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Differential response to combined prochloraz and thyme oil drench treatment in avocados against the control of anthracnose and stem-end rot

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Abstract The ability to meet consumers demand for high-quality standard fruit entails the distribution of unblemished safe fruit free of chemical residues on its edible portion. Therefore, this study was focused on investigating the influence of the combined effect of aqueous plant volatiles with half strength prochloraz solution to control anthracnose and stem-end rot in the green-skinned avocado cultivar (Fuerte). This method was applied due to its practicability on bulk fruits in packhouses and the fruits were subjected to stand-alone and combined treatments to assess the development of the disease after cold storage and observe the elicitation of the residual effect of the treatments on the defence related enzymes in 'Fuerte'. The incidence of stem-end rot was 10% by the combination of prochloraz® (500 μg mL^{-1}; P50) with 0.1% *v*/v thyme oil compared to the 58.8% incidence exhibited by the untreated fruit during storage at 6.5 °C for 14 days followed by 3 days at retail shelf conditions (15 °C) (preventative application). Citral (0.1% *v*/v), P50 (500 μg mL^{-1}) + 0.1% v/v citral and yucca extract alone reduced the stem-end rot incidence to about 25% during storage. More so, thyme oil (0.1% *v*/v) reduced both anthracnose and stem-end rot incidence to 35% after postharvest storage and P50 (500 μg mL^{-1}) + 0.1% v/v thyme oil and 0.1% v/v thyme oil effectively induced the activity of phenylalanine ammonia lyase, chitinase and β-1, 3 glucanase in fruit inoculated with *Lasiodiplodia theobromae* and *Colletotrichum gloeosporioides* through the improvement of quality and firmness of the fruit after storage.

Keywords Fungicide · Citral · Essential oils · Defence · Postharvest diseases

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Introduction

Avocado production in South Africa is export-oriented and the green-skinned cultivar is rapidly gaining popularity in the international markets (Cordes 2016). Due to substantial economic losses resulting from postharvest diseases of horticultural crops, priority has been given to certifying a high fruit quality standard by consumers (Kade and Rolle 2004). Recently, a South African avocado exporter reported a high demand for green-skinned cultivars in the European market compared to previous years. 'Fuerte', a green-skinned cultivar, is a hybrid of Mexican and Guatemalan types with characteristic tolerance to cool temperatures (Crane et al. 2013; Wolstenholme 2013). The skin of 'Fuerte' is smooth, thin and does not stick to the mesocarp when ripe, thus this is one of the fruit characteristics favourable to consumers. Fuerte cultivars are more susceptible to fungal-associated pathogenic attack compared to its counterpart 'Hass' (Schaffer et al. 2013). Generally, avocado fruit is susceptible to various postharvest diseases but anthracnose (*Colletotrichum gloeosporioides*)

(Prusky et al. 2014) and stem-end rot which is associated with several endophytic pathogens, including *Lasiodiplodia theobromae*, are the industry's major problematic diseases (Akgül et al. 2016; Madhupani and Adikaram 2017).

Infections attributed to the fungi remain quiescent until ripening resumes on the harvested fruit (Beno-Moualem and Prusky 2000). Several fungicides are applied at a postharvest stage in packhouses as a measure to control and improve the fruit quality during storage and marketing (Sivakumar and Bautista-Baños 2014). Prochloraz, a non-systemic imidazole fungicide (Fig. 1) is commonly used in agriculture against plant disease caused by viruses, bacteria and predominantly fungi including ascomycetes such as *Colletotrichum* species. In South Africa, prochloraz is an important registered postharvest fungicide that exhibits eradicative activity against phytopathogen and the avocado fruit (Bowyer and Denning 2014). Adoption of a synthetic approach to control the diseases has elevated consumers' concerns about the harmful effect of the chemicals and its possible degradation in the environment and human health (Krystallis and Chryssohoidis 2005). Studies have demonstrated some limitations associated with the use of plant volatiles in vapour form, i.e. cost, phytotoxicity and bulk application of the treatments in packhouses (Ferhat et al. 2006; Lopez-Reyes et al. 2013). On the other hand, induction of defence enzymes and activation of biosynthetic pathways to defend the fruit from potential pathogens have been recognized as important strengths of applying natural alternatives at the pre- and postharvest stages of horticultural crops (Shao et al. 2015; Wu et al. 2017).

