Expression of pathogenesis-related (PR) genes in avocados fumigated with thyme oil vapours and control of anthracnose

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Highlights
- Thyme oil fumigation effectively controlled the anthracnose in avocados.
- Defence enzymes (chitinase and β-1,3-glucanase) were induced by thyme oil fumigation.
- Thyme oil fumigation initiated the expression and activities of defense enzymes.

Abstract

Thyme oil (TO) fumigation (96 μL L^{-1}) to cv. Hass and Ryan avocados significantly reduced anthracnose incidence compared to prochloraz and to untreated control. Also enhanced activities of β-1,3-glucanase, chitinase were noted in both cultivars. TO fumigation induced the expression of both β-1,3-glucanase and chitinase genes in naturally infected fruit of both cultivars, during storage at 7 or 7.5 °C for up to 21 d and during subsequent simulated market shelf conditions at 20 °C for 5 d. However, the impact of TO fumigation on the β-1,3-glucanase gene expression was higher in both cultivars. Higher gene regulation and β-1,3-glucanase, chitinase activities were observed in cv. Ryan compared to Hass. Although TO

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fumigation significantly reduced anthracnose incidence in both naturally infected cultivars, the inhibitory effect was slightly higher in cv. Ryan than Hass. Thus postharvest TO fumigation had positive effects on enhancing anthracnose disease resistance during storage and also gave a residual effect during the simulated shelf life.

Graphical abstract
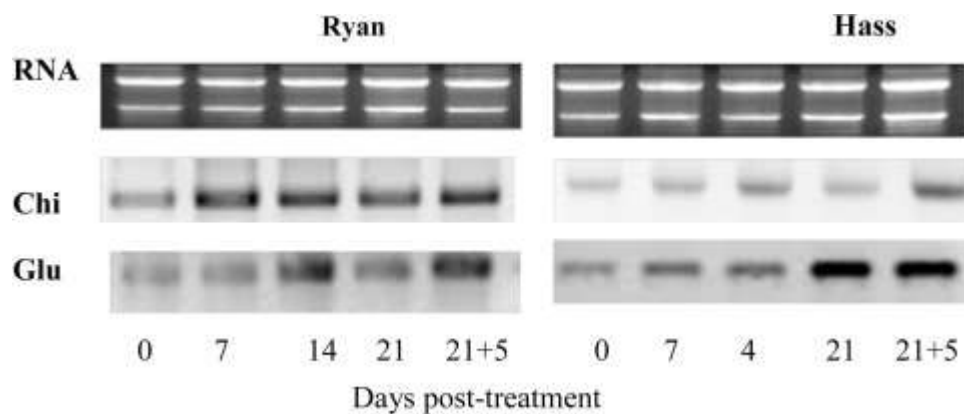
1. Introduction

Avocado fruit (*Persea americana* Mill.) are highly nutritious being rich in oleic, palmitic, linoleic and palmitoleic acids; vitamin A, B, C, E and K; and the minerals, potassium, phosphorus, magnesium and iron (Lu et al., 2009; Yahia, 2010). Anthracnose disease, caused by *Colletotrichum gloeosporioides* Penz. is the predominant postharvest pathogen that can cause severe postharvest losses if not effectively controlled (Sanders and Korsten, 2003 A synthetic non-systemic fungicide prochloraz is used in commercial postharvest applications to control anthracnose disease.. The development of acidified prochloraz treatments has been shown to reduce the concentration of active prochloraz

necessary for control anthracnose (Mavuso and van Niekerk, 2013). However, the disposal of the residual low pH solution remains an industry challenge as well as the increase in consumer concern regarding food safety. The demand for organically produced fruit has brought a need for safer disease control methods specifically for postharvest treatments which are closer to the market and to the point of consumption.

Application of essential oils has received much interest in the food industry due to their antifungal, eco-friendly and biodegradable properties (Tzortzakis and Economakis, 2007). Moreover, application of essential oils in the vapour phase has been shown to be effective at low concentrations due to the volatility and therefore is a more attractive fumigant for postharvest disease (Lopez-Reyes et al., 2010; Laird and Phillips, 2011). One essential oil that has these properties is thyme oil (TO) whose active ingredient is thymol, which is a natural monoterpene phenol derivative. Furthermore, the maximum allowed safe level for flavouring compounds of chemical group C25 (which includes thymol) is 5 mg kg^{-1} for all animal species (EFSA, 2012). In *in vitro* experiments TO vapour completely inhibited the mycelial growth of *C. gloeosporioides* at an application rate of 5 µL on each Petri plate. Its mode of action was shown to involve altering the morphology of the hyphae as well as affecting the viable spores (Sellamuthu, Sivakumar, & Soundy, 2013a). However, fine structures of the fungal intracellular and intercellular hyphae were unaffected by thymol treatments (Svircev et al., 2007).

