (66.7 μL $L^{-1}$) was higher than the commercially adopted prochloraz treatment in both cultivar types after 4 days. Although the incidence of anthracnose increased after 6 days in inoculated fruit subjected to all postharvest treatments, thyme oil (66.7 μL $L^{-1}$) and prochloraz treatments showed similar control effect on cv. Hass. However, after 6 days, thyme oil (66.7 μL $L^{-1}$) showed significantly higher effect on control of anthracnose in cv. Fuerte than the prochloraz treatment (Fig. 1 A and B). The anthracnose severity (lesion diameter) increased with incubation time, and thyme oil (66.7 μL $L^{-1}$) significantly reduced the disease severity than the commercially adopted prochloraz treatment (Fig. 2 A and B).

### *3.4. Effect of essential oils on defence related enzymes activities (PAL, β-1, 3-glucanase chitinase and POD) and phenolic content*

PAL activity in thyme oil treated avocado cultivars showed a higher activity on the 4$^{th}$ day and a slight decrease in activity was observed on day 6. Thyme oil treated avocado cultivars showed significantly ($P < 0.05$) higher PAL activity than the prochloraz treated fruit (Table 4). However, significant ($P < 0.05$) difference in PAL

activity was noted between day 4 and day 6 with respect to the thyme oil or prochloraz treatments. β-1,3-glucanase activity was higher in thyme oil or prochloraz treated avocado cultivars than the untreated control fruit and the higher enzyme activity was noted on day 4 than on day 6 after inoculation (Table 4). Although low chitinase activity was observed in untreated (control) fruit, a significant ($P < 0.05$) increase in activity was observed in thyme oil treated fruit and the activity was higher on day 4 (Table.4). POD activity was higher in avocado cultivars treated with thyme oil or prochloraz than the untreated control fruit (Table 4). The total phenol content in terms of g fresh weight was carried out in all treated and control fruits (Table 4). The level of total phenol g fresh weight was significantly ($P < 0.05$) higher in the treated fruits at 4 and 6 days after inoculation than in the untreated control fruit. Fruit subjected to thyme oil treatment contained higher phenolic content than the fruit treated with peppermint or citronella oils.

### *3.5. Effect of essential oils on antioxidant enzymes or hydrogen peroxide metabolizing activities (SOD and CAT)*

SOD activity was higher on day 4 after inoculation in fruit subjected to different postharvest treatments and a slight decrease in activity was noted on day 6. A significantly ($P < 0.05$) decreased SOD activity was noted in untreated control fruit. Avocado cultivars treated with thyme oil showed significantly ($P < 0.05$) higher SOD activity than the untreated (control) and the other essential oils treatment (Table 5). The CAT activity also showed similar trend as SOD, showing an increase in activity on day 4 and declining on day 6 in both cultivars (Table 5).

## 4. Discussion

It is evident from these investigations that thyme oil effectively reduced the anthracnose incidence and severity in avocado cvs. Fuerte and Hass. Thymol, the active component of thyme oil was similarly reported to reduce spoilage due to decay in sweet cherries (Chu et al., 1999), apricots and plums (Lui et al., 2002), citrus (Plaza et al., 2004), table grapes (Valverde et al., 2005), and strawberries (Wang et al., 2007). Essential oils control postharvest pathogens primarily due to their direct inhibitory effect on pathogen growth (mycelia) and spore germination (Areebola et al., 2010; Yigit et al., 2000) by affecting the active sites of enzymes and cellular metabolism (Juglal et al., 2002). Essential oils also have the ability to change the permeability of the cell membranes of pathogens for cation exchange and affect the ion gradients which will affect the metabolic processes in the pathogens (cells) and ultimately result in cell death (Ultee et al., 1999). It is also reported that essential oil components such as thymol or eugenol showed an ability to increase antioxidant levels (polyphenols, flavoids, anthocyanins) and oxygen absorbance capacity in plant tissues, including in enzymatic and non-enzymatic systems (Jin et al., 2012b; Wang et al., 2007; Wang et al., 2008). The influence of essential oil components on increasing the antioxidant capacity and scavenging activity can cause a great impact on enhancing resistance of plant tissues against pathogens and reducing physiological deterioration. In our investigations thyme oil increased the total phenol content in the inoculated avocado fruit and similar observation was noted in peach fruit inoculated with *Penicillum expansum* and exposed to the natural volatile compounds methyl jasmonate (Jin et al., 2009a). Phenol content plays a major role in plant resistance and defence mechanism against invasion of plant pathogens (Beckman, 2000). In light of our results it

can be suggested that thyme oil has the ability to act as a 'signalling compounds' that triggers a signal similar to a mild stress condition on the fruit. The phenolic compounds are also linked to antioxidant activities (Małgorzata and Irena, 2005). In this investigation the increase of phenolic content occurred concomitantly with an increase in PAL activity and similar results were noted in different horticultural crops (Jin et al., 2009a,b; Kim et al., 2007; Zeng, 2006).

