# The efficacy of combined application of edible coatings and thyme oil in inducing resistance components in avocado (*Persea americana* Mill.) against anthracnose during post-harvest storage.

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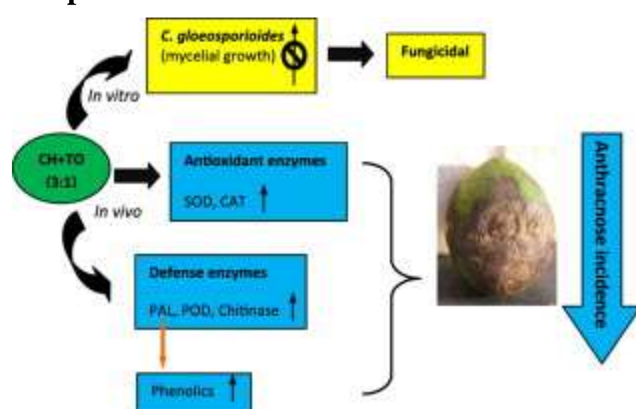
## Highlights

•Thyme oil and chitosan or *Aloe vera* coatings improved the antifungal activity against *Colletotrichum gloeosporioides.*
•Thyme oil and chitosan coating effectively reduced the anthracnose incidence and severity.
•Thyme oil and chitosan coating revealed higher PAL, POD, chitinase, β-1,3-glucanase, CAT and SOD.

## Graphical abstract




## Abstract

Avocado is a high economic fruit. However, major post-harvest losses are encountered throughout the supply chain mostly due to anthracnose disease caused by the fungus

*Colletotrichum gloeosporioides*. Increasing consumer concern regarding food safety and demand for organically produced fruits makes it necessary to search for natural environmentally friendly alternative products and processes for the fruit industry. Antifungal effects of Gum Arabic (GA) (10%), *Aloe vera* (AL) (2%), chitosan (CH) (1%) alone or in combination with thyme oil (1%) were investigated *in vitro.* CH + Thyme oil and AL + Thyme oil [1:1 or 3:1 v/v] showed fungicidal effects while AL, CH, GA and GA + Thyme oil [3:1 v/v] showed fungistatic effects on mycelial growth of *C. gloeosporioides in vitro*. CH and AL coatings alone or in combination with thyme oil [3:1 v/v], either as preventative or curative treatments in comparison with commercial treatment (prochloraz, 0.05%) and untreated control were evaluated on incidence and severity (lesion diameter) of anthracnose *in vivo*. Preventative CH + Thyme oil treatments significantly reduced the severity of anthracnose (8.9 mm) compared to thyme oil (12.7 mm), AL + TO (14.4 mm), CH (17.8 mm), AL (20.6 mm), PZ (18.3 mm) and untreated samples (34.8 mm). As curative method, the CH + Thyme oil combination also reduced the severity of anthracnose by at least 4 mm compared to the other treatments. The total phenols, polyphenol oxidase, phenylalanine ammonia-lyase, β-1,3-glucanase, chitinase, catalase and superoxide dismutase activities, firmness and flesh colour were also determined. Results showed an increase in peroxidase, phenylalanine ammonia-lyase, β-1,3-glucanase, chitinase, catalase and superoxide dismutase activities and total phenolics with reduced loss of firmness and flesh colour following CH + Thyme oil treatments. This investigation recommends CH + Thyme oil [3:1 v/v] combination treatment as a suitable alternative to the currently adopted prochloraz applications in controlling anthracnose disease in avocado fruit during storage.

*Keywords:*Postharvest decay, *Colletotrichum gloeosporioide,* Antioxidant enzymes, Chitosan, Aloe vera, Gum Arabic

## Introduction

The avocado fruit plays an important role in human nutrition due to its nutritional properties such as oleic, palmitic, linoleic, palmitoleic acids, trace amount of stearic acid, vitamin A, B, C, E, K, and high fibre content (Lu et al., 2009; Yahia, 2010). Avocado production in countries like South Africa, Israel and Chile is export driven with the European Union being the biggest market and this entails high fruit quality standards. The common postharvest disease anthracnose (*Colletotrichum gloeosporioides* Penz.) affects the fruit quality, marketability and shelf life of avocados during marketing (Sanders and Korsten, 2003). Both field spraying and postharvest treatments are necessary to achieve high quality fruit. Copper sprays are commonly used in the orchard to control post-harvest diseases (Korsten and Cook 1996; Everett et al. 2005). Limited control of the anthracnose disease can be achieved with an application of preharvest copper oxychloride. The latter application leaves undesirable patches on the fruit surface and it is a time-consuming process to remove them manually in the packhouse prior to packing. At the packhouse, after cooling the fruit is commonly treated with a synthetic non-systemic fungicide prochloraz as a first defence mechanism in the packing line to control anthracnose and it is a common commercial packhouse treatment adopted in South Africa, New Zealand and Australia (Everett et al. 2005; Scheepers et al. 2007; Smith et al. 2011). Postharvest loses due to anthracnose can increase up to 80% if the fruits are not treated with prochloraz at postharvest stage (Bosse et al., 2011).

However, there is a need for safer methods to control postharvest decay development due to an increase in consumer concern regarding food safety and demand for organically produced fruit. The importing countries have enforced stringent regulations regarding the maximum residue limits (MRL) in the skin of the fruit and the MRL for South African avocado is 2 mg $kg^{-1}$ (Mavuso and Van Niekerk, 2013). It is also important to note that

countries like Netherlands and France which are biggest importers of the fruit are more stringent with MRLs below 0.5 mg $kg^{-1}$ (Scheepers et al., 2007). In addition to this, development of fungicide resistant strains (Ippolito and Nigro, 2000), and growing global pressure on the fruit industry to lower the associated environmental pollution footprint have necessitated the need to search for natural novel products to replace the prochloraz fungicide application at postharvest stage.

Commercially, Avoshine® canuba wax coating is used for avocados (Kremer-Kohne and Duvenhage, 1997). Green-skinned cultivars may develop surface discolouration if the proper wax formulation and application methods are not employed. It is essential that the applied wax coating must not leave any deleterious residues or affect the natural glossiness of the fruit (Kruger, 2013), the eating quality or alter the characteristic fruit flavour. The EU does not allow morpholine in wax emulsions (de Boer, 2010). There is some resistance to waxing of fruits including avocados in the EU due consumer pressure.