It was evident from the investigations of van Loon, Rep, and Pieterse (2006) that the resistance inducers can trigger the expression of genes involved in production of pathogenesis related (PR) proteins, synthesis of phytoalexins, and reactive oxygen species (ROS) production. However, the relationship between the TO and the expression of defence related enzyme (PR) genes have not been investigated in fruit during postharvest application. It is therefore important to test natural compounds that can act as resistant inducers or elicitors to

control anthracnose and prolong the shelf life of fruit during marketing and distribution (Landi, Feliziani, & Romanazzi, 2014).

The objective was to investigate the effect of TO fumigation on responses of induced defence and antioxidant enzymes in order to provide the residual effect against latent infections of *C. gloeosporioides* in the fruit tissue. To achieve this objective *in vivo* studies were carried out with TO fumigation to determine its preventive effect on decay development in artificially inoculated fruit. Secondly to determine the induction of the defence related enzymes chitinase and β-1,3-glucanase. Thirdly, to assess the residual effect of TO as a fumigant on gene expression to determine changes in expression of selected defence genes induced in the avocado cvs Hass and Ryan after fumigation and low temperature storage and subsequent shelf life. Fourthly to determine the anthracnose incidence in naturally infected fruit fumigated with TO after low temperature storage and subsequent shelf life.

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* isolate was cultured and maintained on potato dextrose agar (PDA) (Merck, Johannesburg, South Africa) and incubated at 25 °C for 12-13 d. Spore suspensions were prepared according to Bill et al. (2014) and the mycelia fragments were removed from the suspension by filtering through three layers of muslin cloth. Spores were counted using a haemocytometer and adjusted to 1×10^5 spore mL^{-1}.

2.2. Anthracnose incidence and severity after TO fumigation (preventative application)

Freshly harvested, unblemished avocado fruit of cv. Hass and Ryan were obtained from Bassan 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 NaOCl (0.01%), for 5 min and air-dried at room temperature (~25 °C). Subsequently, the fruit were exposed to (i) the commercial treatment (prochloraz 0.05% for 5 min dip); (ii) TO fumigation or (iii) sterile distilled water dip (untreated control). TO fumigation treatment was performed as follows: The TO concentration was predetermined in the *in vitro* experiment (5 μL plate^{-1}) based on the minimal inhibitory concentration (Sellamuthu et al., 2013a). The TO (960 μL) calculated proportionally to the volume of the container (~12 773 cm^3) was introduced into a 10 L translucent plastic container (90% RH) by placing the TO in a Petri plate (65 mm in diameter) lid inside the container. Ten avocado fruit were carefully placed in the container avoiding contact between the fruit and the TO. Immediately the container was sealed with a slip on lid to start the fumigation process. The fruit were exposed to TO vapour for 24 h at 20 °C and thereafter, placed on a sterile paper towel on the bench tops for inoculation (after 0.5 h). Fruit inoculation was performed according to Sellamuthu et al. (2013a) by uniformly wounding with a sterilized needle (1 mm x 1 mm) and inoculating with 20 μL of a spore suspension of *C. gloeosporioides* (10^5 spores mL^{-1}) at the equatorial region. Inoculated and treated fruit were packed in standard corrugated cardboard cartons and held at ca. 20 °C for 5 d.

Each treatment had five replicate boxes each containing ten fruit. 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 d). The disease incidence was determined according to Sellamuthu, Sivakumar, Soundy, and Korsten, (2013b).

2.3. Measurement of active defence response-related enzyme in avocado fruit fumigated with TO (preventive application).

Determination of enzyme assays for β-1,3-glucanase, and chitinase were performed from fruit inoculated with *C. gloeosporioides* and subjected to TO fumigation, commercial treatment (prochloraz) and untreated control fruit. The enzyme activities were conducted according to Sellamuthu et al. (2013b) by obtaining 0.2 g fruit samples from 5 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 ℃ and supernatant were used to determine enzyme activities. For chitinase and β-1,3-glucanase, the samples were extracted by 50 mM sodium acetate buffer (pH 5.0).