Therefore, it can be concluded that thyme oil plays an important role in stimulating the synthesis of phenolic compounds by promoting higher PAL activity. Our data showed that thyme oil induced activities of chitinase, β-1,3-glucanase and POD in avocado fruit. Chitinase and β-1,3-glucanase play an important in role in plant defence mechanisms against fungal pathogens by mediating the biochemical reactions involved in hydrolysing polymers of fungal cell wall (Dumas-Gaudot et al., 1992; Collinge, 1993).

POD was reported to be associated with disease resistance and involved in generating phenolic cross links connecting neighboring or adjoining biopolymer chains (Mohammadi et al., 2002). Our results clearly indicates that thyme oil treatment can induce the activities of POD, PAL, β-1,3-glucanase, and chitinase that play an important role in disease resistance in avocado fruit. Similar results were reported with the application of natural volatile methyl jasmonate on loquat fruit (Cao et al., 2008), and peach (cv. Baifeng) (Jin et al., 2009a).

The antioxidant enzymes were reported to show a positive relationship to antioxidant capacities. SOD, known as a class of metal-containing proteins that mediates the dismutation reaction of $O_2^{\cdot -}$ into $H_2O_2$ and the POD and CAT convert $H_2O_2$ to oxygen and water. According Mittler (2002) these antioxidant enzymes limit the potential for further

free radical production from $H_2O_2$. Our observations revealed that avocado cvs. Fuerte and Hass exposed to the essential oils (thyme, peppermint, and citronella oils) had higher antioxidant enzyme activities (SOD, POD and CAT) than the untreated control fruit. The higher impact on antioxidant enzymes activities by thymol observed in this investigation was similar to the findings of Chanjirakul et al. (2007) and Jin et al. (2012a, b), where the essential oils increased the antioxidant enzyme activities in strawberry and raspberry fruit. Therefore, it could be possible that the thyme oil treatment activated the antioxidant system or secondary metabolites (phenols, catechin and epicatechin), which were responsible for scavenging free radicals and enhancing antioxidant capacity. On the other hand, thymol treatment was reported to enhance higher antioxidant scavenging activities ($DPPH^{\cdot}$, $HO^{\cdot}$ and $O_2^{\cdot -}$) in strawberry fruit (Wang et al., 2007). However, further investigations needs to be carried out to determine the DPPH, superoxide and hydroxyl radicals and the reducing power in essential oil treated fruit (avocado) in comparison to the untreated fruit. Antioxidants are known as efficient free radical scavengers and have the ability to suppress or reduce the reactive oxygen species [ROS; superoxide, hydrogen peroxide ($H_2O_2$) and hydroxyl radical] in fruit tissue and to reduce oxidative stress due to the growth of decay causing pathogen.

In conclusion, postharvest application of thyme oil significantly enhanced the activity of antioxidant and induced defence enzymes, maintained higher levels of phenolic content and thereby enhanced the resistance of fruit tissue against invasion and reduced the spoilage due to anthracnose. The control of anthracnose in infected fruit in this study with thyme oil in volatile phase indicates that the application of thyme oil in vapour phase can be tested in the future in combination with modified atmosphere packaging in order to

provide a satisfactory solution to the organic avocado fruit industry. Similar approach was adapted to control Rhizopus rot, brown rot (*Molnilia fructicola*) in peaches (Areebola et al., 2010) and *Botrytis cenerea* in grapes (Valverde et al., 2005). The use of thyme oil could be reported as cost effective due to its lower price for commercial application (Tripathi and Shukla, 2002). Moreover, thymol is used as a food preservative and the US Food and Drug Administration lists thymol essential oil and thyme (spice) as food for human consumption, as well as recognised as Generally Regarded as Safe (GRAS) food additives (EPA, 2002).