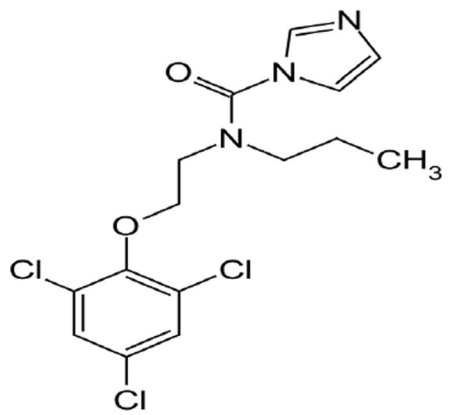

Fig. 1 The molecular structure of prochloraz

Studies have revealed that natural alternatives such as essential oils on wounded fruits could act as elicitor in triggering secondary metabolites involved in defence mechanisms (Ruiz-García and Gómez-Plaza 2013). This has been confirmed in thyme oil which has been suggested to be a relevant natural elicitor used in the management of avocado and mango fruits in South Africa (Bill et al. 2016). Analysis of thyme oil has shown thymol (Fig. 2a) to be the major active constituent present in thyme essential oil (Borugă et al. 2014). Analysis of some chemotypes of aromatic plant and citrus cultivar peels have demonstrated to possess citral (Fig. 2b), a naturally occurring compound of two isomeric aldehydes as the main active component and studies have documented their efficacy in the inhibition of phytopathogen and improvement of fruit quality. Furthermore, citral reduced the incidence of anthracnose and stem-end rot by 75% in the vapour phase (Obianom and Sivakumar 2018).

Elicitors are recognized as compounds involved in triggering chemical defence mechanisms through various biosynthetic pathways such as the shikimic pathway, phenylpropanoid pathway and flavonoid pathways to defend the plant against potential pathogens (Gonzalez-Aguilar et al. 2010; Ruiz-García and Gómez-Plaza 2013; Thakur and Sohal 2013). Initiation of a natural approach to control postharvest diseases of horticultural crops could reduce the prospects of chemical use in disease management by contributing to the development of sustainable agriculture in future. Generally, plant volatiles is active in their volatile phase (Bill et al. 2016), although plant volatiles can be incorporated in wax formulations for fruit coatings. However, the thyme oil or citral application must be successfully integrated into the current avocado packing line as a dip treatment in order to reduce the cost involved in restructuring the packing line. Therefore, the aim of this study is to investigate the efficacy of plant volatiles (Thyme oil or citral) in dip treatments as stand-alone or in combination with half strength prochloraz (500 μg mL^{-1}) on (a) the control of postharvest diseases (anthracnose or stem end rot) in avocados and to (b) investigate the treatment as an inducers of defence-related enzyme activities of 'Fuerte' avocados.

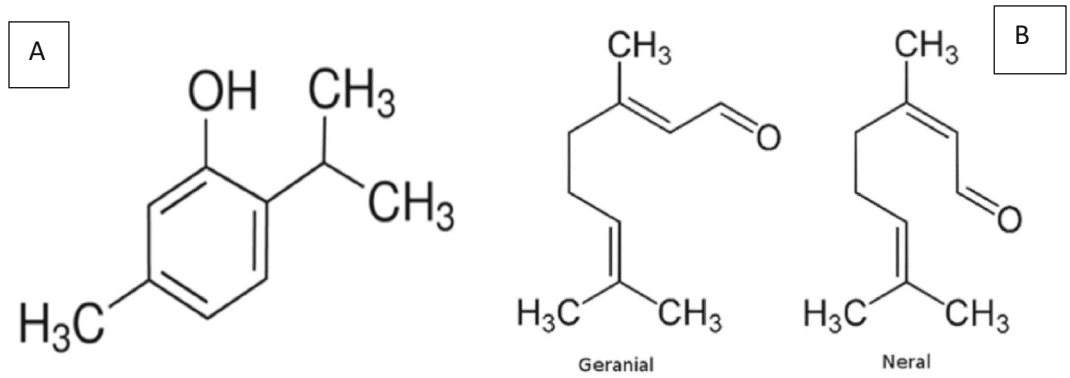

Fig. 2 Chemical structures of (**a**) Thymol (an active component of thyme oil) and (**b**) citral