β-1,3-glucanase activity was determined using a method described according to Abels et al. (1971) and Sellamuthu et al. (2013a) using 100 µL of enzyme extract with 100 µL of 2% (w/v) laminarin (Aldrich, USA). The reaction mixture was incubated for 24 h at 40 ℃. Afterwards, 25 µL 3,5-dinitrosalicyclic reagent was added for reaction. The reaction was stopped by heating the samples in boiling water for 5 min. The amount of reducing sugar was estimated at 540 nm (Zenyth 200 rt Microplate Reader UK-Biochrom Ltd.). β-1,3-glucanase 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^{-1} of protein

Chitinase activity was determined according to the method of Abels et al. (1971) and Sellamuthu et al. (2013b) by mixing 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). Subsequently the reaction

mixture was incubated for 2 h at 40 ºC. After wards 25 µL of 1 M HCl was added in order to terminate the reaction. 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.

The protein content of enzyme extracts was determined according to Bradford method (Bradford, 1976) and all enzyme assays were conducted for each treatment using six replicate per sample.

2.4. Residual effect of TO fumigation on gene expression, enzyme activity, anthracnose incidence and sensory parameters in naturally infected fruit

Naturally infected fruit at commercial maturity were exposed to TO fumigation for 24 h as mentioned in 2.2. Immediately after fumigation, treated and untreated control fruit were stored at 7 ºC for Hass and 7.5 ºC for Ryan to simulate shipping conditions. Five replicate boxes were used and ten fruit were taken after 0, 7, 14, 21 d intervals and 21 d + 5 d at 20 ºC (to simulate market shelf life) of storage. The samples were processed and determined for chitinase and β-1,3-glucanase activity as mentioned in 2.3. For gene expression analysis, the collected fruit samples were also cut into small pieces, frozen in liquid nitrogen, ground into powder and stored at -20 ºC until use.

Total RNA was extracted from 0.3 g of pooled samples of frozen fruit using the total RNA plant mini kit (Zymo Research Corporation, Inqaba Biotech, South Africa). Each RNA sample (1 µg) was analysed by electrophoresis in 1.8% (w/v) agarose and 1.0 (v/v) formaldehyde denaturing gel, and then transferred to Hybond-N membranes (Amersham-Pharmacia Biotech, Buckinghamshire, UK) according to Masek et al. (2005). The membranes were incubated for 4 hours at 42 ºC with a prehybridization solution 50% (v/v) formamide, 5 x SSPE (3 mol L^{-1} NaCl, 0.2 mol L^{-1} NaH_2PO_4, 20 nnol L^{-1} EDTA), 1 x Denhardt solution

(0.02% (w/v) BSA, 0.02% (w/v) polyvinylpyrrolidone 40, 0.02% (w/v) Ficoll) and 0.2% (w/v) (SDS) with 0.2 g L^{-1} salmon sperm DNA as blocking agent, and hybridized overnight with biotin labelled specific probes for chitinase and β-1,3-glucanase (Inqaba Biotec, South Africa) at 42 ºC (Table 1). The sequences used in the primer design were obtained from the NCBI GenBank (Z78202 and U49454 for endochitinase and β-1,3-glucanase respectively). The membranes were washed once at 42 ºC for 30 min and three times at 50 ºC with 0.1% (w/v) SDS and 1 x SSC (8.7 g L^{-1} NaCl, 4.41 g L^{-1} Na citrate) for 30 min (Pombo et al., 2011). Blots were developed using the Biotin chromogenic detection kit (Thermo scientific, USA). Quantification was performed by densitometry using the PD quest Quantity-1 1-D analysis software (BioRad Laboratories Inc., Munich, Germany).

Table 1. Base composition of oligonucleotide primers designed for gene expression analysis of chitinase and β-1,3-glucanase.

Gene name	Oligonucleotide sequence	Melting temperature ($^{\circ}$C)
Chitinase	ACTACGGGCGTGGACCATTC	64
β-1,3-glucanase	TCAAGAGCAGCATAAACACC	58

Anthracnose disease incidence in naturally infected fruit freshly harvested, unblemished avocado fruit of cvs. Hass and Ryan were fumigated with TO as mentioned in 2.2 and stored at 7 ºC for Hass and 7.5 ºC for Ryan to simulate shipping conditions for 21 d and thereafter held at 20 °C, RH 70% to simulate the market shelf conditions. The incidence of anthracnose was recorded at the market shelf condition (after ripening). Untreated control fruit and prochloraz treated fruit were included for comparison. Each treatment had five replicate boxes each containing ten fruit.

