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PREVENTION OF POSTHARVEST DISEASE BY MODUALTION OF NATURAL ANTIFUNGAL COMPOUNDS CONCENTRATION IN RIPENING AVOCADOS

Principal Investigator: Dov Prusky

Grantee Institution: Department of Fruit and Vegetable Storage.
ARO. The Volcani Center, Bet Dagan 50250
Israel

Collaborator: Nimal K. B. Adikaram

Institution: Department of Botany, University of Peradeniya
Sri Lanka

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3. Executive Summary

By means of comparative biochemical approaches, we have elucidated putative defense mechanims controlling postharvest disease in unripe avocado fruits. Levels of the avocado diene and monoene were high in unripe, disase-resistance fruit but decreased during fruit ripening, a time when latent fungal infections cause proliferating lesions that make fruit unmarketable. Such correlative evidence, however, does not prove a role for the diene compound in resistance. To provide more critical test we employed various exogenous treatments that modulated the normal disapearance of the diene during the ripening of avocado fruits and observed wheter the treatment concomitantly affected the development of fungal decay. The most effective measures were antioxidant and high carbon dioxide treatments, which inhibited decreases of the avocado antifungal diene compound during ripening or induced accumulation of higher levels. The fact that modulation of diene levels by these agents led to the predicted changes in fruit resistance constitutes important evidence that the diene is a physiologically important agent affecting resistance to *C. gloeosoporioides.*

4. RESEARCH OBJECTIVES

Fruits are generally protected by differentiated integumentary structures, and their physiology changes markedly during development, particularly when ripening occurs. Pathogens frequently infects unripe fruits but cuase relatively minor damage until ripening, when they cause extensive decay. Such quiescent infections habe been observed in tropical subtropical and deciduous fruits. The resultant decays habe great economic importance, since they reduce the shelf life of fruits during storage and transport. The disease problems habe been further exacerbated by the development of pathogen resistance to fungicides and withdrwal of pesticides on environmental grounds. Consequently there is considerable interest in determinig mechanisms accounting for the natural resistance of unripe fruits to fungal pathogens and extending its effectivness to fruits after harvest. One of the possible mechanisms that may account for such differential resistance during ripening is the level of preformed antifungal compounds. In the present proposal we have determined the importance of preformed antifungal compounds as one of the main factors in the resistance of unripe avocado fruits.

5. Methods and Results

EVALUATION OF ANTIOXIDANT TREATMENTS FOR PREVENTION OF AVOCADO ROT

The use of antioxidants to delay the onset of anthracnose and stem end decay in avocado fruits after harvest.

By Dov Prusky

The use of the antioxidant butylated hydroxy anisole and the fungicide prochloraz for the control of postharvest anthacnose of avocado fruits during storage.

By D. Prusky, H. Ohr, N. Grech, S. Campbell, I. Kobiler, G. Zauberman, and Y. Fuchs.

The Use of Antioxidants to Delay the Onset of Anthracnose and Stem End Decay in Avocado Fruits After Harvest

DOV PRUSKY, Department of Fruit and Vegetable Storage, ARO, The Volcani Center, Bet Dagan 50250, Israel

ABSTRACT

Prusky, D. 1988. The use of antioxidants to delay the onset of anthracnose and stem end decay in avocado fruits after harvest. Plant Disease 72:381-384.

Infiltration of avocado fruits (cultivar Fuerte) with 0.1 mM solutions of an antioxidant (butylated hydroxy toluene [BHT], α-tocopherol, *tert*-butylhydroquinone [TBHQ], epicatechin, or 0.01% gum guaiac) delayed the appearance of anthracnose symptoms for 1-2 days. A mixture of 0.1 mM BHT and either 0.5% ascorbic or 0.1% citric acid enhanced the activity of BHT alone and delayed the appearance of anthracnose symptoms for a further day without affecting fungal growth in vitro. Butylated hydroxy anisole and propyl gallate (0.1 mM) had no effect on disease expression. Treatments of fruits with 0.1 mM epicatechin or a mixture of 0.1 mM BHT and 0.5% ascorbic acid delayed the disappearance of endogenous epicatechin and the antifungal diene, and delayed disease expression; infiltration with 1 mM TBHQ, however, enhanced the disappearance and disease expression. In semicommercial trials, stem end rot in cultivars Ettinger and Fuerte was also delayed for 1-4 days as a result of dip treatment in a mixture of 0.1 mM BHT and either 0.5% ascorbic or 0.1% citric acid.

Additional keywords: antifungal compounds, quiescent infections, postharvest diseases, postharvest treatments

Anthracnose, caused by *Colletotrichum gloeosporioides* Penz., and stem end rot, caused by *Diplodia natalensis* Pole-Evans, are important fruit-rot diseases of avocado in the U.S.A., Israel, and Australia (8,12,17,18). *C. gloeosporioides* infects avocado peel (2) and *D. natalensis* infects the fruit pedicel (1) during the growing season, but the infections remain quiescent until fruit ripen after harvest. Under commercial conditions, decay development can usually be delayed either by storage at low temperature (5-6 C) (13) or by fungicide treatment (7). The latter is applied as preharvest orchard sprays or, more efficiently, as postharvest dip treatments (7).

Prusky et al (10) isolated a preformed antifungal compound from the peel of unripe avocado fruits and identified it as *cis,cis*-1-acetoxy-2-hydroxy-4-oxoheneicosa-12,15 diene (Fig. 1). This compound was shown to be involved in the mechanism for quiescence of *C. gloeosporioides* infections in unripe avocado (10). The antifungal diene also inhibited elongation of germinating *D. natalensis* hyphae with an ED_{50} similar to that of *C. gloeosporioides* (480 μg/ml). It was suggested that lipoxygenase catalyzes the oxidation of the antifungal diene during fruit ripening, resulting in its decrease to subfungitoxic concentrations and, therefore, in its decrease in activation of fungal infections (Fig. 2) (9). Infiltration of fruits with α-tocopherol (BDH), an inhibitor of lipoxygenase activity, inhibited the decrease of the antifungal diene and also the development of anthracnose lesions (9). It was concluded that lipoxygenase activity in the peel of ripening fruits could be regulated by concentration of the natural antioxidant epicatechin (Fig. 2) (11). Reduction in concentration of epicatechin in the peel after harvest was always found to be associated with a decrease in diene concentration and development of anthracnose lesions (*unpublished*).

This contribution reports on exogenous postharvest treatments that inhibited lipoxygenase activity and delayed the activation of quiescent infections and development of anthracnose and stem end decay.

Contribution from the ARO, No. 1961-E, 1987 series.

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MATERIALS AND METHODS

Avocado fruits (cultivars Ettinger and Fuerte) were obtained from an orchard near Rehovot, Israel. A single-spore isolate of *C. gloeosporioides* from decayed avocado fruits was used in all experiments. Cultures of this strain were maintained either on avocado fruits or on potato-dextrose agar (PDA) plates (10). Fruits were inoculated with a 10-μl drop of a spore suspension (10^4 spores/ml) placed at four different positions along both sides of the longitudinal axis of each of 20 freshly harvested fruits and then incubated at 20 C. Fruits were examined daily for disease symptoms using a binocular microscope. Peel darkening exceeding 1 mm in diameter was considered positive for disease expression. The effects of the treatments were expressed as days from inoculation to disease expression. Standard deviation was calculated for 80 infection points per treatment.

Effect of antioxidants on *C. gloeosporioides* decay development. Twenty-four hours after inoculation, opposite halves of the same fruits were infiltrated with either antioxidant or solvent solutions (9). Infiltration was carried out by immersing the appropriate half of the fruit and reducing the pressure to 100 mm Hg for 60 sec. The amount of solution absorbed by each half fruit was 0.20 ± 0.08 g per 100 g of fruit. Fruits were infiltrated with α-tocopherol, butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), gum guaiac, *tert*-butylhydroquinone (TBHQ), and epicatechin in 0.1% ethanol, 0.1% dimethyl sulfoxide (DMSO), and 0.01% Triton X-100. The same detergent-ethanol mixture, without antioxidant, served as a control. In some experiments, ascorbic or citric acid at 0.5% and 0.1%, respectively, were added to BHT as

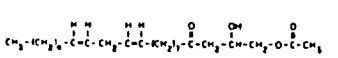

Fig. 1. Structural formula of the antifungal diene from avocado.

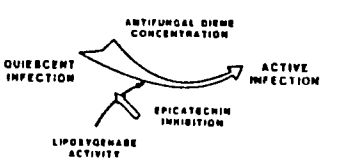

Fig. 2. Sequence of reactions leading to active infections of *Colletotrichum gloeosporioides* in ripening avocado: lipoxygenase-catalyzed decrease in diene concentration facilitates activation of quiescent infection. Epicatechin inhibits lipoxygenase activity.

synergists of antioxidant activity (14). Experiments were repeated at least twice.