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**References**

Abeles, F.B., Bosshart, R.P., Forrence, L.E., Habig, W.H., 1971. Preparation and purification of glucanase and chitinase from bean leaves. Plant Physiol. 47, 129–134.

Adams, R.P., 1995. Identification of essential oil components by gas chromatography/ mass spectroscopy. Allured Publishing Corporation, Carol Stream, Illinois.

Arrebola, E., Sivakumar, D., Bacigalupo, R., Korsten, L., 2010. Combined application of antagonist *Bacillus amyloliquefaciens* and essential oils for the control of peach postharvest diseases. Crop Prot. 29, 369–377.

Assis, J.S., Maldonado, R., Munoz, T., Escribano, M.I., Merodio, C., 2001. Effect of high carbon dioxide concentration on PAL activity and phenolic contents in ripening cherimoya fruit. Postharvest Biol. Technol. 23, 33–39.

Beckman, C.H., 2000. Phenolic-storing cells: key to programmed cell death and periderm formation in wilt disease resistance and in general defense response in plants. Physiol. Mol. Pathol. 57, 101–110.

Beers, R.F., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 95, 133–140.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cao, S.F., Zheng, Y.H., Yang, Z.F., Tang, S.S., Jin, P., 2008. Control of anthracnose rot and quality deterioration in loquat fruit with methyl jasmonate. J. Sci. Food Agric. 88, 1598–1602.

Chanjirakul, K., Wang, S.Y., Wang, C.Y., Siriphanich, J., 2007. Natural volatile treatments increase free-radical scavenging capacity of strawberries and blackberries. J. Sci. Food Agric. 87, 1463–1472.

Chu, C.L., Liu, W.T., Zhou, T., Tsao, R., 1999. Control of post-harvest grey mold rot of modified atmosphere packaged sweet cherries by fumigation with thymol and acetic acid. Can. J. Plant Sci. 79, 686–689.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K., Rasmussen, U., Vad, K., 1993. Plant chitinase. Plant J. 3, 1–40.

Constantine, N.G., Stanley, K.R., 1977. Superoxide dismutases. Plant Physiol. 59, 309–314.

Dafarera, D., Ziogas, B.N., Polissiou, M.G., 2000**.** GC–MS analysis of essential oils from Greek aromatic plants and their fungi toxicity on *Penicillium digitatum*. J. Agric. Food Chem. 2576–2581.

Danderson. M., 1986. Omega* (Prochloraz), A fungicide or post-harvest control of anthracnose, the *Dothiorella/Colletotrichum c*omplex and stem-end rot in avocados. S. Afr. Avocado Growers' Assoc. Yearb. 9, 27–30.

Dumas-Gaudot, E., Grenier, J., Furlan, V., Asselin, A., 1992. Chitinase, chitosanase and β-1,3 glucanase activities in *Allium* and *Pisum* roots colonized by *Glomus* species, Collinge. Plant Sci. 84, 17–24.

EPA RED Facts Thymol http://www.epa.gov/oppsrrd_1/REDs/factsheets/3143facts.pdf (accessed on 22nd. August, 2012).

Ippolito, A., Nigro, F., 2000**.** Impact of preharvest application of biological agents on postharvest diseases of fresh fruits and vegetables. Crop Prot. 19, 715–723.

Jiang, A.L., Tian, S.P., Xu, Y. 2002. Effects of controlled atmospheres with high $O_2$ or high $CO_2$ concentrations on postharvest physiology and storability of "Napoleon" sweet cherry. Acta Bot. Sin. 44, 925–930.

Jin, P., Wang, S. Y., Gao, H., Chen, H., Zheng, Y., Wang, C.Y., 2012a Effect of cultural system and essential oil treatment on antioxidant capacity in raspberries. Food Chem. 132, 399–405.

Jin, P., Wu, X., Xu, F., Wang, X., Wang, J., Zheng, Y., 2012b. Enhancing antioxidant capacity and reducing decay of Chinese bayberries by essential oils. J. Sci. Food Agric. 60, 3769–3775.

Jin, P., Zheng, Y., Tang, S., Rui, H., Wang. C.Y., 2009a. Enhancing disease resistance in peach fruit with methyl jasmonate. J. Sci. Food Agric. 89, 802–808.

Jin, P., Zheng, Y., Tang, S., Rui, H., Wang, C.Y., 2009b. A combination of hot air and methyl jasmonate vapor treatment alleviates chilling injury of peach fruit. Postharvest Biol. Technol. 52, 24–29.