Subsequently, a set of 40 naturally infected fruit per treatment was used for sensory evaluation using an untrained panel. As mentioned by Meir *et al*. (2005). The fruit used for evaluation was left until complete softening (1.5 kg firmness) was achieved. It is at this

degree of firmness that the fruit was considered to have reached normal eating ripeness and the fruit were selected for sensory evaluation. Ten fruit per treatment were placed on white plates and 20 students from the Department of Crop Sciences, Tshwane University of Technology, Pretoria West Campus, were asked to assess the fruit quality for taste, texture, off-flavour development and overall acceptability.

Fruit taste, texture and overall acceptability was assessed from 1 to 10, where 1 = bad and 10 = excellent (excellent 9-10; good 8-9; fair 7-8; poor or unacceptable less than 6) and each member of the panel was requested to indicate the sample they liked best. Off-flavour was also assessed on a structured scale from 1 to 10, where (strong off-flavour 9-10, fair 8-9; good 7-8; acceptable fruit flavour less than 6) 1 = no off-flavour and 10 = very strong off flavour and inedible. The sample presentation was randomized. The tasting panel consisted of 10 female and 10 male, with ages ranging from 20 to 25.

2.5. 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 *Anthracnose incidence, severity and defence-related enzymes after TO fumigation (preventive application)*

Significant differences ($p < 0.05$) were observed on anthracnose incidence and severity between different treatments in both cultivars after 5 d storage at 20 °C. TO

Table 2

 Effect of thyme oil fumigation treatment on the incidence and severity of anthracnose in artificially inoculated avocado fruit cv. Hass and Ryan

Treatment	Incidence of anthracnose (%)		Severity of anthracnose (mm)	
	Hass	Ryan	Hass	Ryan
Untreated control	100 a	89.2 a	25.0 a	18.9 a
Prochloraz 0.05% for 5 min dip	48 b	44.4 b	12.8 b	12.4 b
Thyme oil (1%)	28 c	25.0 c	8.1 c	9.0 c

Means in the same column with different letters are significantly different (P <0.05).

Table 3

Effect of thyme oil fumigation treatment on defence related enzymes in artificially inoculated avocado fruit cv. Hass and Ryan

Treatment	Chitinase activity (nM product acid h^{-1} mg of protein^{-1})		β-1.3-Glucanase activity (µM glucose equiv.h^{-1} mg of protein)	
	cv. Hass	cv. Ryan	cv. Hass	cv. Ryan
Untreated control	0.93 b	1.19 c	5.0 c	5.3 c
Prochloraz 0.05% for 5 min dip	0.97 b	1.62 b	5.5 b	5.7 b
Thyme oil (1%)	1.38 a	1.65 a	6.0 a	6.1 a

Means in the same column with different letters are significantly different ($P < 0.05$).

fumigation was the most effective treatments and significantly ($p < 0.05$) reduced the disease incidence by 72% and 75% compared to the control in Hass and Ryan respectively while the prochloraz dip treatment had 20% and 19.6% more decayed fruit in Hass and Ryan respectively. Furthermore, TO (96 μLL^{-1}) fumigation treatments significantly ($p < 0.05$) reduced the disease severity compared to both the prochloraz and untreated controls (Table 2). Active defence response-related enzymes; chitinase and β-1,3-glucanase enzymes activities significantly ($p < 0.05$) increased in both cultivars during preventive application (Table 3).

3.2. Residual effect of TO fumigation on gene expression, enzyme activity, anthracnose incidence and sensory parameters in naturally infected fruit

The expression levels of the two genes after TO fumigation were compared to the untreated control and defined as a fold-stimulation according to the analysis intervals. In cv. Hass, the chitinase gene expression (mRNA relative intensity) increased by 1.2-fold, 1.5-fold, 1.2-fold and 1.1-fold at 7, 14, 21 d of storage and 21 d of storage + 5 d market shelf after TO fumigation. In cv. Ryan, at 0 and 7 d after TO fumigation the expression, the expression of chitinase (mRNA relative intensity) was noted to increase by 1.3-fold and 1.2-fold respectively while at 14 d onwards the expression increased by 1.1-fold. The up-regulation of the gene in cv. Ryan more or less remained constant from the 21st day until the market shelf. Although, TO fumigation induced the chitinase gene expression in both cultivars, the mRNA relative intensity was higher in cv. Ryan than in cv. Hass at commercial maturity at low temperature storage and after 21 d at 20 °C after 5 d market shelf (Fig. 1 A).