Effect of antioxidants on *C. gloeosporioides* growth in vitro. Antioxidants dissolved in 95% ethanol were amended into PDA. Antioxidant-amended and unamended plates were inoculated with 5-mm-diameter plugs of fungal mycelium sampled from a 7-day-old culture. The diameter of growth developed at 20 C and was checked daily. Presence of ethanol at a concentration of 1% in the media did not affect *C. gloeosporioides* growth.

Extraction and quantitative analysis of the antifungal diene and epicatechin. The antifungal diene was extracted from avocado peels and quantified as described by Prusky et al (10). The lipoxygenase inhibitor, epicatechin, was extracted in 5-mM sodium phosphate buffer (pH 7.2), partially purified by flash chromatography, and quantified by HPLC as described by Prusky et al (9).

Effect of antioxidant and fungicide dip treatment on development of *D. natalensis* decay in semicommercial trials. In these experiments, fruits were not artificially inoculated. To assure a high incidence of stem end rot, avocado cultivars Fuerte and Ettinger were picked early during the harvesting season from orchards known for high incidence of the disease (12,13). The fungicide imidazole (Prochloraz, 45 EC) 1-*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]carbamoylimidazole (7,12) was compared with antioxidant treatments for control of stem end rot. Twenty-four hours after harvest, fruits were dipped for 30 sec in 900 μg/ml prochloraz or in aqueous antioxidant solutions at 0.1 mM BHT and 0.5% ascorbic acid, and 0.1 mM BHT and 0.1% citric acid. After treatment, fruits were air-dried and stored at 20 C or stored at 2 C for 10 days and then transferred to 20 C. Symptoms of Diplodia stem end rot exceeding 1 cm deep in the fruit flesh were considered positive for disease expression. Each treatment was replicated six times with 20 fruits in each replicate. Experiments were conducted twice in each of two harvesting seasons.

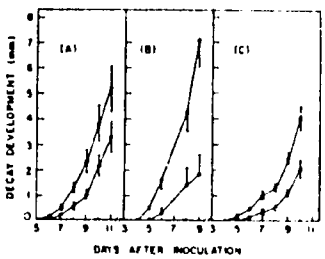

Fig. 3. Effect of antioxidants on decay development of *Colletotrichum gloeosporioides* in avocado fruits stored at 20 C. One-half of the whole fruit was infiltrated 24 hr after inoculation of freshly harvested fruits with (A) butylated hydroxy toluene, (B) α-tocopherol at 0.1 mM, and (C) gum guaiac at 0.01% in 0.01% Triton X-100, 0.1% DMSO, and 0.1% ethanol (●). Control halves (○) were infiltrated with the solvent alone. Decay development is expressed as the diameter of peel darkening at the inoculated site. Darkening of the peel exceeding an area of 1 mm in diameter was considered as symptom expression of disease. Standard deviation is provided in bracket.

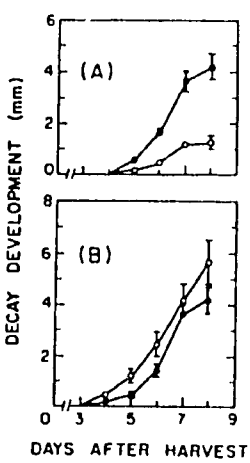

Fig. 4. Effect of *tert*-butylhydroquinone on decay development of *Colletotrichum gloeosporioides* in avocado fruits stored at 20 C. Freshly harvested fruits were inoculated. Twenty-four hours later, one-half of the whole fruit was infiltrated either with the solvent (controls) consisting of 0.01% Triton X-100, 0.1% DMSO, and 0.1% ethanol (○), or with solvent also containing TBHQ (●) at concentrations of (A) 1 mM or (B) 0.1 mM. Decay development is expressed as the diameter of peel darkening at the inoculated site. Darkening of the peel exceeding an area of 1 mm in diameter was considered symptom expression of disease. Standard deviation is provided in bracket.

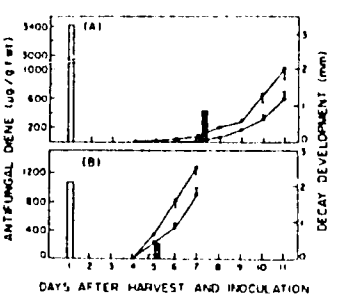

Fig. 5. Effect of epicatechin on the antifungal diene concentration and decay development by *Colletotrichum gloeosporioides* in avocado fruits stored at 20 C. Epicatechin (0.1 mM), in 0.01% Triton X-100, 0.1% DMSO, and 0.1% ethanol (●), was infiltrated into fruit halves 24 hr after inoculation of freshly harvested fruits. Control halves were infiltrated with the solvent alone (○). Concentration of the antifungal diene in crude extracts of peel treated with epicatechin (hatched bar) or the solvent alone (open bar). Two different experiments were carried out (A) in the beginning of the harvest season and (B) 3 mo later. Darkening of the peel exceeding an area of 1 mm in diameter was considered symptom expression of disease. Standard deviation is provided in bracket.

RESULTS

Effect of antioxidants on *C. gloeosporioides* decay development. Infiltration of avocado fruits with 0.1 mM BHT, 0.1 mM α-tocopherol, or 0.01% gum guaiac delayed decay development by *C. gloeosporioides* on the treated side of the fruits (Fig. 3) compared with the control side. Butylated hydroxy toluene delayed disease expression by 1 day, and α-tocopherol and gum guaiac delayed it by 2 days. Inhibition of lesion expansion was observed until 9–11 days after harvest in the antioxidant-treated versus control halves of the fruits. Treatments with 0.1 mM BHA or 0.1 mM propyl gallate were ineffective (results not presented). The effect of TBHQ differed with concentration. *Tert*-butylhydroquinone at 0.1 mM delayed disease expression by 1 day (Fig. 4B), but treatment with 1 mM TBHQ significantly hastened disease expression by 1 day (Fig. 4A). Effects of treatments lasted for at least 8 days after harvest.

The addition of either 0.1% citric or 0.5% ascorbic acid enhanced the activity of BHT. In both treatments disease expression was delayed by 1 day. Eight days after inoculation, the lesion diameter on the BHT-ascorbic acid- and BHT-citric acid treated sides was 1.0 ± 0.2 mm and 0.7 ± 0.3 mm, respectively; in the BHT controls, the lesion averaged 1.8 ± 0.3 mm. Neither 0.1% citric nor 0.5% ascorbic acid affected decay development significantly. Eight days after inoculation, lesion diameter averaged 1.5 ± 0.3 mm on the control side and 1.7 ± 0.5 mm on the ascorbic acid-infiltrated side. On fruits treated with citric acid, the lesion averaged 2.2 ± 0.3 mm on the control side compared with 1.8 ± 0.3 mm on the treated side.

C. gloeosporioides growth was not affected by mixtures of 0.1 mM BHT with either 0.1% citric or 0.5% ascorbic acid added to PDA. After 7 days at 20 C, the diameter of 5 mm fungus circles had grown to 5.8 ± 0.1 mm in the antioxidant-amended and unamended plates.

Effect of antioxidants on epicatechin and diene concentration and on decay development of *C. gloeosporioides*. *Effect of epicatechin treatments on diene concentration and decay development.* Two experiments were carried out during one harvesting season. At the beginning of the season, the concentration of the antifungal diene in the fruit peel was 3,450 μg/g fresh wt 1 day after harvest (Fig. 5A). Infiltration with 0.1 mM

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epicatechin delayed the decrease of the antifungal diene. Seven days after harvest, diene concentration was 450 μg/g fresh wt on the epicatechin-treated side and 60 μg/g fresh wt on the control side. Concentrations of endogenous epicatechin on the epicatechin-treated and control sides were 674 and 451 μg/g fresh wt, respectively, 2 days after treatment. Four days after treatment, epicatechin concentrations were similar in both sides. In the second experiment, 3 mo later, the initial concentration of the diene was 1,100 μg/g fresh wt. Five days later, concentrations of the diene on the epicatechin-treated and control sides of the fruit were 250 and 90 μg/g fresh wt, respectively (Fig. 5B). Epicatechin treatment delayed expression of disease in both experiments by 1 day.

Effect of BHT-ascorbic acid treatment on epicatechin and diene concentration. Infiltration of avocado fruits with a mixture of 0.1 mM BHT and 0.5% ascorbic acid delayed the decrease of endogenous epicatechin concentration that occurs after harvest (Table 1). In the peel on the antioxidant-mixture-treated side, the concentration of epicatechin was higher throughout the experiment. The antioxidant treatment also delayed the diene decrease that occurred in the peel on the control side following the fifth day after harvest. On the seventh day, diene concentration on the control side was about 25% of that on the treated side. Nine days after harvest, disease was expressed on the control side, whereas on the treated side it was expressed on the tenth day.

Effect of TBHQ treatment on epicatechin and diene concentration. Infiltration with 1 mM TBHQ enhanced disease expression by 1 day (Fig. 4) and it also enhanced the decrease of endogenous epicatechin and antifungal diene concentration of the fruit (Table 2). Concentration of epicatechin on the control side had decreased to 288 μg/g fresh wt on the eighth day after harvest, whereas on the treated side it was already 62 μg/g fresh wt. Diene concentration in the TBHQ-treated side of the fruit was lower throughout the experiment. On the eighth day after harvest, it was 520 μg/g fresh wt on the treated side and 2,050 μg/g fresh wt on the control side. On the twelfth day after harvest, disease expression occurred on the TBHQ-treated side, whereas on the control side it occurred on the thirteenth day.