Application of methyl cellulose (Maftoonazad, and Ramaswamy, 2005) or gelatin-starch coatings (Aguilar-Méndez et al., 2008) to avocado fruits have shown beneficial effects especially delaying the ripening behavior. However, it is necessary to investigate the effect of edible coatings on the incidence of decay. Biocoat™ or Natralife™ a mixture of beeswax and olive oil was shown to increase the shelf live with effective control of decay incidence (Báez-sañado et al., 2008). Application of essential oils or their volatile compounds at postharvest stage has been shown to control postharvest diseases in different fruits (Tzortzakis and Economakis, 2007; Berrera-Necha et al., 2008; Regnier et al., 2010). Antifungal activity of thyme oil is well documented and proven to inhibit the fungal growth of *C. gloeosporioides* *in vitro* or *in vivo* in avocado cultivars Hass and Fuerte (Sellamuthu et al., 2013a). Sellamuthu et al. (2013a) also showed that the thyme oil application in vapour phase in modified atmosphere packaging enhanced activities of defence enzymes (PAL, chitinase, 1,3-

β-glucanase, peroxidase), antioxidant enzymes (catalase and superoxide dismutase) as well as high total phenols. Biodegradable polymers are often referred to as edible coatings and are mainly used to improve food appearance and to preserve fruit quality (Ali et al., 2011). Therefore, the incorporation of thyme oil into edible-coatings could be an effective method to control its high volatility thus minimising losses and improving its effectiveness than when applied directly on the surface of the fruit (Cháfer et al., 2012).

Some of the most commonly used edible coatings are chitosan, *Aloe vera* gel and Gum Arabic to improve fruit quality and to suppress decay during postharvest storage (Navarro et al., 2011; Maqbool et al., 2010; Cháfer et al., 2012). Chitosan, a copolymer consisting of β-(1–4)-2-acetamido-D-glucose and β-(1–4)-2-amino-D-glucose units which is derived from chitin has excellent film-forming properties, nontoxic, has antimicrobial activity and is biodegradable (Elsabee and Abdou, 2013). Application of chitosan was observed to be effective in controlling postharvest diseases in strawberries (El Ghaouth et al., 1991), litch (Zhang and Quantick, 1997), sweet cherries (Romanazzi et al., 2000) and papaya (Bautista-Baños et al., 2003), by activating defence-related enzymes such as phenylalanine ammonia-lyase and production of total phenols (Mazaro et al., 2008). Preventative chitosan coatings containing tea tree oil were found to be effective in reducing the incidence of Penicillium *italicum* (blue mold rot) in citrus fruits (Cháfer et al., 2012). Due to its emulsifying properties Gum Arabic is a potential coating component and incorporating either lemon grass or cinnamon oil into Gum Arabic was reported to control *C. musae* in bananas and *C. gloeosporioides* in papayas (Maqbool et al., 2011). *Aloe vera* gel obtained from the leaf pulp of Aloe plants showed antimicrobial properties and has also been identified as a novel coating agent (Marpudi et al., 2013). Growth of yeasts and molds in grapes were inhibited following the application of *Aloe vera* gel during cold storage at 1 °C (Valverde et al., 2013). Up to our

knowledge no work has been reported on the incorporation of thyme oil into edible coatings to control postharvest decay and maintenance of fruit quality in avocados.

This study is comprised of a threefold objective. Firstly to investigate the effect of three different edible coatings incorporated with thyme oil on control of radial mycelial growth *in vitro,* secondly decay inhibition in artificially inoculated fruits (*in vivo*) (curative and preventative) and finally to determine the induction of defence related enzymes chitinase, 1, 3-β-glucanase, PAL, POD, antifungal compound phenol and antioxidant enzymes (catalase and superoxide dismutase.)

## 2. Materials and methods

### *2.1. Pathogen*

*Colletotrichum gloeosporioides* was obtained from the Fruit and vegetables technology laboratories, Tshwane university of Technology, South Africa. The *C. gloeosporioides* isolates were cultured and maintained on potato dextrose agar (PDA) (Merck, Johannesburg, South Africa) and incubated at 25 °C for 12-13 days. Spore suspension was prepared according to Sellamuthu et al. (2013a) and the mycelia fragments were removed from suspension by filtering through three layers of muslin cloth. Spore were counted using a haemocytometer and adjusted to 1 x $10^5$ spore $mL^{-1}$.

### *2.2. Thyme oil and GC-MS analysis*

Thyme (*Thymus vulgaris* L.) oil was purchased 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. The GC/MS analysis was conducted 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) according to Sellamuthu et al. (2013a). Detailed analytical information for the chromatography was mentioned in Sellamuthu et al. (2013a). The identities of the volatiles were confirmed by comparing the collected mass spectra with NIST08 (National Institute of Standards and Technology 08) and also comparison with those published in the literature.

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*2.3. Preparation of edible coating solutions*

Three different edible coatings chitosan, (CH), Gum Arabic (GA) and *Aloe vera* (AV) were prepared different concentrations. Chitosan (deacetylated P95%, and viscosity <_630 mPa s) (Sigma Aldrich, Johannesburg, South Africa) coating was prepared by dispersing chitosan (CH) (1% w/v) in glacial acetic acid (0.5% v/v) at 25 °C (Cháfer et al. 2012) Gum Arabic (GA) (food grade), (Sigma-Aldrich, Johannesburg, South Africa) (10%, w/v) was prepared by dissolving 10 g of GA powder in 100 mL distilled water and stirring the solution with low heat (40 °C) for 60 min (Maqbool et al., 2011). *Aloe vera* coating (AL) (2%, w/v) (Aloway Natural Health Products Pty, Limpopo, South Africa) was made by dissolving 1.85 g *Aloe vera* spray dried powder (Aloway Natural Health Products, Johannesburg, South Africa in 100 mL distilled water, stirring for 60 min and filtered using muslin cloth to remove the undissolved materials (Singh et al., 2013). The concentrations of CH, GA and AV were selected based on our preliminary trials with avocado fruits based on the adherence and steadiness of the coating to the fruit surface.