A significant ($p < 0.05$) up regulation in the β-1,3-glucanase gene was observed in both cultivars. In cv. Hass, the up-regulation was seen after 0, 7, 14, 21 d of storage and 21 d of storage + 5 d market shelf post treatment as 1.9-fold, 2.1-fold, 2.5-fold, 2.3-fold and 2.7-
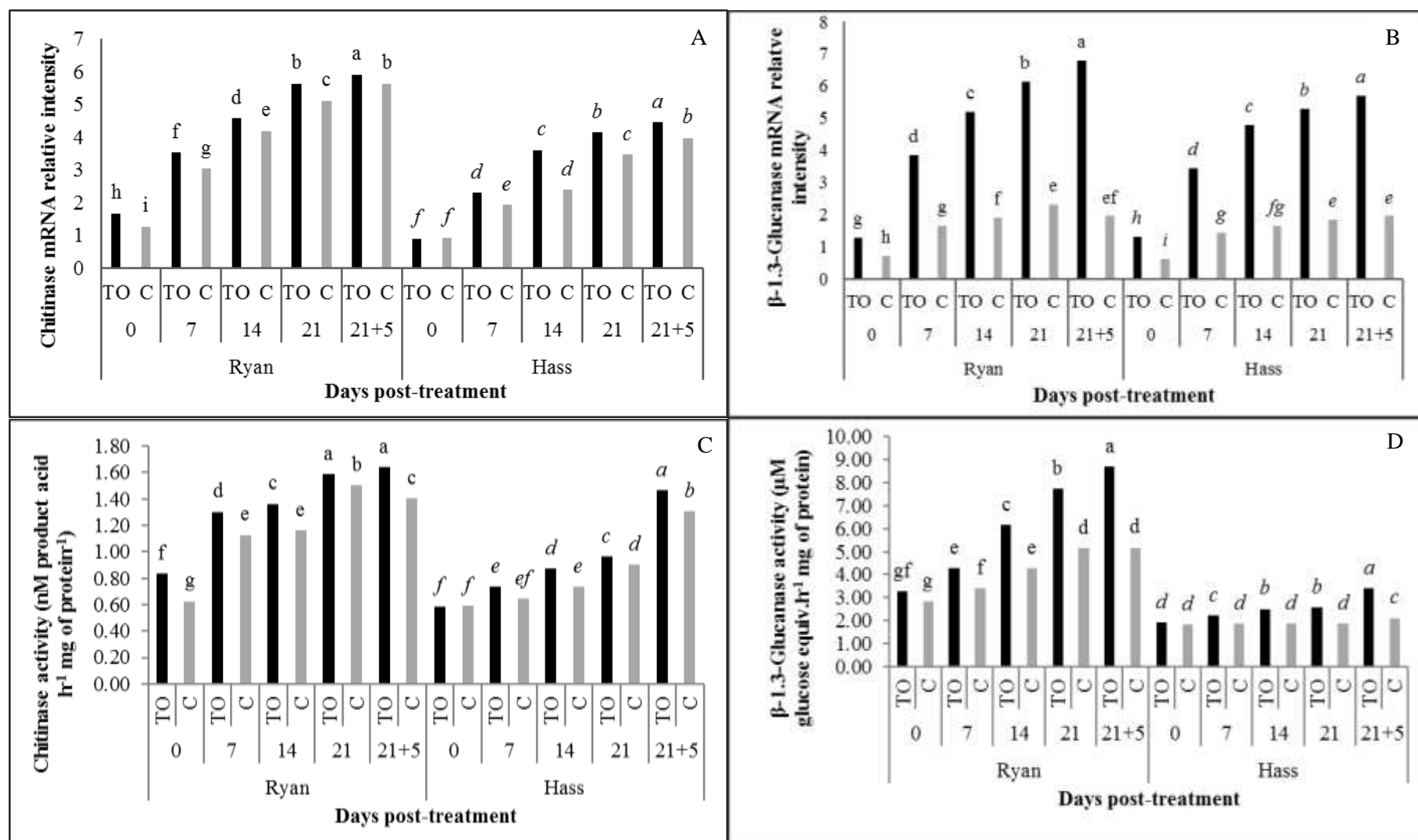
Fig.1. Effect of thyme oil fumigation treatment on chitinase gene expression (A), β-1.3-glucanase gene expression (B), chitinase enzyme activity (C), and β-1.3-glucanase enzyme activity (D) in naturally infected avocado fruit cv. Hass and Ryan during storage. Above each column means followed by a common letter are not significantly different.

fold respectively (Fig. 1 B). Similarly, there was a constant up-regulation of the gene in cv. Ryan by 2.1-fold, 2.7-fold, 3.2-fold, 3.3-fold and 3.5-fold respectively. The mRNA relative intensity (gene expression) of β-1,3-glucanase gene after TO fumigation was higher in both cultivars compared to the mRNA relative intensity of chitinase. In addition the β-1,3-glucanase mRNA relative intensity (gene expression) was much higher in cv. Ryan than in cv. Hass.