Juglal, S., Govinden, R., Odhav, B., 2002. Spice oils for the control of co-occurring mycotoxin-producing fungi. J. Food Prot. 65, 683–687.

Kim, H.J., Fonseca, J.M., Choi, J.H., Kubota, C., 2007. Effect of methyl jasmonate on phenolic compounds and carotenoids of romaine lettuce (*Lactuca sativa* L.). J. Agric. Food Chem. 55, 10366–10372.

Laird, K., Phillips, C., 2011. Vapour phase: a potential future use for essential oils as antimicrobials?. Lett. Appl. Microbiol. 54, 169–174.

Liu, W.T., Chu, C.L., Zhou, T., 2002. Thymol and acetic acid vapors reduce post-harvest brown rot of apricot and plums. HortScience 37, 151–156.

Małgorzata, M., Irena, P., 2005. Antioxidant activity of the main phenolic compounds Isolated from hot pepper fruit (*Capsicum annuum* L.). J. Agric. Food Chem. 53, 1750–1756.

Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7, 405–410.

Mohammadi, M., Kazemi, H., 2002. Changes in peroxidase and polyphenol oxidase activities in susceptible and resistant wheat heads inoculated with *Fusarium graminearum* and induced resistance. Plant Sci. 162, 491–498.

Plaza, P., Torres, R., Usall, J., Lamarca, N., Vinas, I., 2004. Evaluation of the potential of commercial post-harvest application of essential oils to control citrus decay. J. Hortic. Sci. Biotechnol. 79, 935–940.

Regnier, T., Combrinck, S., Du Plooy, W., Botha, B., 2010. Evaluation of *Lippia scaberrima* essential oil and some pure terpenoid constituents as postharvest mycobiocides for avocado fruit. Postharvest Biol. Technol. 57, 176–182.

Sanders, G.M., Korsten, L., 1997. Market survey of stem-end rot and anthracnose on Fuerte avocados and comparison of *Colletotrichum gloeosporioides* isolates from different avocado producing areas. S. Afr. Avocado Growers' Assoc. Yearb. 20, 101–105.

Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol. 299, 152–178.

Tripathi1, P., Shukla, A.K., 2007. Emerging Non-Conventional Technologies for Control of Post Harvest Diseases of Perishables. Fresh produce 2, 111–120.

Tzortzakis, N.G., Economakis, C.D., 2007. Antifungal activity of lemongrass (*Cymbopogon citrates* L.) essential oil against key postharvest pathogens. Innov. Food Sci. Emerg. Technol. 8, 253–258.

Ultee, A., Kets, E. P. W., Smid, E. J., 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Appl. Environ. Microbiol. 65, 4606–4610.

Valverde, J.M., Guillen, F., Martinez-Romero, D., Castillo, S., Serrano, M., Valero, D., 2005 Improvement of table grapes quality and safety by the combination of modified atmosphere packaging (MAP) and eugenol, menthol, or thymol. J. Agric. Food Chem. 53, 7458–7464.

Wang, C, Y., Shiow Y., Wang Jun-Jie., Y, Parry, J., Liangli Lucy, Y., 2007. Enhancing antioxidant, antiproliferation, and free radical scavenging activities in strawberries with essential oils. J. Agric. Food Chem. 55, 6527–6532.

Wang, C.Y., Wang, S.Y., Chen, C.T., 2008**.** Increasing antioxidant activity and reducing decay of blueberries by essential oils. J. Agric. Food Chem. 56, 3587–3592

Wu, F., Jiaping C., Xiaodong Z., Qing L., 2001. Thyme oil to control *Alternaria alternata in vitro* and *in vivo* as fumigant and contact treatments. Food Control 22, 78-81.

Xing, Y., Li, X., Xu, Q., Yun, J., Lu, Y., 2010**.** Antifungal activities of cinnamon oil against *Rhizopus nigricans*, *Aspergillus flavus* and *Penicillium expansum in vitro* and *in vivo* fruit test. Int. J. Food Sci Technol. 45, 1837–1842.

Yigit.F; Özcan, M., Akgül, A., 2000. Inhibitory effect of some spice essential oils on *Penicillium digitatum* causing postharvest rot in citrus. Grasas y Aceites (Sevilla) 51, 237–240.