*2.4. In vitro antifungal effects of edible coatings and TO*

Optimum concentration for thyme oil was selected from previous screening trials from 0.1 to 5% concentrations using the well diffusion technique (Suganya et al., 2012). The antifungal effects of GA, AL, CH and thyme oil alone and in combination were performed based on the inhibition in radial mycelia growth of *C. gloeosporioides in vitro* on PDA media (Maqbool et al., 2011). Mycelia disc (6 mm diameter) cut from the peripheral region of 10-12 days old pure culture of *C. gloeosporioides* was transferred to the centre of a 90 mm Petri dish containing PDA amended with GA (10%) alone, AL (2%) alone, CH (1%) alone or in combination with Thyme oil (1%). For combination treatments, edible coating and thyme oil was mixed in two different ratios i.e. [1:1] and [3:1] and includes; GA (10%) + Thyme oil (1%) [1:1 v/v], GA (10%) + Thyme oil (1%) [3:1 v/v], AL (2%) + Thyme oil (1%) [1:1 v/v], AL (2%) + Thyme oil (1%) [3:1 v/v], CH (1%) + Thyme oil (1%) [1:1 v/v] and CH (1%) + Thyme oil (1%) [3:1 v/v]. Stand alone thyme oil (1%) was included for comparison and sterile distilled water was used instead of coating and thyme oil in the control. Petri dishes were incubated at room temperature (25 ± 2 °C). Radial mycelial growth was measured by measuring the colony diameter along the two axes at right angles to each other using a Vernier calliper (Digimatic; Mitutoyo Co., Japan) in mm on daily basis until control Petri dishes were fully covered (7 days) with mycelia. The fungi toxicity was expressed as percentage inhibition of radial mycelial growth (IMG %) using the formula according to Abdollahi et al. (2011): IMG (%) = [(dc-dt) /dc] x 100, where dc and dt are the radial mycelial growth measurements in control.

In order to distinguish between fungicidal and fungistatic activity of the selected edible coating or edible coating thyme oil combination treatment against the *C. gloeosporioides* the mycelial discs that did not show any growth were transferred to a fresh

poured PDA plate and incubated for 7 days at 25 °C to observe the recovery of the growth. This evaluation was carried out as mycelial growth in millimetres (mm). See Table 2. The fungicidal effect was classified as an absence of growth, whereas any observed growth was classified as fungistatic. The edible coating or edible coating and thyme oil combinations that showed fungicidal effect on *C. gloeosporioides* was further investigated on the control of postharvest disease in artificially infected fruit.

*2.5. Inoculation and measurement of disease progress*

Freshly harvested, unblemished avocado fruits of cv. Hass were obtained from Bassan Fruit Packers (Limpopo Province, South Africa). Fruit at the correct stage of maturity were selected according to a finger feel firmness score 2 (1= hard, 2 = slightly soft just started to ripen, 3= very soft) Sellamuthu et al. (2013a) and thereafter, surface sterilized by dipping in sodium hypochlorite (0.01%), for 5 mins and air-dried at room temperature. Subsequently, fruit inoculation for curative treatment was performed according to Sellamuthu et al. (2013a) by uniformly wounding (2 mm deep and 6 mm wide) the fruit with a sterilised cork-borer and inoculating with 20 μL of a spore suspension of *C. gloeosporioides* ($10^5$ spores $mL^{-1}$) at equatorial region. After inoculation fruits were held at room temperature for 24 h. Thereafter, inoculated fruits were dipped in (i) commercial treatment (prochloraz 0.05% for 5 min dip); (ii) CH; (iii) AL; (iv) TO; (v) CH + Thyme oil (3:1) and (vi) AL + Thyme oil (3:1) and allowed to air dry at room temperature. For preventative treatment, avocados fruits were dipped in (i) commercial treatment (prochloraz 0.05% for 5 min dip); (ii) CH; (iii) AL; (iv) TO; (v) CH+ Thyme oil (3:1) and (vi) AL + Thyme oil (3:1), completely dried (about 3 h at 20 °C ) and subsequently inoculated with *C. gloeosporioides* suspension ($10^5$ spores $mL^{-1}$). Packhouses exporting avocados to the UK do not apply Avoshine® wax coating. Therefore, Avoshine® wax coating was not included in commercial treatment. Inoculated but uncoated

fruits dipped in sterile distilled water (untreated control) were also included for comparison. Inoculated and treated fruits (preventative and curative trials) were packed in standard corrugated cardboard cartons and held at 20 °C for 5 days.

For preventative and curative trials each treatment had 20 randomly selected replicate fruits. The experiment was repeated twice. Observations on disease incidence and severity (lesion diameter in mm) were recorded at the end of the storage time (5 days). The disease incidence was determined according to Sellamuthu et al. (2013a) using the following equation:

$$\text{Disease incidence} = \frac{\text{Number of infected wounds}}{\text{Total number of inoculated fruit}} \text{x } 100$$

*2.6. Measurement of active defence response-related enzyme and total phenolic content in avocado fruit.*

Determination of total phenolic content and enzyme assays for PAL, β-1,3-glucanase, chitinase, POD, SOD and CAT were performed from fruits inoculated with *C. gloeosporioides* and subjected to combination treatments; CH and thyme oil (3:1 v/v), AL and thyme oil (3:1 v/v) thyme oil (1%), stand alone coating treatments; CH and AL, stand alone thyme oil, commercial treatment (prochloraz) and untreated control fruits. The enzyme activities were conducted according to Sellamuthu et al. (2013a) by obtaining 0.2 g fruit samples from 6 fruit (2 mm away from the wound inoculated region) randomly selected from the initial 20 samples and homogenizing with specific buffers. The resulting homogenate solution was centrifuged at 15,000 x g for 30 min at 4 ºC and supernatant were used to determine enzyme activities. Sodium phosphate buffer (100mM, 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 was determined according to Assis et al. (2001), with slight modification reported by Sellamuthu et al. (2013a). 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 ºC. 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 200 rt Microplate Reader UK-Biochrom Ltd.). The specific enzyme activity was expressed as nmol cinnamic acid $h^{-1}$ mg of protein.