Effect of antioxidants and fungicide dip treatment on development of *D. natalensis* decay in semicommercial trials. Dip treatment of Ettinger fruits in a mixture of BHT and either 0.1% citric or 0.5% ascorbic acid delayed symptoms of stem end rot by about 4 days (Fig. 6). After the decay appeared, it increased more rapidly in fruits dipped in BHT and ascorbic acid than in the mixture of BHT and citric acid. Dip treatments of Fuerte fruits (Fig. 7) on a mixture of BHT and citric acid delayed disease expression by 2 days compared with controls. The mixture of BHT and ascorbic acid and the commercial fungicide prochloraz at 900 μg/ml delayed disease expression by only 1 day.

DISCUSSION

The resistance of unripe avocado fruits to attack by postharvest pathogens was associated with the preformed antifungal compound called diene (10). Previous results (9,11) led to the belief that diene decrease was a result of increased lipoxygenase activity. Activity of this enzyme was regulated by the decrease in concentration of the flavan 3-ol inhibitor, epicatechin (11). In the present work, compounds that inhibited lipid peroxidation activity of lipoxygenase (4,16) were tested as possible inhibitors of diene peroxidation. They delayed the development of anthracnose and stem end rot. Infiltration treatments of avocado with α-tocopherol, BHT, TBHQ at 0.1 mM, and 0.01% gum guaiac delayed the expression of anthracnose symptoms by 1–2 days (Figs. 1 and 2).

Treatments with mixtures of 0.1 mM BHT and either 0.5% ascorbic or 0.1% citric acid delayed the expression of disease symptoms by 1 day more than BHT alone. These mixtures did not inhibit the fungus in culture, and their effect must have been on plant metabolism. Plant lipoxygenases are strongly inhibited by some polyphenols

Table 1. Effect of infiltration of avocado fruits (cultivar Fuerte) with a mixture of 0.1 mM butylated hydroxy toluene (BHT) and 0.5% ascorbic acid on the concentration of epicatechin and antifungal diene, and on decay development of *Colletotrichum gloeosporioides* at 20 C

Days after harvest	Epicatechin[a] (μg/g fresh wt)		Diene[a] (μg/g fresh wt)		Decay development (mm)	
	Control	BHT + ascorbic acid[b]	Control	BHT + ascorbic acid	Control	BHT + ascorbic acid
5	913	1,173	1,490	1,500		
6	1,431	1,817	500	1,480		
7	516	786	120	500		
9	12	47			1.9[c]	1.0 *
10					3.3	1.8 *

[a] Samples of partially purified extracts were chromatographed on reverse-phase HPLC columns and peak heights were compared with those of standards.
[b] Fruits were infiltrated with an aqueous solution of 0.01% Triton X-100, 0.1% dimethyl sulfoxide, and 0.1% ethanol. The same solution, without the antioxidant, was infiltrated into control fruits.
[c] Fruits were inoculated by placing 10-μl drops of a spore suspension (10^6 spores/ml) at four different positions along both sides on each of 20 freshly harvested fruits. Decay development was measured as darkening of the peel. Expression of disease was recorded when peel darkening exceeded an area of 1 mm in diameter. * = Means for treated versus control were different at $P = 0.05$.

Table 2. Effect of infiltration of avocado fruits (cultivar Fuerte) with 1 mM *tert*-butylhydroquinone (TBHQ) on the concentration of epicatechin and diene, and on decay development by *Colletotrichum gloeosporioides* at 20 C

Days after harvest	Epicatechin[a] (μg/g fresh wt)		Diene[a] (μg/g fresh wt)		Decay development (mm)	
	Control	TBHQ[b]	Control	TBHQ	Control	TBHQ
1	1,235		720			
4	572	233	910	560		
6	639	273	1,940	510		
8	288	62	2,050	520		
11	28	13	830	440		
12					0.7[c]	1.0 *
13					1.0	2.0 *
14					1.6	3.6 *

[a] Samples of partially purified extracts were chromatographed on reverse-phase HPLC columns and peak heights were compared with those of standards.
[b] Fruits were infiltrated with an aqueous solution of TBHQ in 0.01% Triton X-100, 0.1% dimethyl sulfoxide, and 0.1% ethanol. The same solution, without the antioxidant, was infiltrated into control fruits.
[c] Fruits were inoculated by placing 10-μl drops of a spore suspension (10^6 spores/ml) at four different positions along both sides on each of 20 freshly harvested fruits. Decay development was measured as darkening of the peel. Expression of disease was recorded when peel darkening exceeded an area of 1 mm in diameter. * = Means for treated versus control were different at $P = 0.05$.

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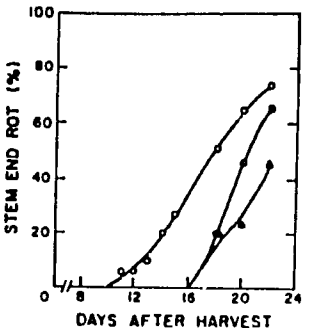

Fig. 6. Effect of postharvest dips in antioxidants on the incidence of *Diplodia natalensis* stem end rot on stored avocado fruit (cultivar Ettinger). Fruits were dipped in a mixture of 0.1 mM BHT and 0.5% ascorbic acid (o) or 0.1 mM BHT and 0.1% citric acid (▲). Controls (●) were dipped in water only. Dipped fruits were stored at 2 C for 10 days and then transferred to 20 C. *D. natalensis* penetration into fruit flesh to a depth of 1 cm was considered rot development.

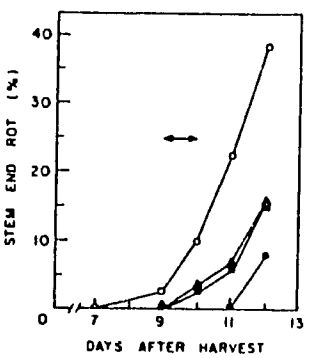

Fig. 7. Effect of postharvest dips in antioxidants and fungicide on the natural incidence of *Diplodia natalensis* stem end rot on stored avocado fruits (cultivar Fuerte). Fruits were dipped in a mixture of 0.1 mM BHT and 0.5% ascorbic acid (■), 0.1 mM BHT and 0.1% citric acid (●), or 900 $\mu g/ml^{-1}$ prochloraz (▲). Control fruits (o) were dipped in water. Fruits were stored at 20 C throughout the experiment. *Diplodia* penetration into fruit flesh to a depth of 1 cm was considered disease expression. Horizontal arrow indicates fruit were completely softened.

(6,15). The inhibition, which is often competitive, is the result of the removal of free radicals required for the reaction (3). When functioning as antioxidants, phenols constitute a trap for free radicals, resulting in their own oxidation to a very stable product (3), preventing epicatechin oxidation and inhibiting lipoxygenase increase and diene decrease. A similar effect was observed with an exogenous treatment of epicatechin, which prevented endogenous epicatechin decrease and delayed the diene decrease.

Tert-butylhydroquinone (0.1 mM) delayed disease expression by 1 day but at 1 mM, epicatechin and antifungal diene decrease and decay development were enhanced (Table 2). Antioxidants at certain concentrations act as prooxidants (5), indicating the importance of optimization of treatments because different concentrations may have opposite effects. This indicates that the regulation of decay development, initiated by the decrease of epicatechin and then of the diene, is a process that might be affected in either way.

The mixture of BHT and ascorbic or citric acid delayed the beginning of stem end rot development in semicommercial experiments by 1–4 days. A mixture of BHT and citric acid was more effective than the commercial fungicide prochloraz used in Israel for this purpose (11).

Treatments of avocado fruits with antioxidants delay the conversion of quiescent infections of *C. gloeosporioides* and *D. natalensis* into active infections and disease. This delay seems to depend on prevention of the decline of the natural mechanisms of resistance.

ACKNOWLEDGMENTS

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The Use of the Antioxidant Butylated Hydroxy Anisole and the Fungicide Prochloraz for the Control of Postharvest Anthracnose of Avocado Fruit during Storage

D. Prusky[1], H. Ohr[2], N. Grech[2], S. Campbell[2], I. Kobiler[1], G. Zauberman[1] and Y. Fuchs[1]. [1]Department of Postharvest Science of Fresh Produce, ARO, The Volcani Center, Bet Dagan 50250, Israel and [2]Department of Plant Pathology, University of California, Riverside, CA 92521, USA.