Zeng, K., Cao, J., Jiang, W., 2006**.** Enhancing disease resistance in harvested mango (*Mangifera indica* L.cv. ‘Matisu’) fruit by salicylic acid. J. Sci. Food Agric. 86, 694–698.

**Table 1.** Effect of essential oils on the inhibition of radial mycelial growth (percentage) of *C. gloeosporioides*.

| Concentration of oil (μL $plate^{-1}$) | Inhibition of radial mycelia growth (%) | | |
|---|---|---|---|
| | **Thyme oil** | **Peppermint oil** | **Citronella oil** |
| 1 | 66.7 ± 1.3e | 33.8 ± 2a | 45.3 ± 2.3b |
| 2 | 77.8 ± 1.5g | 45.8 ± 3.1b | 52 ± 2.3c |
| 3 | 84.9 ± 0.8h | 58.2 ± 1.5d | 60.4 ± 1.5d |
| 4 | 90.2 ± 0.8i | 66.2 ± 3.4e | 68 ± 1.3e |
| 5 | 100 ± 0j | 74.7 ± 1.3f | 75.1 ± 2f |
| 6 | 100 ± 0j | 84.4 ± 2h | 83.6 ± 2h |
| 7 | 100 ± 0j | 89.8 ± 0.8i | 88.9 ± 0.8i |
| 8 | 100 ± $0^{j}$ | 100 ± 0j | 100 ± 0j |

Means ($n$ = 10) followed by same letters in the same column are not significantly different at ($P$ < 0.05). SD±: standard deviation.

**Table 2.** Chemical constituents of thyme oil.

| | Compounds | Retention time | Relative area percentage (%) (RA) |
|---|---|---|---|
| 1 | α-Pinene | 5.567 | 1.24 |
| 2 | Camphene | 5.964 | 0.95 |
| 3 | β-Pinene | 6.768 | 0.20 |
| 4 | β-Myrcene | 7.182 | 1.59 |
| 5 | α-Phellandrene | 7.614 | 0.28 |
| 6 | 3-Carene | 7.808 | 0.18 |
| 7 | 4-Carene | 8.041 | 2.25 |
| 8 | Cymol | 8.434 | 20.68 |
| 9 | γ-Terpinen | 9.567 | 5.23 |
| 10 | (+)-4-Carene | 10.629 | 0.56 |
| 11 | Linalol | 11.174 | 5.08 |
| 12 | Borneol | 13.724 | 2.12 |
| 13 | Terpineol-4 | 14.219 | 1.46 |
| 14 | α-Terpieol | 14.773 | 0.19 |
| 15 | Thymol methyl ether | 15.073 | 0.24 |
| 16 | Benzene, 1-methoxy-4-methyl- | 17.043 | 0.96 |
| 17 | Thymol | 19.631 | 53.19 |
| 18 | Caryophyllene | 24.363 | 1.62 |
| 19 | Alloaromadendren | 25.116 | 0.24 |
| 20 | 4-t-Butylcatechol | 25.302 | 0.38 |
| 21 | Varidiflorene | 27.387 | 0.14 |
| 22 | γ-Cadinene | 28.520 | 0.24 |
| 23 | Spathulenol | 30.558 | 0.16 |
| 24 | Caryophyllene oxide | 30.757 | 0.27 |
| 25 | 3-Hydroxy-4-methylbenzaldehyde | 42.064 | 0.45 |
| 26 | 1,3-Difluoro-5-tripropyl-silyloxybenzene | 44.445 | 0.12 |