β-1,3-glucanase activity was determined using a method described by Abels et al. (1971) with slight modification reported by Sellamuthu et al. (2013a). 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 ºC. After the incubation period, 25 µL 3,5-dinitrosalicyclic reagent was added for reaction. The samples were then heated in boiling water for 5 min to stop the reaction. The amount of reducing sugar was determined at 540 nm (Zenyth 200 rt Microplate Reader UK-Biochrom Ltd.). The enzyme activity was expressed in units with one unit defined as the amount of enzyme necessary to catalyze the formation of 1 µmol glucanase equiv. $h^{-1}$ mg of $protein^{-1}$.

Chitinase activity was determined according to the method of Abels et al. (1971) using a reaction mixture consisting of 600 µL of the enzyme extract and 125 µL of 2% (w/v) dye-labelled chitin azure in 50mM sodium acetate buffer (pH 5.0) and incubating for 2 h at 40 ºC. After incubation the reaction was terminated by adding 25 µL of 1 M HCl. The supernatant was measured at 550 nm (Zenyth 200 rt Microplate Reader UK-Biochrom Ltd.).

One unit was defined as the amount of enzyme necessary to catalyze the formation of 1 nmol product $h^{-1}$ $mg^{-1}$ of protein.

POD activity was estimated according to Jiang et al. (2002) method with slight modification shown by Sellamuthu et al. (2013a). Here 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 ºC. Afterwards, 72 µL of $H_2O_2$ (100 mM) was added and the increase in absorbance at 460 nm for 120 s was measured (Zenyth 200 rt Microplate Reader UK-Biochrom Ltd.). The specific activity of the enzyme was expressed as ΔA460 $min^{-1}$ $mg^{-1}$ of protein.

Total phenolic compounds were determined by the method of Sellamuthu et al. (2013a) by extracting the fruit samples (2 mg) with aceton-water (1:1). Total phenolic compounds were quantified according to Singleton et al. (1999) using Folin-Ciocalteu reagent and Sample extract (9 µL). After incubating the sample-reagent mixture for 3 min, 180 µL $Na_2$ $CO_3$ (7.5%) solution was added to each well and incubated at 50 ºC for 5 min, and the absorbance was measured at 760 nm (Zenyth 200rt microplate reader). Gallic acid was used as standard and results were expressed as milligrams of gallic acid equivalent per gram of fruit.

*2.7. Measurement of antioxidant enzyme activities and total antioxidant activity*

CAT activity was assayed as described Beers and Sizer (1952) with slight modifications reported by Sellamuthu et al. (2013a). For this assay the reaction mixture contained 150 µL 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 200 rt Microplate Reader UK-Biochrom Ltd.). The enzyme activity was expressed as units per mg protein (one unit: catalase converts 1 µmol of $H_2O_2$ $min^{-1}$).

SOD activity was estimated photochemically as described in Constantine and Stanley (1977) with a slight modification shown by Sellamuthu et al. (2013a). The reaction mixture (200 µL) included sodium phosphate buffer (100 mM, pH 7.8), methionine (13mM), 75 µM of nitroblue tetrazolium (NBT), EDTA (10µM), riboflavin (2 µM), 100 µL of enzyme extract. Thereafter, 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 solution was kept under the dark. The enzyme activity was expressed as unit $mg^{-1}$ of protein.

The antioxidant activity, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was determined according to the method of Sellamuthu et al. (2013a). Fruit samples (2 mg) were extracted with methanol-water (60:40). The extract was diluted with extraction solution to obtain different concentration of samples. The stock solution of 250 µL DPPH (90 µmol$L^{-1}$) was placed in microplates and 28 µL sample was added. The mixture was sonicated and held in dark for 60 min. Absorbance was read at 515 nm (Zenyth 200rt microplate reader). The results were reported as milligrams of gallic acid equivalent per gram of fruit.

*2.8. Assessment of flesh firmness and flesh colour*

Flesh firmness was determined on three points at the equatorial point of the fruit using a Chitillon Penetrometer, Model DFM50 (Ametek, Largo, Florida, USA), with an 8 mm diameter flat-head stainless steel cylindrical probe (puncture method) (Woolf et al., 2005) after 5 days and the results were reported in Newtons (N). Fruit firmness of 9.8 N represented soft, ripe fruit (Standard ISO 7619, International Organisation for standardisation). Fruit was cut open into two halves and the flesh colour measurement hue angle ($h°$) was taken from two points in each half fruit with a Minolta Chroma Meter CR0-2000 (Minolta Camera Co. Ltd, Tokyo, Japan). The chroma meter was calibrated with a white standard tile. Flesh

firmness and colour measurements were taken on 20 fruit per treatment soon after observations on disease incidence and severity.

*2.9. Statistical analysis*

A complete randomised design was adopted in this study. Data of the experiment were analysed with the General Linear Models (GLM) procedure in the SAS (Statistical Analysis System) computer program (SAS Enterprise Guide 4.0; SAS Institute, 2006, Cary, NC). Means were separated by LSD (5%). All the experiments were repeated twice.

## 3. Results

*3.1. Composition of Thyme oil*

The results of GC/MS analysis of the thyme oil is shown in Table 1. The thyme oil showed 18 components. Amongst these, a phenolic monoterpene thymol (58.77%) and a terpene hydrocarbon with an aromatic ring (4-isopropyltoluene) cymol (17.82%) were found as major components in thyme oil.

*3.2. Effect of edible coatings alone or in combination with thyme oil on radial mycelial growth of C. gloeosporioides*

Fig. 1 illustrates the effect of edible coatings in combination with thyme oil or as stand alone treatments on the radial mycelial growth of *C. gloeosporioides* after 10 days during *in vitro* experiment. It is clearly evident from Fig. 1 that the stand alone edible coatings showed significantly lower ($P < 0.05$) effect on the inhibition of radial mycelia growth of *C. gloeosporioides* than the combined application of the edible coating and thyme

