

MECHANISM OF INDUCED SUSCEPTIBILITY TO POSTHARVEST DISEASE (*C. gloeosporioides*) THROUGH 1-MCP TREATMENTS

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The preformed (Z,Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (AFD) is the most active antifungal compound in avocado; it affects the quiescence of *Colletotrichum gloeosporioides* in unripe fruit. Ethylene treatment ($40 \mu\text{g.l}^{-1}$) of freshly harvested avocado fruits cv. Fuerte enhanced the expression patterns of genes encoding Δ^{12} fatty acid desaturase, fatty acid elongase, their respective enzymatic activities, and the level of the AFD. Application of the ethylene-action inhibitor 1-methylcyclopropene (1-MCP) after harvest and prior to ethylene treatment resulted in inhibition of fatty acid desaturase, fatty acid elongase and their enzymatic activities and AFD accumulation. 1-MCP treatment of fruit harvested 280 and 360 days after fruit set delayed fruit softening and reduced their AFD content compared with that in untreated fruits. However, decay symptoms caused by *C. gloeosporioides* were differentially affected by 1-MCP treatment after harvest. In the early-harvested fruits, the initial level of the AFD was $1820 \mu\text{g.g}^{-1}$ fresh wt and the inhibition of the AFD synthesis by 1-MCP did not reduce it below its threshold for inhibiting *C. gloeosporioides*. In contrast, in the late-harvested fruits, the initial level of the AFD was only $950 \mu\text{g.g}^{-1}$ fresh wt, and the 1-MCP treatment reduced the level of the AFD below its inhibition threshold, enabling fungal development in unripe fruits. These results demonstrate temporal relationships among fruit firmness, the synthesis of the precursor of AFD, the AFD content, and quiescence of *C. gloeosporioides*, and suggest that in avocado fruits, the processes of fruit ripening and AFD induction are independent processes that are differentially affected by ethylene action during fruit ripening.

Key words: synthesis antifungal compound, decay development

MECANISMO DE INDUCCIÓN DE SUSCEPTIBILIDAD AL ATAQUE DE ENFERMEDAD DE POSCOSECHA (*C. gloeosporioides*) POR TRATAMIENTOS DE 1-MCP

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El compuesto antifúngico preformado (Z,Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (AFD) es el compuesto más activo contra *Colletotrichum gloeosporioides* en fruto no maduro. Tratamientos con etileno ($40 \mu\text{g.l}^{-1}$) para frutos recién cosechados de la variedad Fuerte aumentaron los modelos de expresión de genes que codifican a Δ^{12} ácido graso desaturasa, ácido graso elongasa, y sus respectivas actividades enzimáticas, así como también el nivel del AFD. Tratamientos del inhibidor 1-metilciclopropeno (1-MCP), después de cosecha y previo al tratamiento de etileno, causaron la inhibición de la expresión y la actividad del ácido graso desaturasa, ácido graso elongasa y la acumulación del AFD. Tratamientos con 1-

MCP de fruto cosechado 280 y 360 días después del cuajado del fruto, retrasaron el ablandamiento de los frutos y redujeron la concentración del AFD comparado con el de frutos sin tratamiento. Sin embargo, los síntomas de podredumbre causados por *C. gloeosporioides* se vieron afectados por 1-MCP diferencialmente luego de la cosecha. En frutos cosechados a principio de temporada, el nivel inicial de AFD era $1820 \mu\text{g}\cdot\text{g}^{-1}$ de peso fresco y la inhibición de la síntesis del AFD por parte de 1-MCP no lo redujo por debajo de su límite por inhibir el desarrollo de *C. gloeosporioides*. En contraste, si el fruto fue cosechado a finales de temporada, el nivel original del AFD fue sólo de $950 \mu\text{g}\cdot\text{g}^{-1}$ de peso fresco, y el tratamiento con 1-MCP redujo el nivel del AFD por debajo del nivel mínimo de inhibición, permitiendo el desarrollo fúngico en frutos no maduros. Estos resultados indican la relación temporal que existe entre el estado de maduración, la síntesis del AFD, su contenido y el estado de reposo de *C. gloeosporioides*, y sugieren que en paltas, los procesos de maduración de frutos e inducción de AFD son procesos independientes que son afectados diferencialmente por la acción de etileno durante la maduración del fruto.