**Table 3.** Effect of essential oil vapours treatment on defence related enzymes and phenolic content in avocado fruit.

| Essential oil | Incubation time (d) | PAL activity (nM cinnamic acid $h^{-1}$ mg of $protein^{-1}$) | | Cultivar | | | | POD activity ($\Delta A460$ $min^{-1}$ mg of $protein^{-1}$) | | Total phenol content (gallic acid equiv. g of $FW^{-1}$) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | β-1,3-Glucanase activity (µM glucose equiv. $h^{-1}$ mg of $protein^{-1}$) | | Chitinase activity (nM product $h^{-1}$ mg of $protein^{-1}$) | | | | | |
| | | Fuerte | Hass | Fuerte | Hass | Fuerte | Hass | Fuerte | Hass | Fuerte | Hass |
| Thyme oil | 4 | 23.66 ± 0.60f | 25.42 ± 0.90f | 11.72 ± 0.40f | 12.70 ± 0.70d | 1.08 ± 0.03f | 1.33 ± 0.10g | 11.85 ± 0.60d | 9.14 ± 0.60e | 1.54 ± 0.05f | 2.80 ± 0.20f |
| Thyme oil | 6 | 19.28 ± 0.70c | 20.88 ± 1.0d | 9.53 ± 0.40d | 10.61 ± 0.50c | 0.85 ± 0.02d | 1.13 ± 0.10f | 12.11 ± 0.70d | 9.22 ± 0.50e | 1.41 ± 0.02e | 2.45 ± 0.10e |
| Peppermint oil | 4 | 20.55 ± 0.50d | 19.18 ± 1.30c | 9.05 ± 0.20cd | 10.10 ± 0.80c | 0.79 ± 0.03c | 0.83 ± 0.10d | 10.35 ± 0.60c | 6.5 ± 0.40cd | 1.14 ± 0.10c | 1.99 ± 0.10d |
| Peppermint oil | 6 | 17.15 ± 0.70b | 16.92 ± 0.50b | 6.75 ± 0.30b | 7.70 ± 0.20b | 0.69 ± 0.03b | 0.72 ± 0.03c | 9.53 ± 0.40c | 7.2 ± 1.00d | 1.05 ± 0.10c | 1.77 ± 0.10b |
| Citronella oil | 4 | 18.96 ± 0.20c | 18.60 ± 0.30c | 8.84 ± 0.20c | 9.70 ± 0.60c | 0.78 ± 0.02c | 0.74 ± 0.03cd | 9.82 ± 0.80c | 5.91 ± 0.30bc | 1.14 ± 0.03c | 1.97 ± 0.10cd |
| Citronella oil | 6 | 17.41 ± 0.50b | 16.17 ± 0.90b | 6.51 ± 0.03b | 7.40 ± 0.50b | 0.65 ± 0.02b | 0.69 ± 0.02bc | 7.71 ± 0.40b | 6.09 ± 0.70bc | 1.05 ± 0.10c | 1.92 ± 0.10cd |
| Prochloraz | 4 | 22.50 ± 0.40e | 23.60 ± 0.30e | 12.17 ± 0.60f | 11.90 ± 0.30d | 0.92 ± 0.01e | 1.13 ± 0.04f | 11.56 ± 0.50d | 8.51 ± 0.50e | 1.47 ± 0.10ef | 2.71 ± 0.20f |
| Prochloraz | 6 | 18.62 ± 0.40c | 19.10 ± 0.40c | 10.30 ± 0.30e | 9.80 ± 0.50c | 0.81 ± 0.01c | 0.96 ± 0.02e | 11.86 ± 0.80d | 9.04 ± 0.80e | 1.26 ± 0.10d | 1.99 ± 0.20cd |
| Untreated (control) | 4 | 16.76 ± 0.60b | 17.14 ± 0.70b | 6.91 ± 0.20b | 8.00 ± 0.40b | 0.68 ± 0.02b | 0.62 ± 0.10ab | 7.71 ± 0.40b | 5.12 ± 0.50ab | 0.86 ± 0.02b | 1.51 ± 0.10b |
| Untreated (control) | 6 | 11.94 ± 0.2a | 11.41 ± 1.2a | 5.63 ± 0.4a | 5.9 ± 0.4a | 0.59 ± 0.02a | 0.53 ± 0.03a | 5.40 ± 0.4a | 4.68 ± 0.3a | 0.72 ± 0.1[a] | 1.27 ± 0.10a |

Means ($n$ = 15) followed by same letters in the same column are not significantly different at ($P < 0.05$). SD±: standard deviation. The commercial fungicide prochloraz treatment was a 0.05% for 5 min dip. Fruit were exposed to thyme oil vapour at 66.7 µL $L^{-1}$ and peppermint oil and citronella oil vapours at 106 µL $L^{-1}$ concentration. FW, fresh weight; PAL, phenylalanine ammonia-lyase; POD, peroxidase.