**Table1**

Chemical composition of thyme oil

| Compound | Retention time | Relative area percentage |
|---|---|---|
| α-Pinene | 5.457 | 0.67 |
| Camphene | 5.850 | 0.55 |
| B-Myrcene | 7.000 | 1.02 |
| (+)-4-Carene | 7.850 | 1.05 |
| Cymol | 8.180 | 17.82 |
| D-Limonene | 8.282 | 0.90 |
| Eucalyptol | 8.366 | 0.43 |
| γ-Terpinen | 9.339 | 3.03 |
| α-4-Dimethyl styrene | 10.459 | 0.35 |
| Linalo | 10.933 | 5.64 |
| Borneol | 13.622 | 1.91 |
| Terpineol-4 | 14.126 | 1.33 |
| Thymol methyl ether | 17.014 | 0.47 |
| Thymol | 19.505 | 58.77 |
| ρ-Thymol | 19.754 | 4.17 |
| Caryophyllene | 24.706 | 1.06 |
| Caryophyllene oxide | 31.497 | 0.42 |
| 1,4-Benzenediamine, N,N-dimethyl | 43.388 | 0.41 |

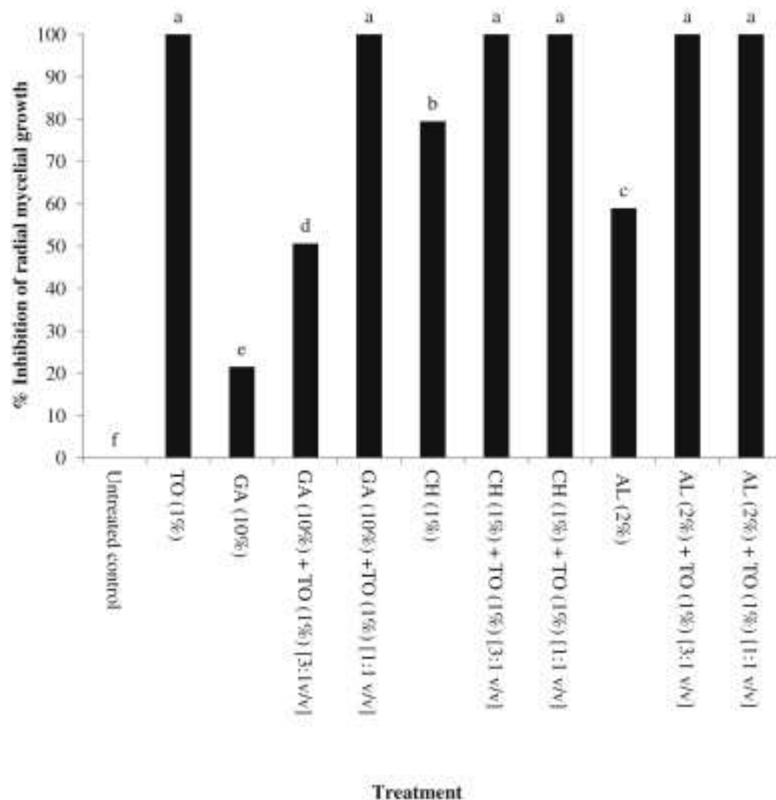


Fig. 1. Effect of edible coatings alone or in combination with and thyme oil on percentage inhibition of radial mycelia growth of *C. gloeosporioides*. Above each column means followed by a common letter are not significantly different at 5% level. TO, thyme oil; GA, Gum Arabic; CH, chitosan; AL, *Aloe vera*.

oil. Among the stand alone edible coating treatments, chitosan coating showed higher inhibitory effect on the radial mycelia growth, while the stand alone GA revealed significantly ($P < 0.05$) lower % inhibitory effect on the radial mycelia growth. Stand- alone thyme oil treatment completely inhibited the radial mycelial growth of *C. gloeosporioides* during *in vitro* as the combination treatments; CH+ thyme oil at both concentrations [(CH + Thyme oil 1:1] and [(CH + Thyme oil 3:1], AL + thyme oil at both concentrations [(AL+ Thyme oil 1:1] and [(AL+ Thyme oil 3:1] and GA + thyme oil at [(GA + Thyme oil 1:1] (Fig. 1). Therefore, incorporating the thyme oil into the edible coating improved their antifungal activity against the *C. gloeosporioides.* Furthermore, thyme oil alone or in combination with all the edible coatings at both concentrations (1:1 or 3:1) except for GA +

**Table 2**

The fungicidal or fungistatic effect of edible coatings alone or in combination with thyme oil on *C. gloeosporioide in vitro*

| Treatment | Radial mycelial growth of *C. gloeosporioide* (mm) |
|---|---|
| Untreated control | 74[a] |
| TO (1%) for 7 days and transferred to freshly poured PDA | 0[i*] |
| GA (10%) for 7 days and transferred to freshly poured PDA | 58[bł] |
| CH (1%) for 7 days and transferred to freshly poured PDA | 15.2[eł] |
| AL (2%) for 7 days and transferred to freshly poured PDA | 30.4[dł] |
| GA (10%) + TO (1%) [1:1] for 7 days and transferred to freshly poured PDA | 5.0[f*] |
| GA (10%) + TO (1%) [3:1] for 7 days and transferred to freshly poured PDA | 36.6[cł] |
| AL (2%) + TO (1%) [1:1] for 7 days and transferred to freshly poured PDA | 0[ł] |
| AL (2%) + TO (1%) [3:1] for 7 days and transferred to freshly poured PDA | 0[i*] |
| CH (1%) + TO (1%) [1:1] for 7 days and transferred to freshly poured PDA | 0[fł] |
| CH (1%) + TO (1%) [3:1] for 7 days and transferred to freshly poured PDA | 0[f*] |

Means in the same column with different letters are significantly different ($P < 0.05$). CH, chitosan; AL, *Aloe vera*; TO, thyme oil. [*] Fungicidal; [ł] Fungistatic; PDA, potato dextrose agar

Thyme oil (3:1) showed fungicidal effects on *C. gloeosporioides* (Table 2). Stand alone edible coatings revealed fungistatic effect on *C. gloeosporioides* (Table 2). Among the stand alone coating treatments, CH showed a significantly higher ($P < 0.05$) fungistatic effect on *C. gloeosporioides* while GA had the least effect. Due to the fungicidal effect recorded during combination treatments; [CH + Thyme oil (3:1) and AL+ Thyme oil (3:1)], theses two treatments were further tested as dip treatments to control anthracnose *in vivo*.