Palabras claves: síntesis de compuestos antifúngicos, desarrollo de podredumbre

Introduction

The pathogen, *Colletotrichum gloeosporioides* Penz. (teleomorph *Glomerella cingulata* (Stonem.) Sacc., et Schrenk) attacks unripe fruit of avocado, *Persea americana* Mill. in the orchard. Germinated spores produce appressoria that breach the cuticle, but the resulting subcuticular hyphae become quiescent and do not develop further until the fruits ripen (Prusky, 1996). The mechanism of resistance of avocado fruits against *C. gloeosporioides* attack was previously shown to be correlated to the presence of fungitoxic concentrations of the preformed antifungal compound, 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (AFD) in the exocarp of the unripe fruits (Prusky and Keen, 1993). The AFD is the most potent of five preformed antifungal compounds detected in avocado fruit peel (Prusky and Keen, 1993). Increased susceptibility to *C. gloeosporioides* is the result of a decline in the AFD concentration to subfungitoxic levels that occurs during fruit ripening (Prusky et al, 1982). The decline in the level of AFD is attributed to its oxidation and metabolism by lipoxygenase, whose activity is inhibited by high concentrations of flavan-3-ol, epicatechin, a natural antioxidant present in the avocado exocarp; during ripening, decreasing concentrations of epicatechin results in increased lipoxygenase activity and increased AFD metabolism. Thus, the concentration of epicatechin appears to play a key role in fruit susceptibility during fruit ripening, by indirectly controlling the levels of the AFD (Prusky et al, 1985).

Although the AFD is a preformed compound, several biotic and abiotic treatments induced its accumulation in unripe fruits, and this increased the resistance of ripening avocado fruit to fungal attack (Prusky et al, 1991). Challenge inoculation of unripe resistant fruit with non-pathogenic strains of *C. gloeosporioides* or *C. magna*, or exposure of the fruit to 30% CO₂ or to a temperature of 4°C for 24 h had enhanced the accumulation of the AFD and fruit resistance to *C. gloeosporioides* (Prusky and Keen, 1993). Challenge inoculation with a pathogenic *C. gloeosporioides* strain, Cg-14 and ethylene treatment transiently enhanced the level of the AFD without affecting fruit resistance (Prusky et al, 1994). The accumulation of the AFD was accompanied by the transcriptional and enzymatic activation of *avfad9* encoding a Δ^9 stearyl-ACP

desaturase (Madi et al, 2003), *avfad12-3* encoding a Δ^{12} fatty acid desaturase (Wang et al, 2004a) and *avfae1* encoding a fatty acid elongase (Wang et al., 2004 b).

The possibility of manipulating the AFD level with ethylene highlights the importance of the ripening hormone in inducing the biosynthetic pathway of the antifungal compound. 1-MCP blocks ethylene by binding to its receptor and has been shown to prevent ethylene action in a range of climacteric and non-climacteric fruits. The extremely efficient inhibition of ethylene action by 1-MCP in avocado fruits (Adkins et al, 2005) makes the latter an important pharmacological tool to study the mechanism of the AFD synthesis induction by ethylene. Because 1-MCP also significantly delays fruit softening and ripening, it enables us to study symptom development in unripe fruits and thus to uncouple fruit softening from the process of activation from quiescence to necrotrophic development by *C. gloeosporioides*.

The objectives of the present study were to test the effect of 1-MCP, an inhibitor of ethylene binding, on the inhibition of the induction of the biosynthetic pathway of the AFD and to analyze its importance in modulating the activation of quiescent infections during fruit ripening and softening.

Materials and Methods

Plant material and fruit treatments

Avocado fruits (*Persea americana* cv. Fuerte) were freshly harvested from the orchards of Israel. For induction studies unripe-mature fruits were treated with 1-MCP immediately after harvest by exposing them to 1-MCP (SmartFresh, Rohm and Hass Co., Philadelphia, PA, kindly provided by Rimi Ltd, Israel) at $270 \pm 20 \text{ nL.l}^{-1}$ for 17 h by placing an open tube of an aqueous solution of the powder at 25°C in the bottom of a 30-L jar which was promptly sealed. The jars were incubated at 20°C , after which there were opened, ventilated and sealed, and ethylene at $40 \mu\text{L.l}^{-1}$ was injected via a rubber gasket and incubation was continued for a further 3 h. With fruits treated only with ethylene, the treatment was initiated 17 h after harvest and continued for 3 h. At specified intervals after the treatments, pieces of exocarp tissue, 1-3 mm thick, 1-2 cm wide and 5-10 cm long, were removed with a fruit peeler. Samples were immediately frozen in liquid nitrogen and kept at -80°C pending further analysis. The analyses of freshly sampled tissues for the AFD level and the enzymatic activities of Δ^{12} fatty acid desaturase and fatty acid elongase were started immediately after the treatments.