Material and methods

Pathogens

C. gloeosporioides and *L. theobromae* were obtained from Westfalia Laboratories, Limpopo. The isolates were continuously sub-cultured and maintained on malt extract agar (MEA) and potato dextrose agar (PDA) respectively at 25–30 °C.

Plant volatiles

Citral was purchased from Sigma-Aldrich (Johannesburg, South Africa), thyme oil from Vital Health Foods, South Africa and Yucca extract from OII-YS, Venture Innovations (Lafayette, LA, USA). Chronos 45 EC (active ingredient Prochloraz; 45,000 μg/ml) was purchased from Makhteshim-Agan SA (Pty) Ltd. South Africa.

In vivo experiment

Freshly harvested 'Fuerte' avocado with accurate maturity index was purchased from Bassan Fruit Packers, (Tzaneen, South Africa). The fruit surface was sterilized by dipping in sodium hypochlorite (0.01% NaOCl) for 5 min and allowed to air-dry before subjecting the fruit to preventative treatments (Bill et al. 2014). These defence inducers were selected from the in vitro tests.

For preventative treatment, the fruit was initially dipped in different defence inducers and thereafter, inoculated the fruit with the *C. gloeosporioides* or *L. theobromae respectively*. Although the pathogen gains entry via direct penetration, the fruit was inoculated in this study to prevent the contamination occurring at the packhouse. The plant volatiles was dissolved in Tween 80 (1% *v*/v) and vortexed to obtain a uniform mixture of the required solution (aqueous treatment). The fruit was immersed in the following aqueous treatments: (a) 0.1% v/v thyme oil alone, (b) 0.1% v/v citral alone, (c) Yucca extract solution (d) Prochloraz® alone (1000 μg mL^{-1}), (e) Prochloraz® (500 μg mL^{-1}; P50) (f) P50 (500 μg mL^{-1}) + 0.1% v/v thyme oil, (g) P50 (500 μg mL^{-1}) + 0.1% v/v citral, (h) P50(500 μg mL^{-1}) + Yucca extract and distilled water as control treatment before allowing to air-dry. Thereafter, the fruit was uniformly wounded (artificial inoculation) with a sterile needle (2 mm × 2 mm) and inoculated with 20 μL of the fungal spore suspension (10^5 spores mL^{-1}) at the equatorial (*C. gloeosporioides*) and stem-end regions (*L. theobromae*) of the fruit. Altogether a set of 100 fruits were inoculated with a specific pathogen and one wound per fruit was created. After inoculation, the spores were allowed to initiate for 6 h before being transferred to a 25 L container (90% RH). A set of 20 fruit was placed in each punnet and held at 6.5 °C for 14 days. Five punnets per treatment were included in this study. After cold storage, the fruits were displayed at simulated market shelf conditions of 20 °C for 3 days (preventative application). On the third day, the anthracnose or stem-end rot incidence, severity and the defence related enzymes were assessed as described by Bill et al. (2016).

Determination of defence-related enzymes in artificially infected fruit after storage

To determine the defence-related enzymes in preventatively treated fruit, methods described by Sellamuthu et al. (2013) was adopted. The enzymes

PAL (phenylalanine ammonia-lyase), Chitinase and β-1,3-glucanase activity were spectrophotometrically analysed from fruit infected with *C. gloeosporioides* and *L. theobromae* and treated with the following: (a) 0.1% *v*/v thyme oil alone, (b) 0.1% v/v citral alone, (c) Yucca extract solution (d) Prochloraz® alone (1000 μg mL^{-1}), (e) Prochloraz® (500 μg mL^{-1}; P50) (f) P50 (500 μg mL^{-1}) + 0.1% v/v thyme oil, (g) P50 (500 μg mL^{-1}) + 0.1% v/v citral, (h) P50 + Yucca extract including the control replicates. The enzymes were analysed by weighing 200 mg of avocado mesocarp obtained from the healthy margin around the inoculated region. Two-milligram sample of the fruit tissue was homogenized in the appropriate buffer and centrifuged at 15,000 g for 10 min at 4 °C. The homogenates were used to determine the enzyme activities in the fruit. PAL activity was determined from 5 replicate fruit per treatment.