The expression of defense related genes, chitinase and β-1,3-glucanase, correlated with their enzyme activities. TO fumigation significantly ($p < 0.05$) increased the chitinase and β-1,3-glucanase enzyme activities during 0, 7, 14, 21 d of storage and 21 d and 5 d market shelf (ripened fruit) (Figures 1 C and D). The increase in chitinase enzyme activity were 1.1-fold (0.09 nM product acid h^{-1} mg^{-1}) after 7 d, 1.2-fold (0.14 nM product acid h^{-1} mg^{-1}) after 14 d, 1.1-fold (0.06 nM product acid h^{-1} mg^{-1}) after 21 d and 1.1-fold (0.16 nM product acid h^{-1} mg^{-1} of protein) after 21d storage + 5 d market shelf) in cv. Hass. In cv. Ryan the chitinase activity increased up to 1.3-fold (0.21 nM product acid h^{-1} mg^{-1} of protein) after 0 d, 1.2-fold (0.18 nM product acid h^{-1} mg^{-1} of protein) after 7 d, 1.2-fold (0.20 nM product acid h^{-1} mg^{-1}) after 14 d, 1.1-fold (0.09 nM product acid h^{-1} mg^{-1} of protein) after 21 d and 1.2-fold (0.23 nM product acid h^{-1} mg^{-1} of protein) after 21 d + 5 d market shelf (Fig 1C). Similar trends was noted with β-1,3-glucanase, activities in both cultivars subjected to TO fumigation prior to storage. β-1,3-glucanase, activities increased significant ($p < 0.05$) by 1.2-fold (0.4 μM glucose equiv.h^{-1} mg^{-1} of protein) after 7 d, 1.3-fold (0.6 μM glucose equiv. h^{-1} mg^{-1} of protein) after 14 d, 1.4-fold (0.7 μM glucose equiv. h^{-1} mg^{-1} of protein) after 21 d and 1.6-fold (1.3 μM glucose equiv. h^{-1} mg^{-1} of protein) after 21 d + 5d market shelf in cv. Hass as shown in Fig. 1D. However, in cv. Ryan higher β-1,3-glucanase activity was noted; 1.1-fold (0.4 μM glucose equiv. h^{-1} mg^{-1} of protein) after 0 d, 1.3-fold (0.9 μM glucose equiv. h^{-1} mg^{-1} of protein) after 7 d, 1.4-fold (1.9 μM glucose equiv. h^{-1} mg^{-1} of protein) after 14 d, 1.5-fold (2.6

μM glucose equiv. h^{-1} mg^{-1} of protein) after 21 d and 1.7-fold (3.5 μM glucose equiv. h^{-1} mg^{-1} of protein) after 21 d + 5 market shelf life following TO fumigation.

