Control of anthracnose disease via increased activity of defence related enzymes in 'Hass' avocado fruit treated with methyl jasmonate and methyl salicylate

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ABSTRACT

Development of anthracnose disease caused by *Colletotrichum gloeosporioides* Penz. is one of the major issues within the avocado supply chain. Exposure to methyl jasmonate (MeJA) and methyl salicylate (MeSA) vapours 10 and 100 µmol l^{-1} was investigated as an alternative solution to commercial fungicide - prochloraz® that is currently being used by the industry. The incidence of anthracnose disease was found to be significantly reduced in 'Hass' avocado fruit treated with MeJA or MeSA vapours, especially at 100 µmol l^{-1}. The mechanism involved enhanced activity of defence related enzymes, i.e. chitinase, *β*-1,3-glucanase and PAL, and higher content of epicatechin.

Keywords: Persea americana Mill., anthracnose, epicatechin, chitinase, *β*-1,3-glucanase, supply chain

1. Introduction

Avocado (*Persea americana* Mill.) is becoming a popular fruit mainly due to its nutritional content (Dreher & Davenport, 2013), in particular being rich in monounsaturated fatty acids (Ozdemir & Topuz, 2004; Lu et al., 2009). The production of avocados in South Africa is mainly export driven, and according to the latest (2015) food trade and supply chain directory (www.foodtradesa.co.za), the European Union, and United Kingdom in particular, is the biggest export market.

Development of postharvest disease, such as anthracnose (caused by *Colletotrichum gloeosporioides* Penz.), is one of the major issues within the avocado supply chain, affecting marketability of the produce. At the moment prochloraz®, a synthetic fungicide, is being used in the packhouses to control anthracnose disease. However, since there is an increasing demand to reduce the use of fungicides (Bill, Sivakumar, Thompson, & Korsten, 2014), there is clearly a need for new techniques that could reduce undesired fungal decay.

One of the possible options to reduce the disease development is via inducing the defence mechanisms in the fruit (Romanazzi et al., 2016). There are numerous methods used to reduce microbial contamination of fresh produce and to extend its storage life (Ramos, Miller, Brandao, Teixeira, & Silva, 2013), however the postharvest use of jasmonates and salicylates seems to be overlooked. These are natural plant signalling compounds that play a role in stimulating natural defence mechanisms against both biotic and abiotic stress, and the information on their use to reduce losses within the fruit supply chain has recently been reviewed (Glowacz & Rees, 2016).

It has been reported that dipping 'Hass' avocado fruit in 2.5 µmol l^{-1} methyl jasmonate solution for 30 s reduced the development of chilling injury in fruit subsequently stored for 2 weeks at 1 °C (Sivankalyani et al., 2015) and 4 weeks at 2 °C (Meir et al., 1996), respectively. It was further confirmed in our trials with methyl jasmonate and methyl salicylate that these compounds have the ability to maintain the postharvest quality of cold stored 'Hass' avocado fruit by altering their fatty acids content and composition (Glowacz, Bill, Tinyane, & Sivakumar, 2017). However, to the best of our knowledge there is no information in the literature on the effects of methyl jasmonate and methyl salicylate on the anthracnose disease susceptibility in 'Hass' avocado fruit, while the ability of these compounds to reduce fungal decay has already been reported for numerous products, e.g. loquat (Cao, Zheng, Yang, Tang, & Jin, 2008; Cao et al., 2008) and mango (Zeng, Cao, & Jiang, 2006).

It is well known that the activity of defence related enzymes, i.e. chitinase and β-1,3-glucanase is enhanced when the produce is challenged by the fungal pathogen (Mauch, Mauch-Mani, & Boller, 1988). These enzymes, acting synergistically, are capable of hydrolysing polymers of fungal cell walls - chitin and β-1,3-glucan respectively, leading to weakened cell wall and cell lysis (Stintzi et al, 1993). Chitinase and β-1,3-glucanase are thus involved in the plant defence mechanisms preventing/delaying the fungal growth and in this way reducing the decay (Theis & Stahl, 2004).

The activity of PAL is often induced by both abiotic and biotic stress (Dixon & Paiva, 1995), e.g. in response to wounding or pathogen attack, where synthesised phenolics could either act directly as defence compounds or indirectly, due to being precursors of lignin and suberin, producing a barrier and strengthening cell walls (Passardi, Penel, & Dunand, 2004), which would prevent the infection and limit pathogen expansion in infected fruit.