Enzyme analysis

The PAL activity in the fruit was determined according to a method modified by Sellamuthu et al. (2013) with slight modification. The reaction was terminated by adding 75 μL of 1 M HCl and the production of cinnamate was measured at an absorbance of 290 nm (Zenyth 200rt Microplate Reader, UK-Biochrom Ltd., Cambridge, UK). The activity of the enzyme was expressed as moles of cinnamic acid produced per mass of protein (nmol kg^{-1} s^{-1}).

The Chitinase activity was performed by the method modified by He et al. (2017). The reaction was terminated by adding 25 μL of 1 M HCl. The supernatant was measured at an absorbance of 550 nm and each unit was defined as the amount of enzyme required to catalyse the rate of production of moles per product mass of protein (nmol kg^{-1} s^{-1}).

The β-1, 3-glucanase activity was determined according to the method adopted by Bill et al. 2016. The amount of reducing sugar was determined at an absorbance of 540 nm and each unit was expressed as the amount of enzyme required to facilitate the formation of glucose equivalent production rate per mass of protein (nmol kg^{-1} s^{-1}).

Protein content from the enzyme extract was determined using the method by Bradford (1976) with bovine serum as the standard.

Total phenolic content

The total phenolic content was determined and quantified by the method of Singleton et al. (1999) using the Folin-Ciocalteu reagent with slight modifications. The absorbance measured at 760 nm and Gallic acid equivalent was used as a standard and the results obtained were expressed as kg of gallic acid equivalent kg^{-1} of fruit.

Statistical analysis

The experiments were performed in a completely randomized design and repeated twice to confirm the preliminary observations. Each treatment, consisting of 20 fruit replicates, was subjected to analysis of variance using the GenStat for Windows (2004) statistical package. Fisher's protected least significant difference (LSD) test was performed at 5% level of significance.

Results

Influence of the plant volatiles in aqueous phase on disease development in the fruit

The incidences of anthracnose were reduced to less than 50% with the following treatments: 0.1% thyme oil (35.29%), P50 + 0.1% *v*/v thyme oil, [Y-extract and 0.1% v/v citral] (47.5%) respectively and Prochloraz (41.15%) after postharvest storage (6.5 °C for 14 days and 3 days at the retail shelf). On the other hand, 1% *v*/v citral and [P50 + 0.1% *v*/v citral] showed 17.6% stem-end rot incidence and [P50 + 0.1% v/v thyme oil] showed prevalent control of stem-end rot (10%) compared to anthracnose after storage (Fig. 3).

Induction of defence-related enzymes and total phenolic content

Apparently, the effect of 0.1% *v*/v thyme was observed through the induction of defence-related enzyme activities in the treated fruit. The PAL activity and total phenolic content were significantly induced by P50 + 0.1% *v*/v thyme oil and 0.1% *v*/v thyme oil in fruit infected with *L. theobromae* and *C. gloeosporioides* respectively, compared to the other treatments adopted in this study (Tables 1 and 2). Similar trend was observed with chitinase and β-1, 3-glucanase activities. It