*3.3. Effect of edible coatings alone or in combination with thyme oil on anthracnose incidence and severity in inoculated avocado fruit*

Significant ($P < 0.05$) differences were found on anthracnose incidence and severity between different treatments. In general, preventative dip treatment with CH or AL incorporated with thyme oil or stand alone treatments showed lower incidence and severity of anthracnose compared to curative treatments (Table 3). Preventative CH + thyme oil and AL + thyme oil combination were the most effective and both combination treatments and significantly reduced the % disease incidence by 70% and 55% respectively. Comparably, when applied as curative treatments CH+ thyme oil or AL+ thyme oil reduced disease incidence by 50% and 40% respectively. Thyme oil stand alone treatment showed significantly reduced anthracnose incidence and severity than the stand alone AL and CH treatments. The results of this study showed that the incorporation of thyme oil to the CH and AL coatings (combination) either as preventative or curative significantly ($P < 0.05$) reduced the incidence and severity of anthracnose than the stand alone thyme oil or CH or AL treatments. This indicated a synergistic effect between the chitosan or *Aloe vera* coatings and thyme oil in inhibiting the anthracnose incidence. Fruits treated preventatively and curatively with prochloraz (fungicide) reduced the anthracnose disease incidence by 50% and 40 % respectively. It is clearly evident from this investigation that the chitosan incorporated with

**Table 3**

Effect of edible coatings alone or in combination with thyme oil on the incidence and severity of anthracnose in artificially inoculated avocado fruit

| Treatment[x] | Incidence of Anthracnose (%) | | Severity of Anthracnose (mm) | |
|---|---|---|---|---|
| | Preventative | Curative | Preventative | Curative |
| Untreated control | $90^{a}$ | $90^{a}$ | $34.66^{a}$ | $34.65^{a}$ |
| Prochloraz | $40^{d}$ | $50^{e}$ | $17.78_{c}$ | $22.79_{c}$ |
| CH (1%) | $50^{c}$ | $65^{c}$ | $18.30^{c}$ | $18.76^{d}$ |
| AL (2%) | $60^{b}$ | $75^{b}$ | $20.63^{b}$ | $26.08^{b}$ |
| TO (1%) | $40^{d}$ | $60^{d}$ | $12.70^{e}$ | $15.36^{e}$ |
| CH (1%) + TO (1%) [3:1 v/v ] | $20^{f}$ | $40^{f}$ | $8.94^{f}$ | $11.21^{f}$ |
| AL (2%) + TO (1%) [3:1 v/v ] | $35^{e}$ | $50^{e}$ | $14.38^{d}$ | $15.25^{e}$ |

Means in the same column with different letters are significantly different ($P < 0.05$). The commercial fungicide prochloraz treatment was a 0.05% dip for 5 min.CH, chitosan; AL, *Aloe vera*; TO, thyme oil.

thyme oil significantly reduced the anthracnose incidence and severity than the currently commercially used prochloraz fungicide treatment.

*3.4. Effect of edible coatings combination with thyme oil on the active defence response-related enzymes activities (*PAL, β-1,3-glucanase, chitinase and *POD) and antioxidant enzymes (SOD and CAT )in inoculated avocado fruit*

As shown in Table 4, active defence response-related enzymes; PAL, POD, chitinase and β-1,3-glucanase and antioxidant enzymes; SOD and CAT activities significantly ($P < 0.05$) increased when the inoculated fruits (*C. gloeosporioides*) were coated with CH + thyme oil coating compared to the stand- alone CH or AL or thyme oil treatments. The observed effect between the CH + thyme oil coating on inducing the defence related and antioxidant enzymes was significantly higher than the AL + thyme oil coating (Tables 4).

Moreover infected fruits (*C. gloeosporioides*) coated with CH coating incorporated with thyme oil revealed significantly ($P < 0.05$) higher PAL, POD, chitinase, β-1,3-glucanase, CAT and SOD than the prochloraz fungicide dipped fruits (Table 4). It is evident from this study that the combined application of chitosan coating and thyme oil enhanced the activities of defence response-related enzymes.

*3.5. Effect of edible coatings in combination with thyme oil on total phenolic content and antioxidant activity in inoculated avocado fruit*

The influence of different treatment on total phenol content and antioxidant activity are shown in Table 5. The total phenol content and the antioxidant activity were significantly ($P<0.05$) higher in CH + thyme oil coated fruit than the AL + thyme oil coating, commercial fungicide treatment (prochloraz) and untreated (control) fruit. The combined application of chitosan and thyme oil significantly ($P<0.05$) improved the total phenolic content and the

**Table 4**

Effect of edible coatings alone or in combination with thyme oil on defence related and antioxidant enzymes in artificially inoculated avocado fruit

| Treatment | Defence related enzymes | | | | Antioxidant enzymes | |
|---|---|---|---|---|---|---|
| | PAL activity (Nm cinnamic acid $h^{-1}$ mg of $protein^{-1}$) | POD activity (A460 $min^{-1}$ mg of $protein^{-1}$) | Chitinase activity ( Nm product acid $h^{-1}$ mg of $protein^{-1}$) | β-1.3-Glucanase activity (μM glucose equiv.$h^{-1}$ mg of protein) | CAT activity (U mg of $protein^{-1}$) | SOD activity (U mg of $protein^{-1}$) |
| Untreated control | 12.86[e] | 1.47[e] | 0.81[e] | 4.06[g] | 1.32[e] | 115.13[e] |
| Prochloraz | 18.10[d] | 1.52[d] | 1.10[d] | 8.71[e] | 1.37[c] | 124.31[d] |
| CH (1%) | 23.96[c] | 1.71[b] | 1.58[b] | 9.29[d] | 1.35[d] | 167.76[b] |
| AL (2%) | 18.92[d] | 1.49[e] | 0.79[e] | 7.14[f] | 1.35[d] | 118.87[e] |
| TO (1%) | 23.36[c] | 1.63[c] | 1.25[c] | 10.66[c] | 1.38[c] | 131.98[c] |
| CH (1%) + TO (1%) [3:1 v/v ] | 38.86[a] | 1.85[a] | 2.52[a] | 14.35[a] | 1.47[a] | 177.63[a] |
| AL (2%) + TO (1%) [3:1 v/v ] | 33.29[b] | 1.73[b] | 1.32[c] | 11.21[b] | 1.42[b] | 129.63[c] |

Means in the same column with different letters are significantly different ($P < 0.05$). The commercial fungicide prochloraz treatment was a 0.05% dip for 5 min. CH, chitosan; AL, *Aloe vera*; TO, thyme oil. PAL, phenylalanine ammonia-lyase; POD, peroxidise; CAT, catalase; SOD, superoxide dismutaste.

antioxidant activity in infected avocado fruits during incubation period than the AL + thyme oil coating.