Finally, epicatechin, an antioxidant present in the peel, is also involved in delaying/preventing the fungal decay via lowering the activity of lipoxygenase during the activation of quiescent infection (Karni, Prusky, Kobiler, & Kobiler, 1989) and slowing the rate of decline of antifungal 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene compound (Ardi, Kobiler, Keen, & Prusky, 1998).

The concentrations of 10 and 100 µmol l^{-1} of MeJA and MeSA were used in majority of the studies reviewed by Glowacz and Rees (2016), e.g. loquat treated with MeJA at 10 µmol l^{-1} (Cao et al., 2008; Cao, Zheng, Wang, Jin, & Rui, 2009), mangos treated with MeJA at 10 µmol l^{-1} and 100 µmol l^{-1} (Gonzalez-Aguilar, Buta, & Wang, 2001) or MeSA at 100 µmol l^{-1} (Han, Tian, Meng, & Ding, 2006), papaya treated with MeJA at 10 and 100 µmol l^{-1} (Gonzalez-Aguilar, Buta, & Wang, 2003), peaches treated with MeJA at 100 µmol l^{-1} (Meng, Han, Wang, & Tian, 2009), pears treated with MeJA at 100 µmol l^{-1} (Zhang et al., 2009), pomegranates treated with MeJA or MeSA at 10 and 100 µmol l^{-1} (Sayyari et al., 2011), and tomatoes treated with MeJA or MeSA at 10 and 100 µmol l^{-1} (Ding, Wang, Gross, & Smith, 2002).

Thus, the objective of this study was to investigate the effect of methyl jasmonate (MeJA) and methyl salicylate (MeSA) vapours exposure at two concentrations of 10 and 100 µmol l^{-1} on i) disease incidence ii) epicatechin content, and iii) activity of defence related enzymes (chitinase, *β*-1,3-glucanase, PAL) in naturally and artificially infected 'Hass' avocado fruit kept at 2 °C for 14 d, followed by 6-7 d shelf-life at 20 °C.

2. Materials and methods

2.1. Plant material and handling

Freshly harvested, unblemished late season 'Hass' avocado fruit were obtained from Koeltehof Packers (Nelspruit, Mpumalanga province, South Africa) at commercial maturity (28-30% DM). Fruit were organised into the following treatment: i) untreated control – fruit that were transported to the laboratory and then left untreated; ii) dipped for 5 min in 0.05 % prochloraz® – fruit were treated at the pack house, i.e. the commercial treatment, prior to being transported to the lab; iii) fruit that were transported to the laboratory and then exposed to methyl jasmonate (MeJA) or methyl salicylate (MeSA) vapours at two concentrations of 10 and 100 µmol l^{-1} for 24 h at 20.0±0.5 °C.

After placing the fruit in a 10 l air-tight container, the appropriate volume of MeJA or MeSA to reach the desired concentration of 10 and 100 µmol l^{-1}, respectively was deposited on the Petri dish at the bottom of the container (Gimenez et al., 2016), using the system set up previously designed for the thyme oil fumigation (Bill, Sivakumar, Beukes, & Korsten, 2016). The container was immediately hermetically-sealed and solutions were left to evaporate over the 24 h period. Control and prochloraz® treated fruit were also kept in similar sealed containers. Thereafter half of the fruit were wounded and inoculated at the equatorial region with 20 µl of *C. gloeosporioides* spore suspension (10^5 spores ml^{-1}) as previously described (Bill et al., 2016). Both naturally and artificially infected 'Hass' avocado fruit were subsequently kept at 2.0±0.2 °C for 14 d followed by 6-7 d shelf-life at 20.0±0.5 °C, RH 70 %.

2.2. 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 suspension was prepared following a method of Bill et al. (2016). The mycelia fragments were removed from the suspension by filtering through three layers of muslin cloth. Spores count was determined using a haemocytometer and adjusted to 10^5 spores ml^{-1}. Fruit were prepared for artificial infection by disinfecting the place of inoculation with 70% ethanol (left to dry for ~30 min). The inoculation was then performed by uniformly wounding the fruit with a sterile needle (1 mm x 1 mm) and transferring 20 µl of spore suspension (10^5 spores ml^{-1}).

2.3. Disease incidence

At the 'ripe and ready to eat' stage (firmness near to 6.7 N, which has been defined by Arpaia, Collin, Sievert, and Obenland (2015) as the optimal eating firmness) fruit were assessed for signs of rotting (anthracnose), by giving them a score of 0 or 1 – no/ signs of rotting, respectively. In case of stem-end rot being noticed in naturally infected fruit, the note of it was taken. Disease incidence was expressed as the proportion (%) of fruit showing signs of rotting out of the total number of fruit in each treatment.