ABSTRACT

Prusky, D., Ohr, H., Grech, N., Campbell,S., Kobiler, I., Zauberman, G., and Fuchs, Y. 1994. The use of the antioxidant Xedaphen-20 and the fungicide Prochloraz for the control of postharvest anthracnose of avocado fruit during storage. Plant Dis. 78:000

The incidence of post-harvest anthracnose of avocado caused by *Colletotrichum gloeosporioides* was reduced significantly after dip or spray treatment with the anti-oxidant butylated hydroxy anisole (BHA) or BHA + prochloraz. In small, medium and semi-commercial experiments in California on cv. Hass or in Israel on cv. Fuerte there was a significant reduction of decay by a single treatment with BHA at 1200 µg ai/ml and by BHA at 1200 µg ai/ml + prochloraz at 250 µg ai/ml. Prochloraz at 1000 µg ai/ml alone did not always reduce the incidence of decay while BHA or BHA + prochloraz reduced decay consistently. The effect of BHA + prochloraz was longer lasting than BHA alone. It is suggested that the antioxidant might reduce postharvest decay in avocado by modulating the natural fruit resistance.

Additional keywords: antifungal compounds, quiescent infections, postharvest diseases, postharvest treatments.

Anthracnose, caused by *Colletotrichum gloeosporioides (Penz.) Penz. Sacc. in Penz.*, and stem end rot caused by *Diplodia natalensis* Pole-Evans, *Dothiorella gregaria* Sacc. or *D. aromatica* (Sacc.) Petr. & Syd. are the most important fruit rot diseases of avocado *Persea americana* Miller in the USA, Israel, Australia and South Africa (1,3,9,10,11). In avocado, anthracnose in its early stages is essentially a cosmetic disease. The peel of Hass avocados turns black as they ripen masking early disease symptoms. When avocados are ripe enough to eat the disease has not progressed beyond the peel into the fruit to any great degree and is removed with peeling. The Fuerte avocado is shipped from Israel to Europe where the blemishes of early disease development do not alarm the consumer. In California, postharvest decay in avocado was not considered a significant problem until prolonged rain during the winter of 1992-93 resulted in an increase in its incidence. *C. gloeosporioides* infects the avocado peel and *D. natalensis* infects the fruit pedicel during the fruit development season, but because avocados do not ripen on the tree the infections by both pathogens remain quiescent until after harvest when the fruit ripens. Under commercial conditions, decay development can be delayed by storing fruit at a temperature of 5-6 C during short-duration shipment or storage. When the fruit must be held for longer periods, (e.g., 20-24 days as with the South African fruit), a combination of low temperature and fungicide treatments are needed (1). Fungicides are applied as preharvest sprays or, more efficiently, as postharvest treatments (1,3).

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The regulations limiting the use of agrichemicals in local and export markets have stimulated the search for new alternatives to postharvest fungicides. Biological control has been suggested as a possible alternative to fungicides but in spite of the significant amount of work invested in their development, there are no commercial products on the market for the biological control of postharvest diseases (2). Prusky and Keen (5) have suggested a new approach for the prevention of postharvest disease; it involves the enhancement of the natural resistance of fruits. Prusky et al. (6,7) isolated an antifungal compound from the peel of unripe avocado fruit and identified it as *cis,cis*-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene, that prevents the development of *C. gloeosporioides* and *D. natalensis* beyond the initial infection in unripe avocados. The antifungal diene is apparently oxidized by lipoxygenase during fruit ripening which allows the fungi to resume colonization of the fruit tissues(7). Lipoxygenase activity in the peel of ripening fruit is regulated by the concentration of the natural antioxidant, epicatechin (4). Infiltration or dip treatment of avocado fruit with several antioxidants such as α-tocopherol, butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA) and *tert*-butylhydroquinone (TBHQ), that inhibited lipoxygenase *in vitro*, inhibited the decrease of the antifungal diene and also inhibited development of anthracnose. Prusky has described the possibility of using antioxidants to delay the onset of postharvest diseases in avocado (4). Although BHA and BHT are common food additives commercial formulations of these antioxidants suitable for agricultural use have only recently been developed. A commercial formulation of BHA was tested and found to be

effective, in several trials, in preventing postharvest diseases in avocados cv. Fuerte (10).

Below we report on several small and medium scale experiments, and semi-commercial level treatments with the antioxidant, BHA. Experiments were carried out in Israel and the USA, using two different avocado cultivars.

MATERIALS AND METHODS

General. Avocado fruit, cv's Hass and Fuerte, hand harvested by commercial crews were obtained from groves around Rancho California and in Israel respectively. All disease in these experiments resulted from natural infections. Chemicals used were prochloraz (Prochloraz 50 WP) Nor Am and butylated hydroxy anisole (BHA) (Xedaphene-20, 20% ai) Xeda International. Data analysis was done using Duncan's multiple range test.

Small- and medium-scale experiments with Hass cultivar in California. Fruit were dip treated within 4-5 h of harvest or after being precooled for 24 h at 5 C in a laboratory cold room. Fruit were dipped for 30 sec in 1200 µg ai/ml aqueous BHA, 1000 µg ai/ml prochloraz, or 1200 µg ai/ml BHA + 250 µg ai/ml prochloraz. A 250 µg ai/ml prochloraz control was not included because of Prusky's results that concentrations at 300 µg ai/ml or lower were ineffective (9). After treatment, fruit were either air-dried and stored at 20 C for 11 days or stored at 5 C for 14 days and then transferred to 20 C for another 11 days. Symptoms of anthracnose exceeding 1 cm in diameter and the stem end rot in the fruit peel were considered positive for infections. In small-scale experiments, each treatment was replicated four times with 10 fruit in each replicate. In medium-scale experiments, each treatment

was replicated five times with 20 fruit in each replicate. Experiments were conducted three times during the harvesting season.

Semi Commercial-scale experiments with cultivar Fuerte in Israel. Experiments were carried out in three different packing houses, Mehadrin, Granot and HaEmek, representing the main southern, central and northern avocado growing areas in Israel, respectively. All fruit was brought to the respective packing house on the day of harvest, cooled at 5 C overnight and treated commercially 1 day later. In Mehadrin the fruit was washed, brushed and air dried before the treatment. In Granot and HaEmek, the fruit were only brushed before treatment. Materials were applied through A transverse pipe fitted with six nozzles covering the complete 1.5-m width of the packing line. The spray nozzles were conical and placed 15 cm apart at a height to allow overlapping of the individual spray patterns. Spray pressure was maintained at 4 atmospheres for a flow rate of 188 L/h and an effective rate of 0.5 L per metric ton of fruit. Following treatment the fruit were waxed and sorted under commercial conditions. Treatments were BHA at 1200 µg ai/ml, prochloraz (45 EC)at 900 µg ai/ml, BHA at 1200 µg ai/ml + prochloraz at 225 µg ai/ml or water for the control. These identical treatments were used in each packing house.

In the first trial at Mehadrin approxamately 650 kg of fruit were treated with prochloraz, prochloraz + BHA or water, packed as described above and stored for 14 days at 5 C, then transferred to 20 C and evaluated for disease after 8 days. Six replications of 20 fruit each were used to evaluate each treatment. In the second test the fruit were treated and stored at 5 C for either 7 or 14 days, then

transferred to 20 C and evaluated for disease after 4 and 6 days. Ten replicates of 14 fruit each were used to evaluate the treatments.

At Granot the fruit were treated with prochloraz + BHA, sorted according to weight and packed commercially 18 fruit/carton (each about 220 g) and 14 fruit/carton (each about 280 g). There were 10 replicates (cartons) in each treatment. The fruit was stored either at 20 C or at 5 C for 21 days and then 20 C. Those fruit stored only at 20 C were evaluated after 9 and 12 days while those stored initially at 5 C were evaluated after they had been stored at 20 C for 7 and 9 days. The evaluation times were dictated by the disease progress.

Sorted as described before

At HaEmek, fruit from two different groves, Shaar Haamakim and Daliah, were treated or not with BHA + prochloraz, the fruit was ~~stored~~ at either 2 C or 5 C for 14 days then transferred to 20 C and evaluated after 9 days.

RESULTS

Small-scale experiments

With fruit treated within 5 h after harvest the decay incidence in untreated fruit after 11 days at 20 C averaged nearly 60% (Fig 1 A, B) whereas the incidence among fruit treated with 1000 µg ai/ml of prochloraz alone averaged 76%. In contrast the decay incidence among fruit treated with 1200 µg ai/ml BHA and the mixture of 1200 µg ai/ml BHA + 250 µg ai/ml prochloraz was significantly lower at 10% and 30% respectively after 9 days in storage. However, 2 days later, only fruit treated with the mixture had a significant reduction of decay, averaging 22% in two groves (Fig 1 A, B).

With fruit from a third grove, the decay incidence among the controls after 11 days at 20 C was only 10% and each of the treatments reduced the decay by 60-70%(Fig 1 C).

With pre-cooled fruit, disease incidence in untreated fruit averaged 10, 14 and 33% after 7, 9 and 11 days respectively. By contrast fruit treated with BHA + prochloraz averaged 0, 2 and 6% respectively. The difference between the control and respective treatments were significant at P=0.05(Fig 2).