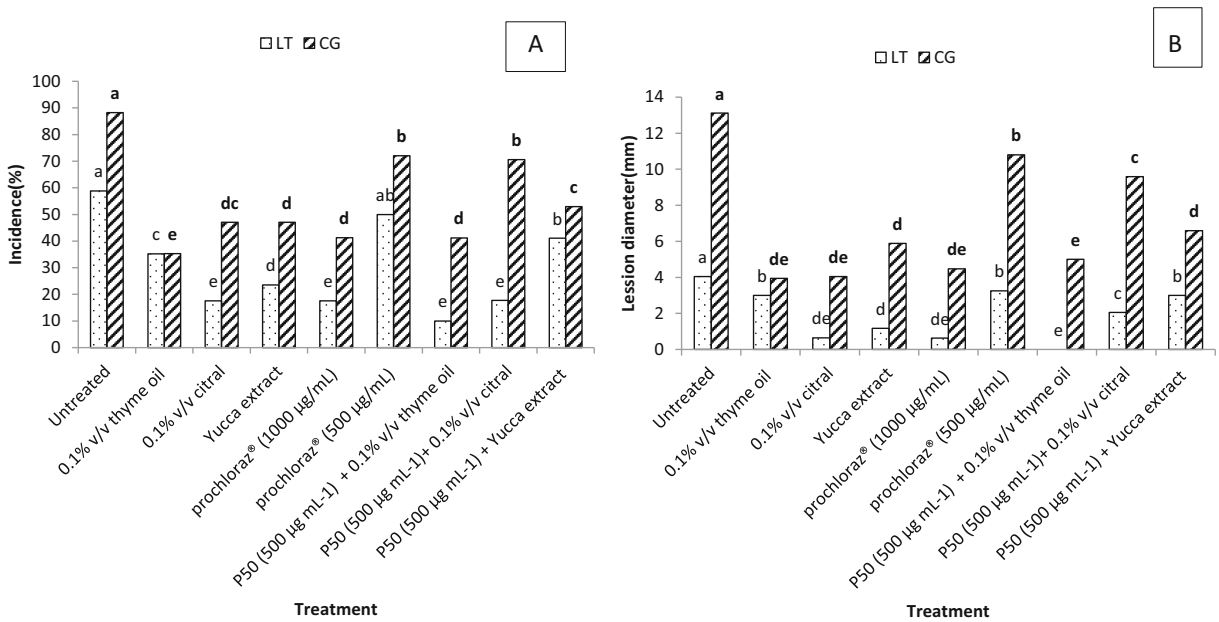

Fig. 3 Antifungal activity of defence inducers on the [a] incidence and [b] severity of stem-end rot and anthracnose in artificially infected 'Fuerte' avocadoes after 14 days cold storage at 6.5 °C and 3 days the retail shelf at 20 °C. LT: *L. theobromae*; CG: *C. gloeosporioides.* Above each bar, means followed by a common letter are not significantly different at 5% level of the treatment

is also evident that the activity of the PAL, chitinase, β-1, 3-glucanase and the total phenolic content were highly induced by the presence of 0.1% *v*/v thyme in fruits inoculated with both *C. gloeosporioides* and *L. theobromae* but it was remarkable in *C. gloeosporioides* inoculated fruit subjected to 0.1% v/v thyme oil compared to *L. theobromae* inoculated fruit that was subjected to [P50 + 0.1% v/v thyme oil] respectively. Similar results were observed with respect to preventively applied 0.1% v/v thyme oil or P50 + 0.1% v/v thyme oil on chitinase and β-1,3-glucanase activities in fruit inoculated with *C. gloeosporioides* and *L. theobromae* respectively. Fruit infected with *C. gloeosporioides* was firmer

Table 1 Influence of postharvest treatments on the defence-related enzymes in 'Fuerte' avocadoes infected with *Colletotrichum gloeosporioides*

Treatment	Total phenolic content (kg GA* equiv. kg^{-1} FW) $\times 10^{-3}$	PAL activity (nmol kg^{-1} s^{-1}) $\times 10^{-2}$	Chitinase activity (nmol kg^{-1} s^{-1}) $\times 10^{-2}$	β-1.3- glucanase activity (nmol kg^{-1} s^{-1})
Untreated	45.02e	0.69f	0.12e	0.17f
0.1% *v*/v thyme oil	102.28a	1.33a	0.39a	1.10a
0.1% v/v citral	59.32d	0.87d	0.18c	0.78c
Yucca extract	54.83d	0.89d	0.17 cd	0.62e
Prochloraz (1000 µg mL^{-1})	82.09b	1.09b	0.18c	0.94b
P50 (500 µg mL^{-1}) + 1% v/v thyme oil	65.64c	0.97c	0.23b	0.90b
P50 (500 µg mL^{-1}) + 1% v/v citral	45.83e	0.84e	0.16d	0.72d
P50 (500 µg mL^{-1}) + Y- extract (26.5 mL L^{-1})	47.74de	0.85de	0.16d	0.81c