2.4. Physical properties of the fruit

Firmness was determined along the equator of the fruit using a Chatillon Penetrometer, Model DFM50 (Ametek, Largo, Florida, USA) with an 8 mm diameter flat-head stainless steel cylindrical probe (Mpho, Sivakumar, Sellamuthu, & Bautista-Banos, 2013) to ensure that only ripe fruit are being assessed.

2.4. Biochemical analysis

2.4.1. Epicatechin content

Epicatechin was determined following the method used by Guetsky et al. (2005), with some modifications. Freeze-dried samples (20 mg) were homogenised in methanol: water (1:1; v/v) solution. Thereafter, the Eppendorf tubes were centrifuged for 10 min at 14 000 x *g*. The supernatant was transferred to the new tubes, and subsequently filtered through a 0.45 µm membrane (Nylon syringe filter, PerkinElmer™, China) prior to injection. The analyses were carried out using a Flexar™ HPLC system (PerkinElmer, USA) consisting of a Flexar Isocratic LC Pump Platform and a variable wavelength Flexar UV/ ViS LC detector. Separation was done on an Analytical C18 column (100 x 4.6 mm; 5 µm) at 25 °C, using water/methanol (25:75 v/v) as a mobile phase, with a flow rate of 1 ml min^{-1}. Chromatographic peak of the epicatechin was identified by comparing the retention time with that of the pure epicatechin (HPLC grade) standard at 320 nm.

2.4.2. Activity of defence related enzymes

Enzyme activity assays for chitinase, β-1,3-glucanase and PAL were performed as previously described (Sellamuthu, Sivakumar, Soundy, & Korsten, 2013). Protein concentration was determined by the method of Bradford (1976).

2.5. Statistical analyses

Avocado fruit were organised into six treatments, each treatment had six replicate boxes containing eighteen fruit, i.e. 108 fruit per treatment, equalling to a total of 648 fruit per trial. The experiment was conducted twice with similar results. Data are presented as mean values from a fully randomised design. The significance of main effect was established using ANOVA. Duncan's multiple range test was used to compare individual treatment mean values. All statistical analyses were performed using GenStat 18th Edition software (VSN International Ltd, UK).

3. Results and discussion

3.1. Disease incidence

Anthracnose disease was observed in more than 50 % of the naturally infected untreated fruit (Fig. 1A), whereas its occurrence was significantly (P<0.05) reduced in fruit treated with prochloraz® (16.9 %), MeJA at 10 μmol l^{-1} (25.7 %) and 100 μmol l^{-1} (12.4 %), or MeSA at 10 μmol l^{-1} (7.9 %) and 100 μmol l^{-1} (8.9 %). The stem-end rot incidence was noted to be within the range of 2.5-10 %, with no significant difference between the treatments.

In the case of artificially infected fruit, development of anthracnose disease was observed in nearly all untreated fruit (Fig. 1B); disease incidence was significantly (P<0.05) reduced in all treated fruit, being the lowest in those treated MeJA at 100 μmol l^{-1} (41.0 %), or MeSA at 10 μmol l^{-1} (35.9 %) and 100 μmol l^{-1} (43.6 %).

Reduced disease incidence in fruit exposed to MeJA and MeSA is in agreement with previous reports, where disease incidence was reduced, due to delayed infection, in loquat fruit exposed to MeJA at 10 μmol l^{-1} for 24 h prior to being stored at 20 °C (Cao et al., 2008a,b) and mango fruit treated with SA solution at 1000 μmol l^{-1} for 2 min prior to being stored at 13 °C (Zeng et al., 2006), confirming the ability of jasmonates and salicylates to enhance disease resistance in the fruit.

3.2. Physical properties of the fruit

All the fruit used in our trials were in the firmness range from 6.4 to 8.5 N - previously defined as ripe fruit by Gamble et al. (2010), i.e. near to 6.7 N, which according to Arpaia et al. (2015) is the optimal eating firmness.