Medium-scale experiments

Fruit precooled at 5 C for 24 h before treatment followed by 17 days of storage at 5 C, showed no decay at the time of transfer to 20 C (Fig 3). Six days later however, decay incidence among the control fruit was nearly 10% whereas treated fruit averaged 4-5%. After an additional 4 days at 20 C no differences in decay development were observed among fruit treated with BHA, prochloraz or a mixture of both. All the treatments reduced the incidence of decay by about 45% compared with the control (Fig 3)

Semi-commercial experiments

In trials at Mehadrin, Israel after storage for 14 days at 5 C followed by transfer to 20 C the incidence of decay in untreated fruit after 8 days at 20 C was 12±1%. In contrast fruit treated with 900 µg ai/ml prochloraz or with 1200 µg ai/ml BHA + 225 µg ai/ml prochloraz had a decay incidence of 6±1%.

In experiment 2 at Mehadrin (Table 1) as storage time at 5 C increased the incidence of decay increased (Table 1). The treatment with BHA reduced the incidence of decay compared with the control; BHA

+ prochloraz showed the greatest reduction of decay but was not always significantly different from prochloraz alone.

At Granot, Israel with fruit of two different sizes, after 9 days in fruit initially stored at 20 C the disease incidence in the 220 g size control was 7 times that of treated fruit. In the 280 g size, the control had 1.6 times the decay of treated fruit after 9 days (Table 2). Three days later, the incidence of decay in both sizes had increased, but treated fruit still showed significantly lower decay incidence than the untreated controls. In fruit stored 21 days at 5 C before storage at 20 C, 220 g control fruit had 2.2 times the decay of treated fruit while 280 g control fruit had 3.2 times the decay of treated fruit. Again, 2 days later, the incidence of decay in all fruit had increased but treated fruit still fared better than controls. Fruit of 280 g average size showed an overall higher incidence of decay than fruit of an average size of 220 g, but treated fruit always showed a significant decrease in decay symptoms (Table 2).

After 14 days storage at 2 C or 5 C followed by 9 days of storage at 20 C, the treated fruits from Shaar Haamakim and Daliah, Israel showed a significantly lower decay development at both storage temperatures relative to the controls (Table 3).

DISCUSSION

Postharvest diseases in avocado have been reduced significantly in South Africa and Australia by postharvest dip treatment with the fungicide, Prochloraz (1,3,9). (3) Prochloraz This treatment was effective against the three major post-harvest diseases anthracnose, stem end rot and (1,3,9) *Dothiorella* rot. In Israel, Prochloraz was also found to be effective

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in preventing post-harvest decay, at 900 µg ai/ml, but was ineffective at 300 µg ai/ml or less(9,10).

A second method to control postharvest disease in avocado fruit is the application of antioxidants, which delay the loss of anti-fungal compounds in the peel and hence the conversion of quiescent infections of *C. gloeosporioides* into active infections (5). A commercial formulation of BHA, Xedaphen-20, used to inhibit lipid peroxidation in many foods was used to prevent the oxidation of the antifungal diene (4). In the present work, BHA at 1200 µg ai/ml, alone or in a mixture with low concentrations of prochloraz at 225 to 250 µg ai/ml, provided some control of postharvest decay in cultivars Fuerte and Hass respectively.

In each of the various experiments, a single treatment with BHA at 1200 µg ai/ml delayed the onset of disease. The mixture of BHA at 1200 µg ai/ml and prochloraz at 225 µg ai/ml reduced decay development 2-4 times more than the BHA alone during short storage conditions.

Prochloraz at 900 µg ai/ml is an effective treatment for the control of postharvest decay in avocado (9,10). In California, however, at 1000 µg ai/ml, prochloraz failed to control disease and BHA at 1200 µg ai/ml was less effective on non pre-cooled fruit with a high amount of infection (Fig 1 A, B) than in pre-cooled fruit (Fig 2, 3) or fruit with less infection (Fig 1 C). Thus when favorable conditions for latent fruit infections exist in the avocado groves pre-cooling the fruit before treatment could enhance the effectiveness of the anti-oxidant and the fungicide.

The application of low dosages of fungicide in combination with BHA have enhanced post-harvest disease control in avocados, possibly by an additive or synergistic effect. The mixture was also observed to

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be effective in experiments in which the fruit was stored for almost 21 days, suggesting that, even at low concentrations, the fungicide in combination with BHA has an effect on disease development.

Our results indicate that it is feasible to reduce postharvest disease in avocado by modulation of the natural resistance of the fruit. In the present case, the application of an antioxidant that prevented the conversion of a quiescent infection into an active infection was enough to delay decay development. A limitation of the use of the antioxidant is that the treatment should be applied within a day of harvest. Previously the efficacy of the treatment decreased if the treatment was applied later than 24 h after harvest(4,10).

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FIGURES

Figure 1. Effect of postharvest dip treatment of avocado cv. Hass on the percentage of decay development. Fruit from three groves in southern California (represented in A, B and C) were harvested and treated within 5 h in the laboratory with 1000 µg ai/ml prochloraz, 1200 µg ai/ml BHA or BHA 1200 µg ai/ml + prochloraz at 250 µg ai/ml and compared to untreated fruit. Fruit were stored at 20 C and scored for disease incidence at 7, 9 and 11 days. Different letters above bars (within each observation day) indicate significant differences ($P<0.05$) as determined by Duncan's multiple range test.

Figure 2. Effect of postharvest dip treatment of avocado cv. Hass on the percentage of decay development. Fruit from a grove in southern California were harvested and cooled at 5 C for 24 h before dip treatment in BHA at 1200 µg ai/ml + prochloraz at 250 µg ai/ml and compared to untreated fruit. Fruit were stored at 20 C and scored for disease incidence at 7, 9 and 11 days. Different letters above bars (within each observation day) indicate significant differences ($P<0.05$) as determined by t test ~~Duncan's multiple range test~~.

Figure 3. Effect of postharvest dip treatment of avocado cv. Hass on the percentage of decay development. Fruit from a grove in southern California were harvested, cooled for 24 h at 5 C and dip treated in the laboratory with prochloraz at 1000 µg ai/ml, BHA at 1200 µg ai/ml or BHA at 1200 µg ai/ml + prochloraz at 250 µg ai/ml and compared to untreated fruit. Fruit were stored for 17 days at 5 C, then transferred to 20 C and scored for disease incidence at 4, 6 and 10 days. Different letters above bars (within each observation day) indicate significant differences ($P<0.05$) as determined by Duncans's multiple range test.

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Table 1. Effect of BHA and prochloraz on post harvest decay by *C. gloesporiodes* and *D. natalensis* of Fuerte avocados stored 7 or 14 days post treatment at 5 C followed by transfer to 20 C for 6 days.

	Storage at 5 C			
	7 days		14 days	
	Storage at 20 C			
	4 days	6 days	4 days	6 days
Control	16.7 a	53.2 a	20.5 a	77.7 a
BHA, 1200 µg ai/ml	2.1 b	24.0 b	11.2 b	41.3 b
prochloraz, 900 µg ai/ml	2.1 b	16.7 b	1.0 c	21.4 c
BHA, 1200 µg ai/ml + prochloraz, 225 µg ai/ml	0 b	6.3 c	3.1 c	18.8 c

Means of 10 replications of 14 fruit each.
Numbers within columns followed by different letters indicate significant differences between treatments ($P<0.05$) using Duncan's multiple range test.

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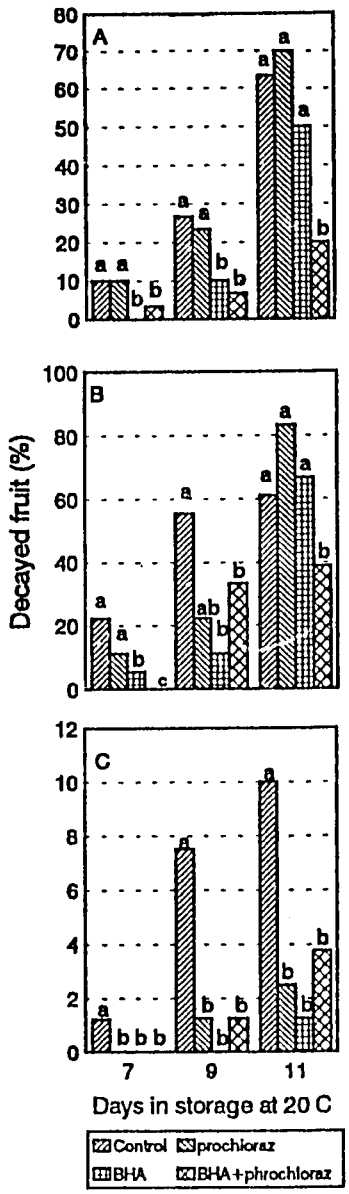

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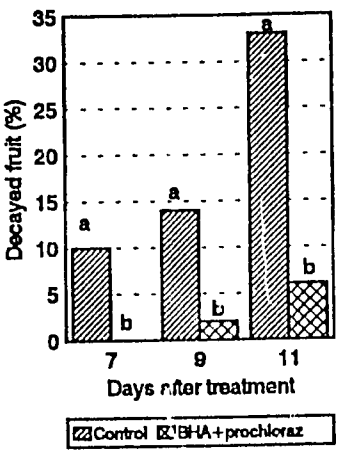