Means in each column with the same letters are not significantly different at 5% level of the treatment, Fisher''s LSD

GA, Gallic acid; *FW*, Fresh weight; *PAL*, Phenylalanine ammonia-lyase

Table 2 Influence of aqueous postharvest treatments on the defence-related enzymes in 'Fuerte' avocadoes infected with *Lasiodiplodia theobromae*

Treatment	Total phenolic content (kg GA equiv. kg^{-1} FW) × 10^{-3}	PAL activity (nmol kg^{-1} s^{-1}) × 10^{-2}	Chitinase activity (nmol kg^{-1} s^{-1}) × 10^{-2}	β-1.3- glucanase activity (nmol kg^{-1} s^{-1})
Untreated	35.58c	0.19d	0.09c	0.05d
0.1% v/v thyme oil	43.85b	0.24c	0.10b	0.13c
0.1% v/v citral	52.19a	0.30b	0.13b	0.16b
Yucca extract	50.08ab	0.22c	0.12b	0.14c
Prochloraz (1000 μg mL^{-1})	46.41b	0.25c	0.12b	0.14c
P50 (500 μg mL^{-1}) + 1% v/v thyme oil	56.10a	0.43a	0.27a	0.20a
P50 (500 μg mL^{-1}) + 1% v/v citral	54.39a	0.27c	0.14b	0.16b
P50 (500 μg mL^{-1}) + Y- extract (26.5 mL L^{-1})	49.18b	0.22c	0.12b	0.14c

Means in each column with the same letters are not significantly different at 5% level of the treatment, Fisher''s LSD

GA, Gallic acid; *FW*, Fresh weight; *PAL*, Phenylalanine ammonia-lyase

compared to *L. theobromae,* but predominant in fruits dipped in 0.1% v/v thyme oil and 0.1% v/v citral for both infections.

For anthracnose infected fruit, considerably higher firmness was observed in 0.1% v/v thyme oil (2.05 kg) and 0.1% v/v citral (1.48 kg), hence the least firmness was obtained by fruits dipped in P50 + yucca extract (0.89 kg). Stem-end rot infected fruit were firmer when dipped in 0.1% v/v thyme oil (0.98 kg), 0.1% v/v citral (0.99 kg) and P50 + 0.1% v/v citral (0.93 kg), while the least firmness was obtained by fruits dipped in yucca extract (0.801 kg) and P50 + yucca extract (0.804 kg) (Fig. 4).

Discussions

It is evident from this study that thyme oil (0.1% *v*/v) in aqueous phase alone resulted in high stem-end rot incidence and severity, and the combined application of thyme oil (0.1% v/v) in aqueous phase with prochloraz® (500 μg mL^{-1}; P50) as a dip treatment in packhouse tank enabled significant control of stem-end rot. This is the first report on the combination of thyme oil vapours in the aqueous phase with prochloraz® (500 μg mL^{-1}) application. Application of thyme oil in the aqueous phase is beneficial as a dip treatment since it can be easily implemented on the packing line with the