3.3. Fruit biochemistry

3.3.1. Epicatechin content

Epicatechin content in the skin of naturally infected untreated 'Hass' avocado fruit was significantly lower than in their counterparts treated with prochloraz®, MeJA at 100 μmol l^{-1}, or MeSA at 10 μmol l^{-1} and 100 μmol l^{-1} (Fig. 2A). In the case of artificially infected fruit, the lowest epicatechin content was also observed in untreated samples, being significantly lower than in all the other treatments (Fig. 2B). Interestingly, the highest epicatechin content was observed in fruit exposed to MeSA at 100 μmol l^{-1}, regardless whether fruit were naturally or artificially infected.

One of the findings of this research is the fact that exposure of 'Hass' avocado fruit to MeJA and MeSA at 10 μmol l^{-1} and 100 μmol l^{-1} prior to cold storage led to higher content of epicatechin. Ardi, Kobiler, Keen, and Prusky (1998) have previously suggested that epicatechin is involved in the resistance of avocado fruit to *C. gloeosporioides*, i.e. the cultivars of avocado that are more resistant to fungal decay, were reported to have high epicatechin content, which declines with a slower rate (Prusky, Kobiler, & Jacoby, 1988). Epicatechin is also involved in slowing the rate of decline of antifungal diene compound via lowering/delaying the expression of the LOX genes (Prusky, Alkan, Mengiste, & Fluhr, 2013), which in fact has been observed in our research (Glowacz, Bill, Tinyane, & Sivakumar, 2017). These results are also in agreement with previously reported findings (Sivankalyani et al., 2015) where avocado fruit were dipped for 30 s in MeJA solution at 2.5 μmol l^{-1}, and subsequently stored at 1 °C, and those of Cao, Zheng, Wang, Jin, and Rui (2009) where loquat fruit were exposed to MeJA at 10 μmol l^{-1} for 24 h prior to storage at 1 °C, and thus highlighting the ability of these treatments to alter membrane stability, making the fruit less susceptible to fungal attack and subsequent disease development (Guestsky et al., 2005).

3.3.2. Activity of defence related enzymes

The activity of PAL was only increased in fruit treated with MeJA or MeSA at 100 μmol l^{-1} (data not presented) regardless whether fruit were naturally or artificially infected, but not in those treated at 10 μmol l^{-1}, which suggests that phenolics content could be higher in these samples. A substantial increase in the activity of PAL has in fact been previously reported in several fruit exposed to jasmonates or salicylates e.g. in mangos treated with SA at 1000 μmol l^{-1} and inoculated with *C. gloeosporioides* (Zeng et al., 2006), in loquat treated with MeJA at 10 μmol l^{-1} and inoculated with *C. acutatum* (Cao et al., 2008b). Phenolic compounds improve the antioxidant capacity and reactive oxygen species (ROS) scavenging capacity, thus they could contribute to reduced susceptibility to diseases. Phenolics could either act directly as defence compounds or indirectly, due to

being precursors of lignin, by producing a barrier and strengthening cell walls (Passardi, Penel, & Dunand, 2004), which would prevent the infection of wounds by fungal pathogens and limit their expansion in infected fruit.

The mechanism of improved host resistance against pathogens in fruit treated with jasmonates and salicylates also involves increased activity of defence-related enzymes, i.e. chitinase and *β*-1,3-glucanase (Yao & Tian, 2005; Cao et al., 2008a; Xu & Tian, 2008; Yu, Shen, Zhang, & Sheng, 2011). The activity of chitinase was indeed found to be significantly increased in MeJA and MeSA exposed fruit, regardless whether the fruit were naturally (Fig. 3A) or artificially (Fig. 3B) infected, especially at 100 μmol l^{-1}. Similarly, the activity of *β*-1,3-glucanase was significantly increased in naturally infected fruit exposed to MeJA and MeSA at 10 μmol l^{-1} and 100 μmol l^{-1} (Fig. 4A), but not in their artificially infected counterparts, where only exposure to MeJA or MeSA at 100 μmol l^{-1} led to a significant increase in the activity of *β*-1,3-glucanase (Fig. 4B).

4. Conclusion

The incidence of anthracnose disease was found to be significantly reduced in fruit treated with MeJA or MeSA vapours, especially at 100 μmol l^{-1}. The results obtained in this research highlight the fact that increased activity of chitinase, *β*-1,3-glucanase and PAL, and higher content of epicatechin, are all involved in enhancing resistance of 'Hass' avocado fruit to *C. gloeosporioides* via exposure to MeJA or MeSA vapours. Therefore, exposure to MeJA or MeSA vapours prior to cold storage is a promising alternative to commercial fungicide - prochloraz® that is currently being used by the industry.

Conflict of interest

None.

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