Effect of 1-MCP on fruit ripening and decay development.

Twenty-four hours after harvest, control and treated fruits were inoculated with *C. gloeosporioides* by placing 10 μL of conidial suspension (10^6 conidia per ml) at six spots spaced along the fruit, three on each side, and 10 replications (fruits) were used for each treatment. Following inoculation, the fruits were incubated at 22°C , 95% RH for 24 h then transferred to ambient RH (70-80%) at 22°C until fruit ripening and symptom development. Symptoms of decay, indicated by peel browning were assessed by daily measurements.

Firmness, an inverse parameter of ripening, was measured by recording the force (in newtons) required to penetrate the fruit with a conical probe (5 mm diameter and 4 mm length) with a Chatillon Digital Force Gauge, Model DFGS50 on two opposite sides of every fruit, and 10 replications (fruits) were used for each treatment.

AFD extraction and quantification

The preformed AFD was extracted and quantified as described by Prusky et al. 1982.

Expression studies of *avfad12-3*

Expression studies were carried out with Northern blotting analysis following the methods of Wang et al. 2004a, 2004b.

Δ^{12} fatty acid desaturase and elongase enzymatic assay

The desaturation assay of Δ^{12} fatty acid desaturase was performed according to Miquel and Browse (1992). For the very-long-chain fatty acid elongase activity was tested according to the method of Bernasconi *et al.* (2000).

Results

Effects of ethylene and 1-MCP on the AFD level, Δ^{12} fatty acid desaturase activity and *avfad12-3* expression

Exposure of freshly harvested avocado fruits to ethylene increased the levels of the AFD from 1100 to 1590 $\mu\text{g.g}^{-1}$ fresh wt by 24 h after the treatment. However, a similar ethylene exposure that was applied following treatment with 270 nL.l^{-1} of 1-MCP did not change the level of AFD (Fig. 1A). Exposure of freshly harvested fruits to ethylene enhanced Δ^{12} fatty acid desaturase activity 3.3-fold and 3.8-fold by 6 and 24 h, respectively, after the treatment (Fig. 1B). Expression of *avfad12-3* following ethylene treatment was enhanced at 6 h after the ethylene treatment and at 24 h no transcript was detected (Fig. 1C). However, pre-ethylene treatment of the fruit with 1-MCP, blocked the ethylene-induced Δ^{12} fatty acid desaturase activity and *avfad12-3* expression (Fig. 2B). Control fruits treated with 1-MCP alone did not show Δ^{12} fatty acid desaturase activity or *avfad12-3* expression (Fig. 1B, C).

Effects of 1-MCP on fatty acid elongase activity and *avfae* expression

Exposure of avocado fruit to ethylene increased the enzymatic activity of fatty acid elongase in the peel of freshly harvested avocado fruits by 6 to 7 fold at 6 or 24 h, after the ethylene treatment. Fatty acid elongase expression probed with *avfae1* in Northern blot analysis detected transcript accumulation 6 and 24 h after ethylene treatment. Application of 1-MCP before the ethylene prevented both the ethylene-induced fatty acid elongase activity and *avfae1* transcript accumulation, compared to the control.

Effects of 1-MCP on AFD level, fruit firmness and *C. gloeosporioides* development

Fruits harvested approximately 280 days after fruit set softened after 13 days of storage at 22 C, compared with 8 d for those harvested 80 days later (Fig. 2A). Treatment with 1-MCP in freshly harvested fruits prevented the decline in fruit firmness in both batches. The concentration of the AFD in the late-harvested fruits was 950 $\mu\text{g.g}^{-1}$ fresh wt compared with almost double that in fruits harvested 80 days earlier. 1-MCP treatment reduced the level of the AFD in fruits of both dates: in those harvested 280 days after fruit set the treatment reduced the AFD level from approx. 1400 almost to 800 $\mu\text{g.(g fresh wt)}^{-1}$ 8-10 days after treatment, and in those harvested 80 days later the AFD declined from 650-790 to 380-420 $\mu\text{g.g}^{-1}$ fresh wt 4-6 days after the treatment (Fig. 2B).