Although the effectiveness of the TO fumigation (96 μL L^{-1}) after 21 d and 5 d of market shelf life at 20 °C showed higher gene expression in chitinase and β-1,3-glucanase activities in both cultivars, in cv. Rayan the TO fumigation had higher gene up regulation and
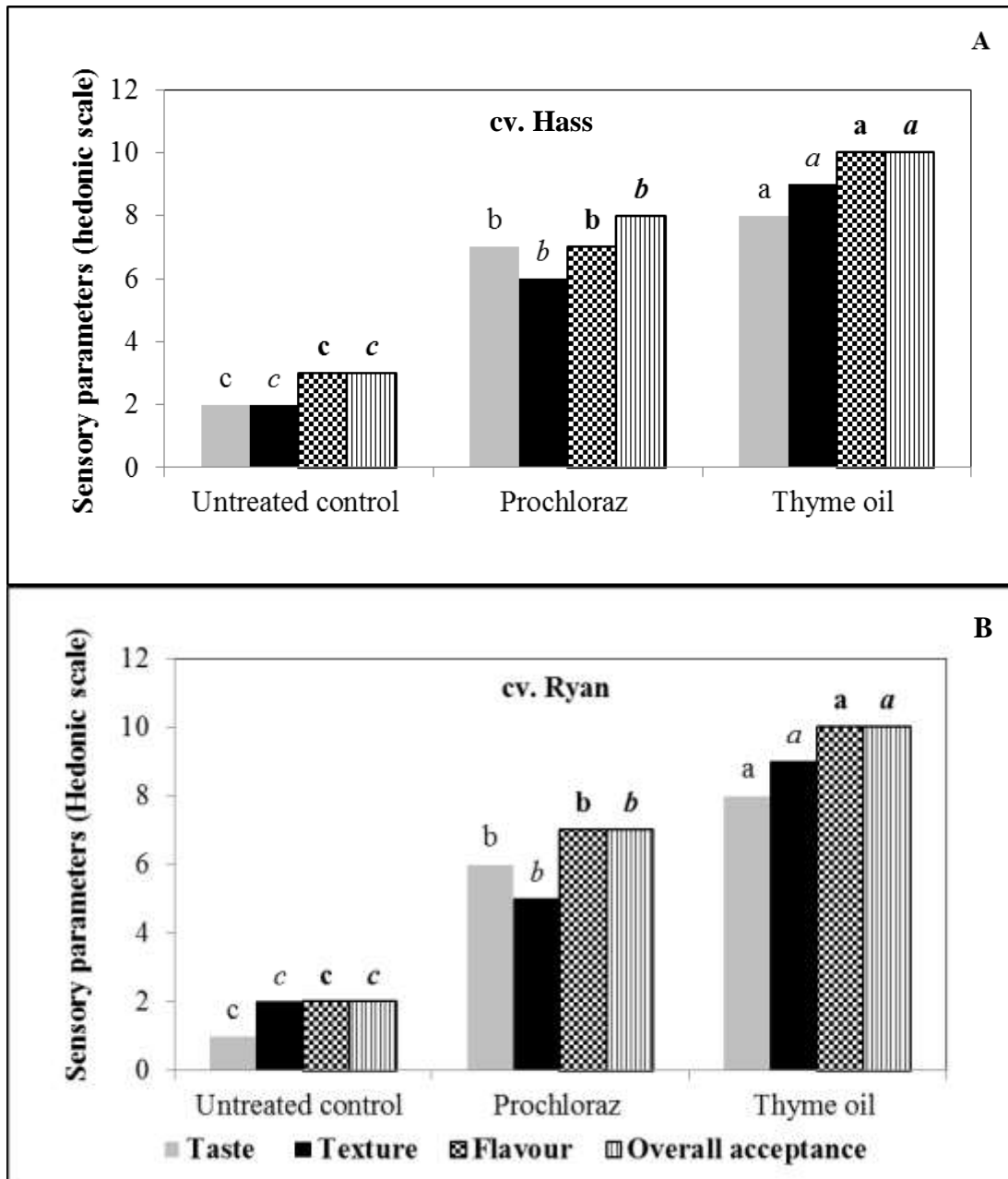

Fig. 2. Effect of thyme oil fumigation treatment on sensory parameters of naturally infect avocado cultivars (A) Hass and (B) Ryan. Above each column means followed by a common letter are not significantly different.

related induced defence enzymes activities. Moreover, it is also evident that the TO fumigation had a great impact on β-1,3-glucanase activity compared to the chitinase activity on treated fruit after 21 d storage and thereafter after 5 d at 20 °C. It is evident that the incidence of anthracnose was significantly ($p < 0.05$) less in the TO fumigation treatments in comparison to the other treatments adopted in this investigation for the naturally infected cv. Hass and Ryan. The TO treatment yielded 70% to 85% decay free fruit in cv. Hass and Ryan respectively after ripening (21 d low temperature storage and 5 d at 20 °C) (data not presented) compared to the untreated control fruit. The TO fumigation treatment also significantly retained the taste, texture and flavour of the fruit compared to both the prochloraz and the untreated control treatments in both cultivars (Figures 2A and B).