**Table 4.** Effect of essential oil vapours treatment on antioxidant enzyme activities in avocado fruit.

| Essential oil | Incubation time (d) | Cultivar | | | |
|---|---|---|---|---|---|
| | | SOD activity (U mg of protein$^{-1}$) | | CAT activity (U mg of protein$^{-1}$) | |
| | | **Fuerte** | **Hass** | **Fuerte** | **Hass** |
| Thyme oil | 4 | 149.58 ± 1.20f | 164.31 ± 4.40f | 1.25 ± 0.01e | 2.10 ± 0.20d |
| Thyme oil | 6 | 135.38 ± 1.30d | 147.72 ± 1.70c | 1.01 ± 0.10d | 1.65 ± 0.10c |
| Peppermint oil | 4 | 139.63 ± 1.10e | 153.23 ± 2.07d | 1.00 ± 0.02d | 1.44 ± 0.10c |
| Peppermint oil | 6 | 127.47 ± 1.01b | 134.18 ± 1.10ab | 0.77 ± 0.10b | 0.99 ± 0.20b |
| Citronella oil | 4 | 138.63 ± 1.10e | 151.97 ± 1.60d | 1.02 ± 0.10d | 1.44 ± 0.10c |
| Citronella oil | 6 | 126.52 ± 0.70b | 134.66 ± 0.90b | 0.87 ± 0.04c | 0.95 ± 0.20b |
| Prochloraz | 4 | 148.77 ± 1.60f | 159.43 ± 1.40e | 1.3 ± 0.10e | 1.57 ± 0.10c |
| Prochloraz | 6 | 134.72 ± 1.10cd | 144.66 ± 1.10c | 0.91 ± 0.10c | 1.40 ± 0.10c |
| Untreated (control) | 4 | 133.35 ± 1.00c | 144.82 ± 3.00c | 0.89 ± 0.04c | 1.15 ± 0.20b |
| Untreated (control) | 6 | 121.17 ± 0.20a | 130.66 ± 0.60a | 0.57 ± 0.10a | 0.64 ± 0.10a |

Means ($n$ = 15) followed by same letters in the same column are not significantly different at ($P < 0.05$). SD±: standard deviation. The commercial fungicide prochloraz treatment was a 0.05% for 5 min dip. Fruit were exposed to thyme oil vapour at 66.7 μL $L^{-1}$ and peppermint oil and citronella oil vapours at 106 μL $L^{-1}$ concentration. SOD, superoxide dismutaste; CAT, catalase.