Decay development was initiated by *C. gloeosporioides* earlier in the late-harvested fruits (Fig. 2C), but the effects of 1-MCP treatment differed between early- and late-harvested fruits. Whereas 1-MCP completely prevented decay development in early harvested fruits (Fig. 2C), decay development in late-harvested fruits did not differ significantly from that in untreated ones (Fig. 2C).

Discussion

The level of the AFD in avocado fruits depends on the balance between its synthesis and breakdown. The concentration of the AFD decreased during fruit development from about 3500 $\mu\text{g} \cdot (\text{g fresh wt})^{-1}$ in the peel of young fruits that weighed 50 g to 700 $\mu\text{g} \cdot (\text{g fresh wt})^{-1}$ in full-size fruits that weighed 200 g. This decline in AFD concentration was possibly the result of reduced synthesis and enhanced catabolic processes. In young fruits, the synthesis of the AFD is higher than that in mature fruits (Wang et al, 2004ab). Both Wang *et al.* (2004) and Madi *et al.* (2003) suggested that the AFD can be synthesized after harvest following induction by either biotic or abiotic factors. However, the overall decrease in the AFD concentrations suggests that the catabolic processes are enhanced to a greater extent than the biosynthetic ones. Lipoxygenase was hypothesized to catalyze the oxidation and the consequent metabolism of preformed AFD, whose activity is regulated by epicatechin, an inhibitor of lipoxygenase activity (Prusky et al, 1983): faster decrease of epicatechin during the ripening of avocado fruits enabled higher lipoxygenase activity, and this resulted in increased susceptibility to *C. gloeosporioides* decay, and the transition from the quiescent to the necrotrophic-development stage of *C. gloeosporioides*.

The biosynthesis of antifungal compounds in avocado fruits may result from general activation of fatty acid biosynthetic pathways that start from acetate and/or from intermediate precursor molecules such as oleic acid or linoleic acid, and are followed by other secondary process that are activated by non-specific factors. Madi et al. (2003) and Wang *et al.* (2004) suggested that in mature fruits, ethylene could induce the level of AFD by facilitating enhanced transcription of the Δ^9 and Δ^{12} desaturation steps, and the elongation from the putative precursor, linoleic acid. The present findings show that treatment with 1-MCP, an inhibitor of ethylene binding to its receptors, prevented the ethylene-induced transcription of fatty acid desaturase and fatty acid elongase, both of which are needed for the transformation of linoleic acid to the AFD. The effect of 1-MCP on genes which may be directly involved in AFD synthesis demonstrates the importance of ethylene as an inducer of *avfad1* and *avfae* gene expression. This, in turn, implies that ethylene production at different periods of fruit life might modulate the biosynthetic pathway of the AFD. The decline in AFD levels that followed 1-MCP treatment in harvested fruits at different harvesting times indicates that synthesis of the AFD was inhibited, which accounts for the decrease by 200 to 400 $\mu\text{g} \cdot \text{g}^{-1}$ fresh wt of the AFD level, compared with that in untreated fruits.

One interesting question is why ethylene did not induce the Δ^{12} desaturation steps, the fatty acid elongation, and the AFD enhancement during fruit ripening after harvest, when the endogenous ethylene bursts during the climacteric phase raised the ethylene concentration as high as 150 $\mu\text{g} \cdot \text{ml}^{-1}$ per kg.h. One key factor in determining the host response to the triggering of the biosynthetic process of the AFD by ethylene is the physiological and biochemical stage of the fruit after harvest. Ethylene treatments applied during first 2 days after harvest and before the

climacteric respiration phase enhanced AFD synthesis, whereas, in contrast, treatments applied to ripe fruit after the climacteric stage failed to enhance the AFD. A similar lack of host responsiveness at the post-climacteric phase was observed for production of reactive oxygen species following fungal inoculation or the induction of resistance by exogenous application of H_2O_2 .