*3.6. Effect of edible coatings alone or in combination with thyme oil on fruit firmness and flesh colour*

Significantly ($P < 0.05$) higher fruit firmness (18.1 N) was retained in fruits dipped in CH+ thyme oil coating followed by the AL + thyme oil (15.5 N) and prochloraz fungicide (15.1 N) treatments (Fig. 2). No significant differences ($P > 0.05$) in firmness were noticed

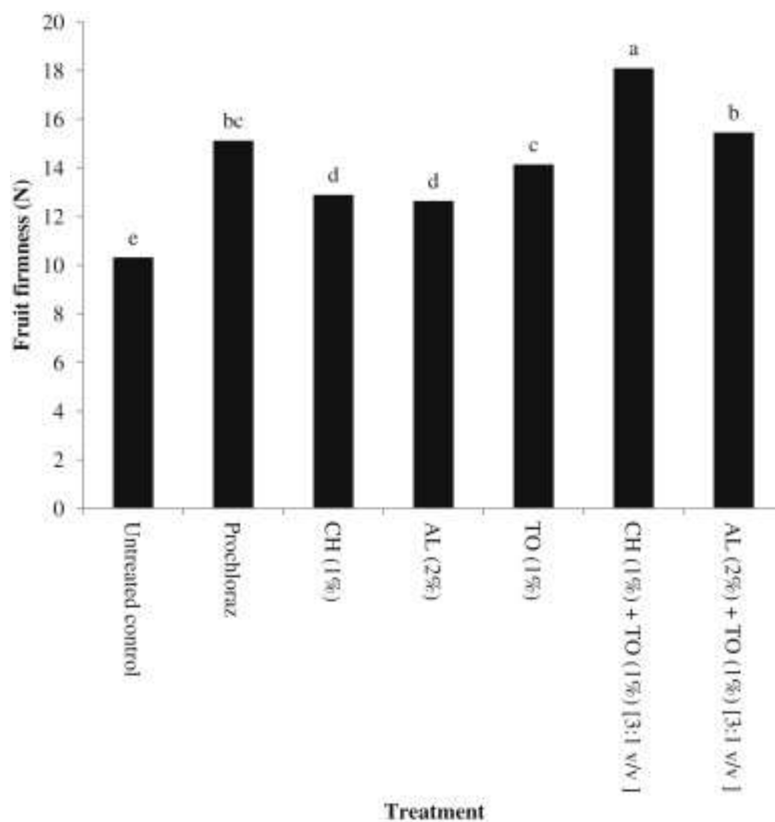


Fig. 2. Effect of edible coatings alone or in combination with thyme oil on fruit firmness in artificially inoculated avocado fruit. Above each column means followed by a common letter are not significantly different at 5% level. The commercial fungicide prochloraz treatment was a 0.05% dip for 5 min. TO, thyme oil; GA, Gum Arabic; CH, chitosan; AL, *Aloe vera*.

between stand alone CH and AL treatments as well as stand alone TO treatment. The untreated control fruits showed the lowest firmness values of 10.3 N as a result of excessive softness due to faster ripening. Higher $h°$ values indicate that the colour of the mesorcap is maintained within the yellowish-green spectrum. Uncoated control fruit showed lower $h°$ value (more yellow) indicating faster ripening of mesorcarp. In fruits treated with CH coating + thyme oil or CH coating or AL + thyme oil coating and AL coating, ripening was delayed and underwent slower changes in $h$ ° (Fig. 3). However, $h$ ° value in fruits subjected to the prochloraz fungicide treatment were lower than the fruits coated with CH + thyme oil coating or AL + thyme oil or dipped in thyme oil stand alone treatment indicating that theses fruits are less ripened than the prochloraz treated fruits.

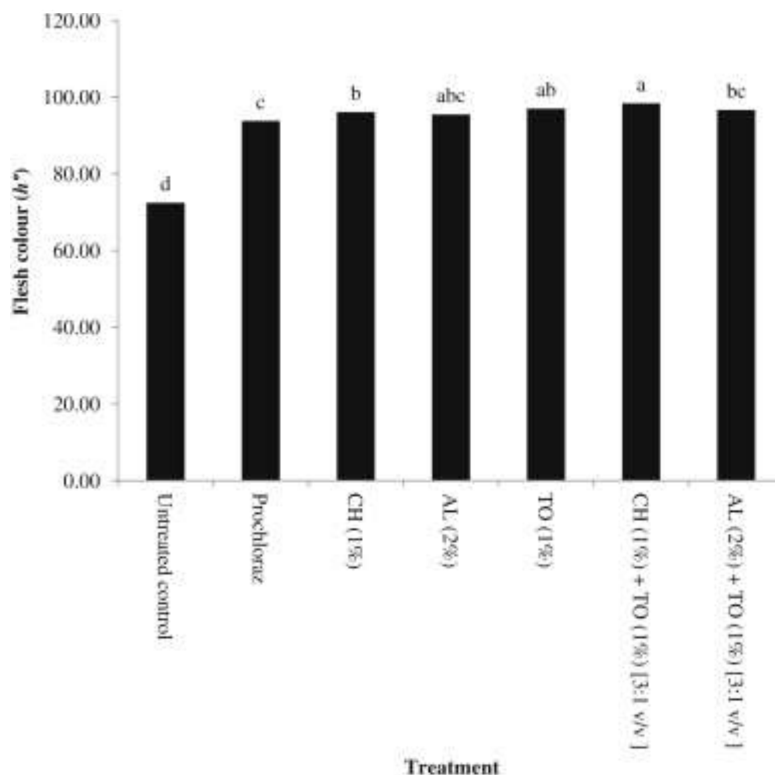


Fig. 3. Effect of edible coatings alone or in combination with and TO on flesh colour in artificially inoculated avocado fruit. Above each column means followed by a common letter are not significantly different at 5% level. The commercial fungicide prochloraz treatment was a 0.05% dip for 5 min. TO, thyme oil; GA, Gum Arabic; CH, chitosan; AL, *Aloe vera*.