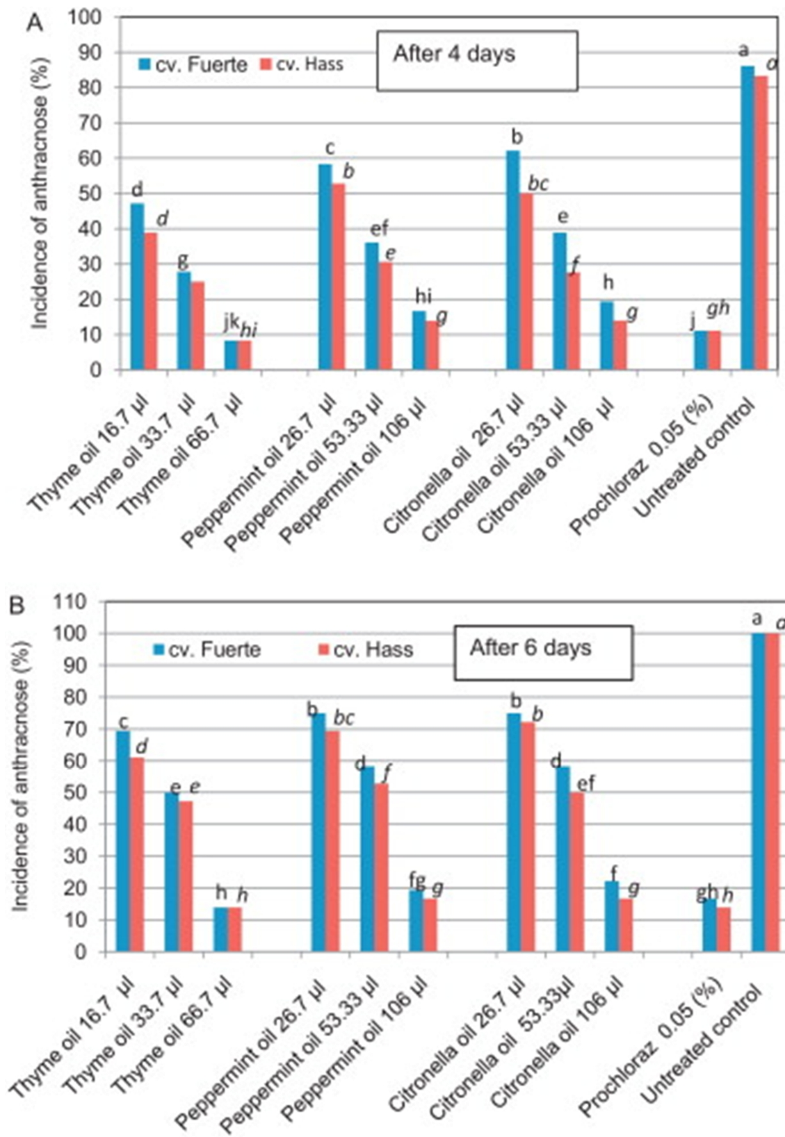


**Fig. 1**. Effect of essential oils on incidence of anthracnose disease in inoculated avocado fruits (cvs. Fuerte and Hass) (A) after 4 days and (B) after 6 days.

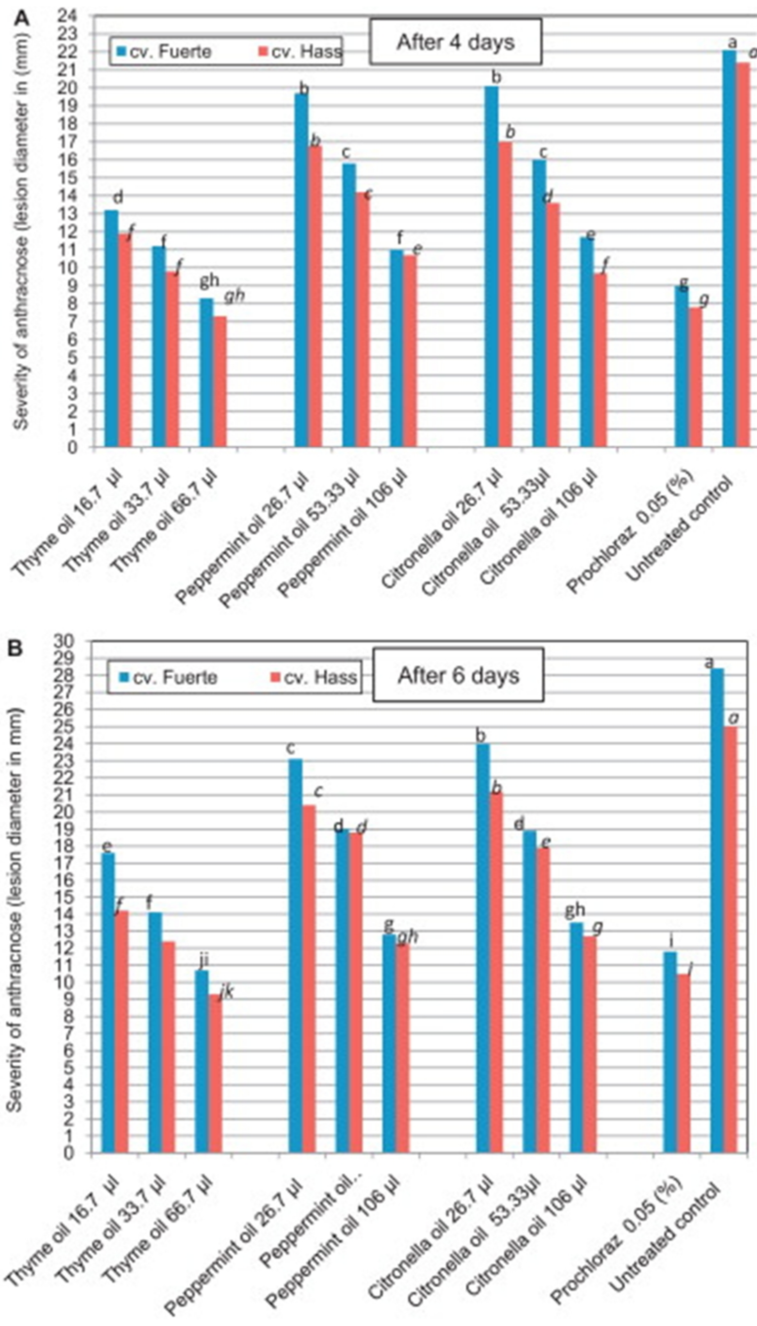


**Fig. 2**. Effect of essential oils on severity of anthracnose disease in inoculated avocado fruits (cvs. Fuerte and Hass) (A) after 4 days and (B) after 6 days.