Discussion

In the present study the application of TO as a fumigant to prevent infection significantly reduced the incidence and severity of anthracnose in both artificially inoculated and naturally infected avocado cvs. Hass and Ryan. Similarly, Feng et al. (2011) reported a significant reduction in infection and disease severity as a result of TO fumigation of cherry tomatoes. Apart from directly inhibiting pathogen growth and spore germination by affecting the active sites of enzymes and cellular metabolism (Arrebola et al., 2010), it is evident from our findings that the TO fumigation increased the enzyme activities of the defence enzymes β-1,3- glucanase and chitinase. Similar induced defence responses had been shown by chemical elicitors such as chitosan, methyle jasmonate and salicylic acid (Ding et al., 2002). Our data also showed that the TO fumigation improved the activities of chitinase, β-1,3-glucanase in avocado fruit compared to the commercial prochloraz treatment. β-1,3-glucanases are PR-2 proteins that catalyze endotype hydrolytic cleavage of 1,3-β-D

glycosidic linkages in β-1,3-glucans present in the cell wall of many pathogenic fungi (Thanseem, Joseph, & Thulaseedharan, 2005). Also chitinases catalyze the cleavage of the bond between C1 and C4 of two consecutive N-acetyl-D-glucosamine monomers of chitin which is also common component of fungal cell walls as well as the exoskeleton of arthropods (Bartnicki-Garcia, 1968).

Both β-1,3-glucanase and chitinase genes are known to be regulated in a development- and organ-specific pattern, but stress conditions such as challenge infection by fungi and elicitor treatment also induces their expression (Graham and Sticklen, 1994). Djami-Tchatchou, Straker and Allie, (2012) reported that β-279 glucanases and chitinases genes, which are involved in defense response, are expressed in response to *C. gloeosporioides* infection in cv. Fuerte avocado fruit. The current study found that chitinase and β-1,3-glucanase genes activation was apparent from different time points until 21 d storage plus 5 d simulated market shelf life after TO fumigation in ripe and naturally infected avocado fruit and the impact was higher on the β-1,3-glucanase gene expression.

Significantly early and higher β-1,3-glucanase and chitinase mRNA levels and enzyme activities were shown in avocado cv. Ryan compared to cv. Hass. Furthermore our investigation also revealed that after 21 d storage and at 5d market shelf life at 20 °C (ripened) the mRNA levels and the β-1,3-glucanase activities were higher in cv. Ryan. The mRNA levels of β-1,3-glucanase and the enzyme activities were showed to be higher than the untreated control fruit especially at the ripened stage at the market shelf condition in cv. Ryan. In this respect, a study by Lawrence et al. (2000) on tomatoes found that pathogen induced expression of PR genes were higher with stronger induction in the resistant cultivars compared to susceptible cultivars . However, detail investigation is needed to prove the differences in susceptibility to anthracnose of the two cultivars. It is also evident from this investigation that the markedly higher expression of β-1,3-glucanase gene and its activity

with the activity of chitinase helped to lower the anthracnose incidence during TO fumigation.

As reported in this investigation regarding the TO fumigation inducing higher mRNA levels of β-1,3-glucanase and enzyme activity in cv. Ryan, similar findings were stated by Landi, Feliziani and Romanazzi (2014) in elicitor-treated (chitosan, benzothiadiazole and calcium and organic acids) strawberries cv. Camarosa. Also ozone-induced changes in PR protein gene expression levels in a tobacco cultivar showed a delayed and lower expression of the chitinase gene compared to the β-1,3-glucanase gene (Ernst et al., 1992) and this relates to our observations on the expression of PR protein gene expression and enzyme activity in avocados in this investigation. The application of resistance inducers was shown to stimulate induced defence mechanisms that helped to protect the fruit against the invading pathogen (Landi et al., 2014). The benefit of applying TO fumigation is initiating the induced defence response at the enzymatic and transcript level that make the fruit less susceptible to anthracnose development after infection. Although different responses were observed to TO fumigation regarding the PR protein gene expression and enzyme activities between the two cultivars, the current TO fumigation resulted in an effective control of anthracnose in both cultivars compared to the currently used prochloraz treatment. The results of these investigations and the previous findings of Sellamuthu et al. (2013b) clearly show that the TO application at volatile vapour phase effectively controlled the anthracnose incidence and severity without causing any phytotoxic effects on the fruit while improving its organoleptic properties (taste, texture and flavour). Consequently, the TO treated fruit showed higher overall acceptance according to the untrained testing panel. However, TO is listed as a minimum risk pesticide and exempt from pesticide residue tolerance requirements (EPA, 2015). Selamuthu et al. (2013b) reported that the GC/MS data of TO treatment include 53.9%

relative area (RA) thymol. Therefore, TO fumigation can be used as an alternative bio-fungicide to replace the currently used prochloraz fungicide application in packhouses.

In conclusion the TO fumigation for 24 h as an elicitor offers great practical potential in reducing the anthracnose incidence during the postharvest supply chain through the induction of the defence mechanism of the fruit.

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