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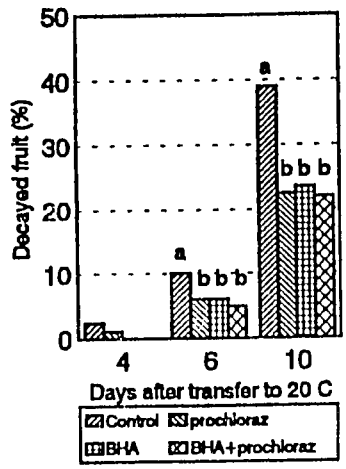

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Agricultural Research Organization, The Volcani Center, Bet Dagan; and Department of Agricultural Botany, The Hebrew University of Jerusalem, Rehovot, Israel

Involvement of Epicatechin in Cultivar Susceptibility of Avocado Fruits to *Colletotrichum gloeosporioides* after Harvest[1]

D. PRUSKY, I. KOBILER and B. JACOBY

Authors' addresses: D. PRUSKY and I. KOBILER, Department of Fruit and Vegetable Storage, The Volcani Center, P.O. Box 6, Bet Dagan 50250 (Israel). B. JACOBY, Department of Agricultural Botany, The Hebrew University of Jerusalem, Rehovot 76100 (Israel).

With 2 figures

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Abstract

Avocado cultivars were defined as susceptible and resistant to *Colletotrichum gloeosporioides* depending upon the length of the incubation period of the disease after fruit softening. In the susceptible cultivars Fuerte, Horshim, Vurtz, Rincon and Benik, epicatechin concentration of the peel decreased to 60—130 $\mu g \cdot g^{-1}$, fr. wt. at fruit softening and symptoms appeared on the same or one day later. In the resistant cultivars Hass, Nabal, Netaim and Pinkerton, epicatechin concentration was still 632—1740 $\mu g \cdot g^{-1}$ fr. wt. when fruit softening and symptoms appeared only 4—10 days later. When susceptible Fuerte fruits became soft the concentration of the antifungal compound 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene, had decreased to 120 $\mu g \cdot g^{-1}$ fr. wt. and symptoms appeared. In resistant Hass fruits, the antifungal diene was still 238 $\mu g \cdot g^{-1}$ fr. wt. at fruit softening; and it had further decreased to 159 $\mu g \cdot g^{-1}$ fr. wt. when symptoms appeared, four days later. A modified atmosphere and 0.2 M $CaCl_2$ infiltration both delayed softening of Fuerte fruits; but symptom appearance on these fruits was related to diene decrease and not to fruit softening.

The results are discussed in relation to the hypothesis that the susceptibility of avocado cultivars to post-harvest decay by *C. gloeosporioides* is related to the degradation of the antifungal diene, catalyzed by avocado lipoxygenase, the activity of which is regulated by the decline of its inhibitor epicatechin.

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Zusammenfassung

Beteiligung von Epicatechin in der Sortenanfälligkeit von Avocadofrüchten gegenüber *Colletotrichum gloeosporioides* nach der Ernte

Avocadosorten wurden als anfällig oder nichtanfällig gegenüber *Colletotrichum gloeosporioides* anhand der Dauer der Inkubationsperiode der Krankheit nach dem Weichwerden der Frucht unterteilt. Bei den anfälligen Sorten Fuerte, Horshim, Vurtz, Rincon und Benik verringerte sich die Schalenkonzentration vom Epicatechin auf 60—130 $\mu g \cdot g^{-1}$ Frischgewicht (Fr. Gew.) zur Zeit des Weichwerdens, und Befallssymptome waren am selben oder am darauf folgenden Tag zu erkennen. Bei den nichtanfälligen Sorten Hass, Nabal, Netaim und Pinkerton dagegen lag die Epicatechinkonzentration noch bei 632—1740 $\mu g \cdot g^{-1}$ Fr. Gew. und Befallssymptome waren erst 4—10 Tage später sichtbar. Beim Weichwerden von Fuerte-Früchten hatte sich die Konzentration der antipilzlichen Substanz 1-Acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-dien auf 120 $\mu g \cdot g^{-1}$ Fr. Gew. verringert und die Befallssymptome erschienen. Bei den nichtanfälligen Hass-Früchten betrug die antipilzliche Dienkonzentration noch 238 $\mu g \cdot g^{-1}$ Fr. Gew. beim Fruchtweichwerden; vier Tage später, als erste Symptome sichtbar wurden, hatte sich die Konzentration auf 159 $\mu g \cdot g^{-1}$ Fr. Gew. weiter verringert. Eine modifizierte Atmosphäre und 0,2 M $CaCl_2$-Infiltration verzögert zwar das Weichwerden von Fuerte-Früchten, aber das Erscheinen von Symptomen war mit der Dienkonzentrationsabnahme und nicht mit dem Fruchtweichwerden verbunden.

Die Ergebnisse werden in Zusammenhang mit der Hypothese diskutiert, daß die Anfälligkeit von Avocadosorten gegenüber *C. gloeosporioides*-Fäule nach der Ernte mit dem Abbau des antipilzlichen Dien korreliert, das wiederum durch die Avocadolipoxygenase katalysiert wird, deren Aktivität durch die Abnahme ihres Inhibitors Epicatechin reguliert wird.

The preformed antifungal compound 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene is apparently responsible for the quiescence of *Colletotrichum gloeosporioides* Penz. infections in unripe avocado fruits (PRUSKY *et al.* 1982). Activation of quiescent infections seems to be dependent on the post-harvest decrease of the antifungal diene that is catalyzed by endogenous lipoxygenase (PRUSKY *et al.* 1983) and regulated by the enzyme's inhibitor, epicatechin (PRUSKY *et al.* 1985). ZAUBERMAN and SCHIFFMANN-NADEL (1977), investigated factors determining the length of the incubation period of *Fusarium* sp., another quiescent fungal infection on avocado fruit. They suggested that the incubation period is related to the duration from harvest to fruit ripening (softening); namely the earlier the fruit softens the shorter is the incubation period of infective fungi. Since the concentrations of the antifungal diene decrease in many avocado cultivars simultaneously with fruit softening, it has been difficult to determine whether renewed development of quiescent fungi depend only on the diene decrease or also on fruit softening. The present work was designed to investigate the relative importance of fruit softening and the processes leading to diene decrease in the resistance of avocado cultivars to post-harvest development of *C. gloeosporioides*. It also proposes criteria for classifying avocado cultivars according to their differential post-harvest susceptiblity.

Materials and Methods

General

Avocado fruits of cultivars Fuerte, Vurtz, Rincon, Horshim, Benik, Pinkerton, Hass, Netaim and Nabal were obtained from orchards near Rehovot, Israel. Firmness, an inverse parameter of

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ripening, was measured by recording the force (kg) required to penetrate the fruit skin and flesh with a conic probe (5 mm in diameter and 4 mm in length). The average of two determinations on each of five fruits is reported. The fruit was considered soft when firmness reached ≤ 1 kg.

A single spore isolate of *C. gloeosporioides* from avocado fruits was used for inoculation in all experiments (PRUSKY *et al.* 1982). This isolate was maintained by inoculation of avocado fruits every 10 days or on Petri dishes with potato dextrose agar incubated at 25°C. Freshly harvested fruits were inoculated by placing a 10 μl drop of a spore suspension (10^6 spores·ml^{-1}) at three different spots along the longitudinal axis of each of five fruits. These fruits were incubated at 20°C in high humidity. Disease expression was defined as the occurrence of darkening over an area of 5 mm in diameter. The incubation period was defined as the number of days taken for these symptoms to be observed in 50% of inoculated sites.

Extraction and quantitative analysis of epicatechin and the antifungal compound

Epicatechin and the antifungal diene in avocado peel were extracted according to the methods described by PRUSKY *et al.* (1982, 1985) and determined by HPLC. For epicatechin determination, 100 μl aliquots of the 1.0 ml concentrated flash-chromatographed material from the crude extract, obtained from 10 g fr. wt were chromatographed (PRUSKY *et al.* 1985). For diene determination, 100 μl aliquots of the 1.0 ml concentrated biologically active fraction isolated by flash chromatography from an extract of 30 g fr. wt., were chromatographed (PRUSKY *et al.* 1982). Epicatechin was monitored with a UV detector at 275 nm and the antifungal diene with a differential refractive index detector. Calculations of concentrations of epicatechin and the diene compound were based on the comparison of the HPLC peak areas of the compounds with those of standards. The average of three separate determinations of each compound is reported.

Fruit Treatments

Treatments affecting fruit ripening were applied to Fuerte fruits. Fruit firmness was determined at different intervals after harvest in uninoculated fruits.

Modified atmosphere treatment. — A modified atmosphere was obtained by storing avocado fruits continuously in closed 2-l jars with a small hole in the lid. The jars were incubated at 20°C and gas samples were taken every hour. A concentration of 8% CO_2 and 13% O_2 was reached after 8 h. These concentrations with a standard deviation of 0.5% was maintained during the whole experiment. Control fruits were kept at the same temperature, under atmosphere conditions.