## 4. Discussion

It is evident from our study that chitosan coating incorporated with thyme oil effectively reduced the anthracnose incidence and severity in avocado ‘Hass’. Essential oils are regarded as low risk targets for the development of microbial resistance and can therefore contribute to a longer useful lifespan of currently used fungicides (Wilson et al., 1997; Tatsadjieu et al., 2009). The antifungal activity of thyme oil is well documented and proven to inhibit the fungal growth of *C. gloeosporioides in vitro* or *in vivo* in avocados (‘Hass’ and ‘Fuerte’) (Sellamuthu et al., 2013a). Thyme oil directly inhibits pathogen growth and spore germination by affecting the active sites of enzymes and cellular metabolism (Arrebola et al., 2010). The presence of phenolic rings and hydroxyl groups on the phenol rings of thymol and cymol, (active volatile compounds) in thyme oil enhanced its antimicrobial activity (Bagamboula et al., 2004). Thyme oil (thymol active ingredient) was reported to reduce postharvest 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), strawberries (Wang et al., 2007) and kiwi (Shirzad et al., 2011). Phenol content was reported to play a major role in plant resistance and defence mechanism against invasion of plant pathogens (Beckman, 2000). The improvement of antioxidant capacity and scavenging activity by the influence of thyme oil has shown to enhance resistance of avocado fruit tissues (‘Hass’) against *C. gloeosporioides* (Sellamuthu et al., 2013a). CH coating was also reported to induce disease resistance against postharvest fungal diseases (El Ghaouth et al., 2000; Ben-Shalom et al., 2003; Romanazzi et al., 2003). CH coating was also shown to intensify the total antioxidant capacity via increasing the phenolic compounds in treated apricot fruit (Ghasemnezhad et al., 2010).

Furthermore, CH coating was reported to control anthracnose during postharvest storage in papaya (*C. gloeosporioides*) (Bautista-Baños et al., 2003; Ali et al., 2010),

mangoes (*C. gloeosporioides*) (Zhu et al., 2008) and bananas (*C. musae*) (Maqbool et al. 2010). Moreover the incorporation of essential oils into CH coating was shown to improve the CH coating's antifungal properties (Kanatt et al., 2008; Kyu Kyu Win et al., 2007, Perdones et al., 2012). However, there is no information reported regarding the combined inhibitory effect of CH coating and thyme oil in controlling anthracnose in avocado during postharvest storage. Our results clearly stated that the increase of total phenolic compounds in fruits coated with CH coating incorporated with thyme oil. Similarly thyme oil in combination with modified atmosphere packaging was shown to increase the concentrations of total phenols and flavonoids (catechin), in avocado cultivars (Sellamuthu et al., 2013a). Chitosan coating showed film forming properties and was reported to create a modified atmosphere around the fruits (Sivakumar et al., 2005). The phenylalanine ammonia-lyase (PAL) is the primary enzyme involved in the biosynthesis of phenols (Cheng and Breen, 1991). CH coating has the ability to induce the PAL enzyme activity (Romanazzi et al., 2002). However, our data confirms that the incorporation of thyme oil into CH coating enabled to enhance the synthesis of phenolic compounds by significantly inducing the PAL activity.

Different researchers have demonstrated the association between the higher activity of chitinase and 1.3,β-glucanase (PR proteins) and enhanced disease resistance against postharvest decay. CH coating was reported to improve the chitinase and 1.3, β-glucanase activities in oranges, strawberries and raspberries (Fajardo et al., 1998; Zhang and Quantick, 1998). Our data showed that the incorporation of thyme oil into CH coating improved the activities of chitinase, β-1,3-glucanase and POD in avocado fruit than the stand alone CH coating or thyme oil treatment. Chitinase and β-1,3-glucanase play a major role in plant defence mechanisms against fungal pathogens by facilitating the biochemical reactions involved in hydrolysing polymers of fungal cell wall (Dumas-Gaudot et al., 1992; Collinge,

1993). POD was associated with disease resistance and involved in synthesis of phenolic cross links connecting adjoining biopolymer chains (Mohammadi et al., 2002). Similar results were reported with the combined application of CH-coating and natural volatile methyl jasmonate on cherry tomatoes infected with *Alternaria alternate* (Cao et al., 2008), and peach (cv. Baifeng) (Jin et al., 2009).

The antioxidant enzymes were reported to show a positive relationship with plant resistance to pathogens. Reactive oxygen species (ROS), $O_2^{\cdot -}$ and $H_2O_2$ were reported to be involved during the early stage of defence mechanism that correlate with plant resistance to pathogens (Torres et al, 2002). However, according to Baker and Orlandi (1999) during latter stage of pathogenesis increased production of ROS can contribute to cell degeneration. SOD, 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. Therefore, the increased antioxidant enzyme activities (SOD, POD and CAT) in thyme oil incorporated CH coating would have delayed the degeneration of infected cells by *C. gloeosporioides* and protected the cell membrane structure and function of the fruit tissue, maintained higher levels of phenolic content (antioxidant capacity). All these biochemical changes could contribute to enhance the resistance of fruit tissue against invasion of *C. gloeosporioides* and to slow down the spread of anthracnose.

Loss of firmness affects the fruit quality during marketing. CH coating was reported to retain fruit firmness by inhibiting the macerating enzyme activity such as polygalacturonase, pectate lyase, and cellulase in tomatoes (Reddy et al., 2000) and peaches (Atkinson et al, 2012). Thyme oil in combination with modified atmosphere packaging was also noted to retain the fruit firmness by delaying the ripening (Sellamuthu et al., 2013b). However, in this study thyme oil incorporated CH coating enabled to reduce the incidence of anthracnose and thereby further maintained the fruit firmness and delayed the ripening which was shown by the delayed pulp colour (yellow, higher *h* °) development. In a similar study

with oranges, the combination of chitosan with either bergamont oil or tea tree oils was also found to delay the fruit ripening and consequently the loss of firmness (Cháfer et al., 2012).

In conclusion the thyme oil in CH coating (combination treatment) offers great practical potential in reducing the anthracnose incidence during the postharvest supply chain. However, this application must be tested in the future in naturally infected fruit in order to provide an effective decay control measure to the organic avocado fruit industry.

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