1-MCP is considered an excellent tool for the prevention of fruit senescence during storage, which enables extended marketing of fruits (Adkins et al, 2005). In avocado fruits the relation between fruit softening and decay development is a key factor in determining susceptibility to *C. gloeosporioides*. Symptoms of *C. gloeosporioides* usually are initiated as a result of the decline in the level of the AFD that occurs in parallel to fruit ripening and softening. Because 1-MCP affects both fruit ripening as well as AFD synthesis, differing fruit responses to fungal attack could be observed. When 1-MCP was applied 280 days after fruit set, *i.e.*, during the normal commercial harvesting period, it significantly inhibited fruit ripening and AFD synthesis. However, because the initial level of the AFD was high enough, at $1820 \mu\text{g} \cdot (\text{g fresh wt})^{-1}$, the decline by approx. $400 \mu\text{g} \cdot \text{g}^{-1}$ fresh wt in its concentration did not affect symptom development in the same time frame as that in which fruit ripening is prevented. However, when the 1-MCP was applied to fruits that had been harvested 360 days after fruit set, by which time the natural level of the AFD was significantly lower, at $950 \mu\text{g} \cdot \text{g}^{-1}$ fresh wt, the decline in the AFD level resulted in the occurrence of decay symptoms in firm fruits. The ED_{50} level of AFD for inhibition of *C. gloeosporioides* germination was previously found to be around $450 \mu\text{g} \cdot \text{ml}^{-1}$, which suggests that reduction of the AFD concentration below its inhibitory threshold could lead to infections by *C. gloeosporioides* (Prusky et al, 1982). Because avocado fruits are consumed only when they have fully softened, 1-MCP-treated fruits would exhibit enhanced disease incidence before softening. Our early data indicate that AFD synthesis occurs continuously during the whole period of fruit growth and present data suggest that it occurs also after harvest. The balance between the rates of metabolism and catabolism is determined by the expression of each particular process during fruit development. Sub-fungitoxic concentrations, resulting from either reduced synthesis or enhanced catabolism of the AFD, trigger the transition from quiescent to actively developing infections, and the occurrence of visible symptoms of *C. gloeosporioides* infection.

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Figures

Fig. 1. Effects of ethylene and its inhibitor 1-MCP on the AFD levels (A), the Δ^{12} fatty acid desaturase activity (B), and transcript expression of *avfad12-3* (C) on exocarp of avocado fruit cv. Fuerte.

Fig. 1 Efecto del etileno y su inhibidor 1-MCP en la concentración del AFD (A), la actividad de la Δ^{12} fatty acid desaturase (B), y el nivel de expreson de *avfad12-3* (C) en la pulpa de avocado variedad Fuerte.

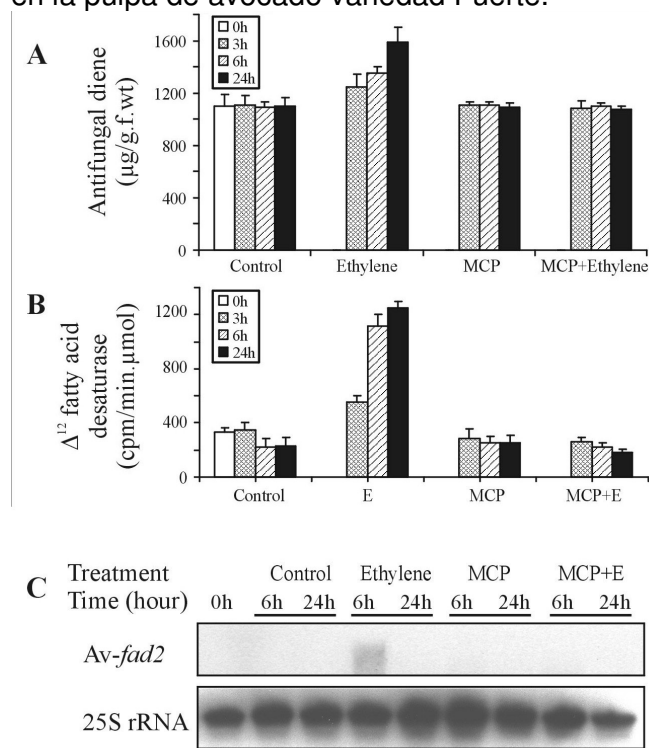


Fig. 2. AFD levels, fruit firmness and *C. gloeosporioides* symptom development on avocado fruit cv. Fuerte harvested at two periods after fruit set. Avocado fruits harvested between the 260 and 360 days after fruit set were exposed to 1-MCP at $270 \pm 20 \text{ nl.l}^{-1}$ for 17 h.

Fig. 2. Niveles del AFD, firmeza de la fruta y desarrollo de la podredumbre causada por *C. gloeosporioides* en frutas de avocad variedad Fuerte en periodos despues de la floracion. Frutas cultivadas entre los 260 y los 360 dias despues de la floracion fueron tratads con $270 \pm 20 \text{ nl.l}^{-1}$ de 1-MCP por 17 horas.