$CaCl_2$ treatment. — Fruits were completely immersed in 0.2 M $CaCl_2$ or H_2O and infiltrated by reducing the ambient pressure to 150 mm Hg for 2 min. The amount of the solution infiltrated was 0.5 ± 0.2 g per 100 g of fruits. Fruits were then stored at 20°C.

Each experiment affecting fruit ripening was repeated at least twice.

CO_2 and O_2 Determination

CO_2 and O_2 were determined by gas chromatography according to EAKS (1966). The instruments were calibrated for each set of samples by injecting 10 ml of standardized CO_2-nitrogen and O_2-nitrogen mixtures.

Results

The relation between fruit softening, epicatechin decrease and incubation period of *C. gloeosporioides* in avocado cultivars

When the concentration of epicatechin was determined in the peel of nine avocado cultivars during post-harvest ripening, it was possible to divide the cultivars into two groups (Table 1). One group included Fuerte, Rincon, Horshim, Vurtz and Benik, where the epicatechin concentration at softening ranged between 60 and 130 μg·g^{-1} fr. wt. In these cultivars symptoms appeared on the

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Table 1

Comparison of fruit softening, epicatechin concentration and incubation period of *Colletotrichum gloeosporioides* in several avocado cultivars stored at 20°C after harvest

Cultivar	Days to softening*	Epicatechin at softening** ($\mu g \cdot g^{-1}$ fr. wt.)	Incubation period (days)
Fuerte	11	60	11
Rincon	10	70	10
Vurtz	15	100	15
Benik	16	110	16
Horshim	9	130	10
Netaim	10	636	15
Hass	16	1500	24
Pinkerton	8	1740	16
Nabal	14	822	24

* Softening was considered firmness ≤ 1 kg.
** The average of three separate determinations is reported.

same or one day after fruits became soft. These cultivars were considered to be susceptible. The second group of cultivars included Netaim, Hass, Pinkerton and Nabal, where epicatechin concentration at softening ranged between 636 and 1740 $\mu g \cdot g^{-1}$ fr. wt., and symptoms appeared 5—10 days later. These were considered resistant cultivars.

Epicatechin and diene decrease in a susceptible and a resistant cultivars

The time sequences of epicatechin and diene decrease were determined in the peel of one susceptible (Fuerte) and one resistant (Hass) cultivar (Table 2). Epicatechin concentration in the peel of Fuerte fruits decreased to 12 $\mu g \cdot g^{-1}$ fr. wt. concomitantly with fruit softening. In cv. Hass, 10 days after harvest,

Table 2

Fruit firmness, epicatechin and antifungal diene concentrations and symptom appearance of *Colletotrichum gloeosporioides* on the peel of cvs Fuerte and Hass avocado fruits stored at 20°C

Days after harvest	Fuerte				Hass			
	Firmness (kg)	Epicatechin* ($\mu g \cdot g^{-1}$ fr. wt.)	Diene	Symptom appearance	Firmness (kg)	Epicatechin* ($\mu g \cdot g^{-1}$ fr. wt.)	Diene	Symptom appearance
1	13.7	433	1420	–				
2					12.3	519	620	–
4	5.2	344	540	–				
6	0.5	12	120	+				
7					8.5	591	821	–
10					1.0	456	238	–
14					0.5	15	159	+

* The average of three separate determinations of each compound is reported.

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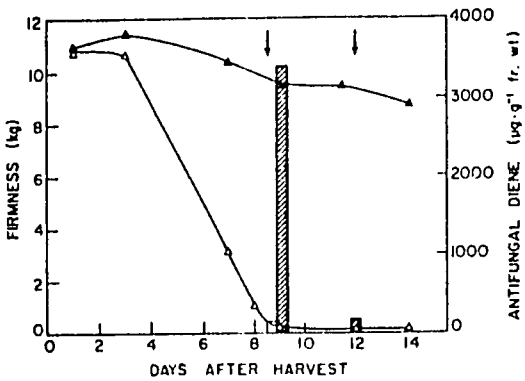

Fig. 1. Effect of modified atmosphere on fruit firmness (▽---▼), concentration of the antifungal diene (□, ▨) and symptom appearance (↓) of *Colletotrichum gloeosporioides* in Fuerte fruits stored at 20°C. The modified atmosphere was obtained by storing freshly harvested fruits in jars with a small outlet, in which the atmosphere contained 9% CO_2 and 13% O_2 (▼, ▨). Untreated fruits (▽, □) were stored at normal atmosphere

when the fruit was completely soft (firmness ≤ 1 kg), the epicatechin concentration was still 456 $\mu g \cdot g^{-1}$ fr. wt. Four days later, epicatechin decreased to 15 $\mu g \cdot g^{-1}$ fr. wt.

Diene concentration in cv. Fuerte was 1420 $\mu g \cdot g^{-1}$ fr. wt. one day after harvest and then decreased to 120 $\mu g \cdot g^{-1}$ fr. wt. on the 6th day, when symptoms appeared. In the peel of cv. Hass fruits, the diene concentration was 620 $\mu g \cdot g^{-1}$ fr. wt. 2 days after harvest and it decreased to 238 $\mu g \cdot g^{-1}$ fr. wt. in soft fruits, 10 days after harvest. Symptoms appeared 4 days later, when the diene concentration had decreased to 159 $\mu g \cdot g^{-1}$ fr. wt.

The fruit peel of all the resistant cultivars is thicker than that of the susceptible ones. It was determined whether this affects the incubation period of *C. gloeosporioides*. Fruits from resistant and susceptible cultivars, Hass and Fuerte respectively, were inoculated when the diene concentration had decreased to subfungitoxic levels. Under these conditions the incubation period was 2 days in both groups of cultivars.

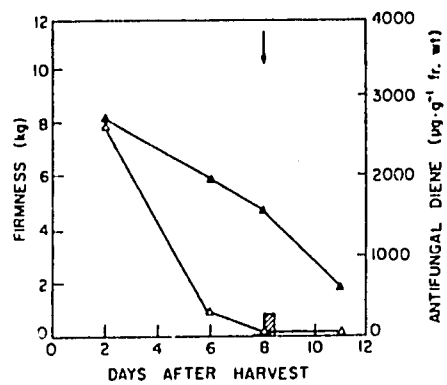

Fig. 2. Effect of infiltration with 0.2 M $CaCl_2$ on fruit firmness (▽-- ▼), concentration of the antifungal diene ((□, ▨) and symptom appearance (↓) of *Colletotrichum gloeosporioides* in Fuerte fruits stored at 20°C. Fruits were infiltrated with $CaCl_2$ (▼, □) or water (▽, ▨) 24 h after harvest

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Effect of modified atmosphere and $CaCl_2$ on fruit firmness, diene concentration and symptom appearance

Untreated Fuerte fruits softened nine days after harvest, together with disease symptom appearance. The diene concentration in these fruits was 116 $\mu g \cdot g^{-1}$ fr. wt. (Fig. 1). At the same time modified atmosphere-treated fruits were still firm and symptomless, and their diene concentration was 3415 $\mu g \cdot g^{-1}$ fr. wt. After three additional days, symptoms of *C. gloeosporioides* appeared on modified-atmosphere treated fruits, and the diene concentration had decreased to 160 $\mu g \cdot g^{-1}$ fr. wt., but fruit firmness was still 8.5 kg.

Treatments with $CaCl_2$ prevented softening of Fuerte fruit. Their firmness was 4.8 and 0.3 kg respectively in treated and untreated fruits on the 8th day after harvest when *C. gloeosporioides* symptoms appeared in treated as well as untreated fruits (Fig. 2). Upon symptom appearance, diene concentration was 5 $\mu g \cdot g^{-1}$ fr. wt. in calcium-treated and 75 $\mu g \cdot g^{-1}$ fr. wt. in untreated fruits, where symptoms had appeared.

Discussion

BINYAMINI and SCHIFFMANN-NADEL (1972) and SCHIFFMANN-NADEL *et al.* (1970) reported that the avocado pathogens *C. gloeosporioides* and *Diplodia natalensis* remained quiescent in firm fruits, and that rot development started concomitantly with or after fruit softening. The present comparison of nine avocado cultivars showed, that the renewal of quiescent *C. gloeosporioides* development after harvest and appearance of decay were not necessarily related to fruit softening. Cultivars that are generally considered as susceptible showed symptom development coincident with fruit softening, but those that are considered resistant showed decay symptoms only 4—10 days after fruit softening.