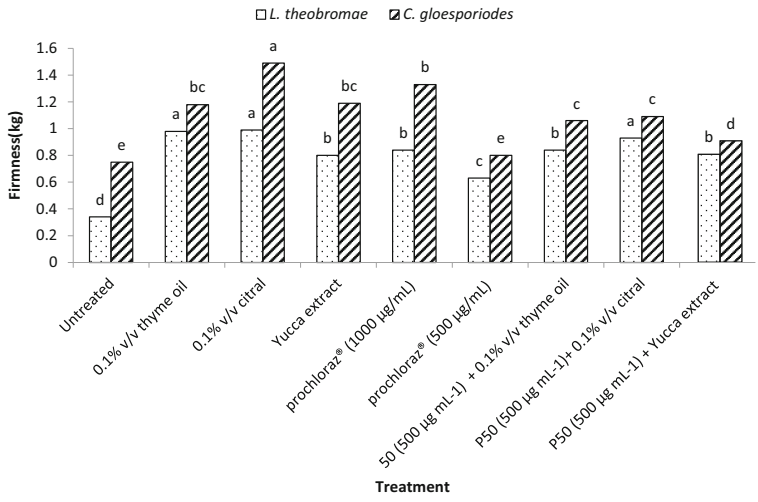

Fig. 4 Fruit firmness of 'Fuerte' avocadoes infected with *Lasiodiplodia theobromae* and *Colletotrichum gloeosporioides.* Firmness collated after 14 days cold storage and 3 days the retail shelf at 20 °C. Above each bar, means followed by a common letter are not significantly different at 5% level of the treatment

fungicide treatment. Bill et al. (2016) and Perumal et al. (2017) demonstrated that thyme oil concentration in vapour form inhibited the growth of artificiality inoculated *C. gloeosporioides* in avocado fruits through the reduction of the anthracnose incidence during storage and similar correlation was observed in this study but in aqueous form. The outcome of this study demonstrated that the aqueous treatment of thyme oil (0.1% v/v) and prochloraz® (500 μg mL^{-1}) delayed the anthracnose and stem-end rot incidence hence provided protection against fruit decay during storage.

The induction of total phenols, PAL and PR-proteins (chitinase and β-1, 3-glucanase) in the fruit might be attributed to the eliciting influence of the natural fungicides applied on the fruit before inoculation of the fungal spore suspension during storage in this study (Shao et al. 2015; He et al. 2017; Perumal et al. 2017).

Similar results have been demonstrated in studies performed on mango (Perumal et al. 2017), avocado (Glowacz et al. 2017), banana (Sun et al. 2013) and passion fruit (Parkinson et al. 2015) with natural fungicides. However, chitinase, β-1, 3-glucanase are inductive and secreted by fungi in cultures enriched with chitin and glucan compounds as its carbon source. Chitinase, β-1, 3-glucanase and PAL activity is important in plant responses in the events of pathogen attack, temperature, wounding as defence mechanisms. Chitinase and β-1, 3-glucanase have been established to play a significant role in plant defence mechanism through degradation of the fungal cell walls (Manjula and Podile 2005) and a similar trend was observed in this study through the reduction of disease incidences. Importantly, PAL is a key precursor involved in the phenylpropanoid pathway which is essential in modulating primary and secondary metabolisms such as phenols, flavonoids and its derivatives in plants (Kim and Hwang 2014). The enzyme activity of PAL was high in this study and its role in biosynthetic pathway might have synthesized the stimulation of phenolic content in artificially infected fruits treated with thyme oil(0.1 *v*/v) and P50 + 0.1 v/v thyme oil. Ebrahim et al. (2011) suggested β-1, 3-glucanase as a direct plant response against fungi through hydrolysis of the fungal cell walls in permutation with chitinase thereby initiating lysis of fungal cells (Sharma et al. 2011; Balasubramanian et al. 2012). Additionally, reports show that chitinase and β-1, 3-glucanase are available at lower concentrations in fruit (Balasubramanian et al. 2012) but could be radically induced through the application of plant volatiles as observed in this study. Naturally, *C. gloeosporioides* (anthracnose) attacks fruits through direct penetration (Prusky and Lichter 2008) but this result and other studies on anthracnose control by thyme oil shows that artificial inoculation of the fungi could be managed by the plant volatile (thyme essential oil) either in aqueous or vapour form at the postharvest stage.

Conclusion

The presence of thyme oil as a combined treatment with half strength prochloraz have helped to decrease the stem-end rot incidence and improved the fruit quality. Integration of natural plant volatiles such as essential oils as a combined treatment with reduced fungicide application could be a potential solution for commercial adoption in packhouses to extend and maintain the fruit quality during storage especially for green-skinned avocado cultivars such as Fuerte, Pinkerton, Ryan, and Edranol. Furthermore, application of higher concentration of Yucca extract alone or in combination with plant volatiles on naturally infected fruit should be investigated on avocado cultivars in relation to the metabolic pathways induced during storage.

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Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of Interest.

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