Development of *C. gloeosporioides* decay on ripening avocado fruits was usually found (PRUSKY *et al.* 1982) to occur when the preformed antifungal diene had decreased to less than 200 $\mu g \cdot g^{-1}$ fr. wt. Oxydation of the diene seems to be catalyzed by lipoxygenase (PRUSKY *et al.* 1983) and regulated by the decrease of the enzyme's endogenous inhibitor, epicatechin, during ripening (PRUSKY *et al.* 1985). The present results show that epicatechin concentrations in cultivars of the susceptible group, decreased to levels of 60—130 $\mu g \cdot g^{-1}$ fr. wt. when the fruits softened, while in resistant cultivars, in the same experiment, epicatechin concentrations ranged still between 636 and 1740 $\mu g \cdot g^{-1}$ fr. wt. at the time of softening. In a separate experiment with the susceptible and resistant cultivars Fuerte and Hass, diene concentration was determined in addition to epicatechin. At softening, the diene concentration in the resistant cultivar was also higher than in the susceptible one, and above the subfungitoxic level (Table 2). These results seem to indicate that the process leading to increased lipoxygenase activity and diene decrease, are responsible for symptom appearance (PRUSKY *et al.* 1982) and not fruit softening. Further support for the lack of a relationship between fruit softening and disease appearance comes from results of experiments with modified atmosphere and with calcium treatments of Fuerte fruits. Both treatments delayed fruit softening, as already reported by SPALDING and REEDER (1975) and

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TINGWA and YOUNG (1974). However, this delay did not prevent symptom appearance; the latter concurred with diene decrease to subfungitoxic levels.

In addition to delayed diene disappearance, resistant avocado cultivars differ from sensitive ones in their thicker peel. Thickness of avocado fruit peel cultivars ranges between less than 1 and over 2 mm (HODGSON 1950). Thin-peeled cultivars are considered those with less than 1 mm thick, while thick-peeled cultivars over 1 mm thickness. When fruits of thick- and thin-peeled cultivars were inoculated with *C. gloeosporioides*, after diene had decreased to subfungitoxic levels, the incubation period was identical. This indicated that diene concentration, and not peel thickness *per se*, is responsible for differential post-harvest resistance of avocado cultivars.

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Involvement of epicatechin in the regulation of lipoxygenase activity during activation of quiescent *Colletotrichum gloeosporioides* infections of ripening avocado fruits

LEAH KARNI†, D. PRUSKY†§, ILANA KOBILER†, EINAV BAR-SHIRA† and D. KOBILER‡

† *Department of Fruit and Vegetable Storage, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel, and* ‡ *Israel Institute for Biological Science, P.O. Box 19, Nes Ziyyona 70450, Israel*

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Avocado lipoxygenase activity increased from 20 to 40 μmole O_2 h^{-1} g^{-1} fresh weight of peel in unripe fruits during the first hour after harvest, and in ripening fruits 4 to 5 days after harvest. Concomitantly, the concentration of the antifungal compound *cis, cis*-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene decreased from approx. 1700 to 200 μg g^{-1} fresh weight of peel. The concentration of epicatechin in the peel was inversely correlated with lipoxygenase activity in each case, and decreased significantly when lipoxygenase increased. The amount of lipoxygenase in the crude extract of the fruit peel during ripening, which was determined by the ELISA technique, ranged between 10 and 30 μg g^{-1} fresh weight of peel during ripening, and was not correlated with the changes in the enzyme activity.

The results suggest that the differences in lipoxygenase activity are not correlated with changes in the amount of this enzyme, but rather result from changes in the concentrations of its inhibitor, epicatechin.

INTRODUCTION

Colletotrichum gloeosporioides Penz. attacks avocado fruits during the growing season, but the infection remains quiescent until the fruit ripens after harvest [*2*]. Prusky *et al.* [*9*] isolated *cis, cis*-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene, a preformed antifungal compound present in the peel of unripe avocado fruits. It was suggested that this compound is responsible for the quiescency of *C. gloeosporioides* infections in unripe avocado fruit.

Lipoxygenase (LOX linoleate:oxygen oxidoreductase EC 1.13.11.12) was shown to catalyse the oxidation of the antifungal diene in an *in vitro* system [*8*]. The specific activity of LOX in avocado peels rose by 80% during ripening [*8*]. In the same work

§To whom correspondence should be addressed.

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Abbreviations used in text: ACE, avocado crude extract; ALOX, avocado lipoxygenase; BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; LOX, lipoxygenase (EC 1.13.11.12); PBS, phosphate buffered saline; SBLOX, soybean lipoxygenase.

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there was an indication of the possible function of LOX in reducing the concentration of the antifungal diene during fruit ripening; infiltrating eicosa-tetraynoic acid and α-tocopherol, which are known to inhibit LOX *in vitro* into the fruit delayed development of disease symptoms.

It was further found that LOX activity in the peel of ripening fruits can be regulated by the presence of the endogenous antioxidant epicatechin [*10*, *11*]. *C. gloeosporioides* symptom appearance after fruit harvest was related to the reduction of the epicatechin concentration [*11*]. Furthermore, the decrease of epicatechin to a non-inhibitory concentration was faster in susceptible than in resistant cultivars [*10*].

The present study was designed to test whether an increase in LOX activity during ripening indeed results from degradation of its endogenous inhibitor epicatechin or from an increase of the amount of enzyme in the fruit. The use of an immunological technique for quantitation of LOX in crude extracts of avocado peel (ACE), enabled us to probe the relationships among LOX activity, LOX amount and epicatechin concentration during fruit ripening.

MATERIALS AND METHODS

Avocado fruits [*Persea americana* Miller var. *drymifolia* (Schlect and Chamb) Blake] of the cultivar Fuerte were obtained from an orchard at Ayanot, Israel.

A single spore isolate of *C. gloeosporioides* from decayed avocado fruits was used to inoculate fruits in all experiments [*8*]. Darkening of the peel at the inoculation sites in excess of 5 mm diameter was considered a symptom of disease.

Firmness of the avocado fruits was determined by recording the force (kg) required to penetrate the fruit skin and flesh with a 5 mm diameter, 4 mm long conic probe [*3*]. The average value from two determinations on each of five fruits is reported.

Extraction and assay of lipoxygenase

Peels (1 to 2 mm thick) of avocado fruits stored at 20 °C were extracted at various times after fruit harvest, as described by Prusky *et al.* [*11*] with some modifications. Twenty five grams of peel were homogenized in 50 ml of ice cold 5 mM sodium phosphate buffer, pH 7·2, containing 2 % Triton X-100. The extract was filtered through four layers of cheesecloth and centrifuged at 20000 *g* for 15 min. The supernatant was filtered through Miracloth (Calbiochem, La Jolla, CA), kept on ice, and used as an enzyme source.

Lipoxygenase activity was determined by monitoring the uptake of O_2 with a Y.S.I. Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) in the presence of linoleate at 36 °C. Buffered 7·5 mM linoleate solution, containing Tween 20, was prepared as described by Ben-Aziz *et al.* [*1*]. A volume (2·5 ml) was placed into the reaction vial with 0·5 ml of enzyme solution. Oleic acid (Sigma) was used as the control substrate [*5*]. Enzyme activity was expressed as μmole O_2 h^{-1} g^{-1} fresh weight.

Preparation of lipoxygenase-specific antibodies

Lipoxygenase from avocado peel, purified approximately 35-fold [*5*], was used for the immunization of five female Balb/c mice. Five subcutaneous injections of 50 μg enzyme per mouse, emulsified in complete Freund's adjuvant (Disco), were given at 2-week

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intervals. One week after the last injection, all mice were injected with 5×10^8 NSO myeloma cells, to produce ascitic fluids. The ascites were tested for the presence of enzyme antibodies by ELISA.

Competitive immunoassay for LOX quantitation

The murine ascitic fluids were used as the source of LOX antibodies and the optimal parameters for a solid phase ELISA were determined. A standard competitive immunoassay based on the ELISA was established.

A commercial preparation of soybean lipoxygenase (SBLOX, Sigma grade V) was absorbed by plates (Dynatech) of 96 wells, by adding to each well 50 µl of a 10 µg ml^{-1} SBLOX solution in 50 mM carbonate buffer, pH 9·6, and incubating the plates for 60 min at 37 °C. After washing the plates twice with saline, the remaining non-specific binding sites were blocked by adding to each well 100 µl of 2% BSA in 100 mM tris buffer, pH 7·2. After incubation for 60 min at 37 °C, the plates were washed four times with saline and immediately used for the immunoassay.

Samples to be assayed were prepared by serial two-fold dilutions of the ACE (designated as "sample free antigen") in 2% BSA-100 mM Tris buffer, pH 7·2, and added to the wells (50 µl per well). Ascitic fluids containing anti-LOX antibodies were diluted (1:200) in 2% BSA-100 mM tris buffer, pH 7·2, and added to the wells (50 µl). After incubation for 90 min at 37 °C, the plates were washed five times with saline. Fifty microlitres per well of rabbit antimouse IgG antibodies conjugated to alkaline phosphatase (Sigma, diluted 1:500 in 2% BSA-tris buffer) were added and the plates were incubated for 60 min at 37 °C. After washing the plates five times with saline, 50 µl of freshly prepared 1 mg ml^{-1} solution of alkaline phosphatase substrate (Sigma, p-nitrophenyl phosphate, disodium) in diethanolamine buffer, pH 9·8, was added to each well. The enzyme reaction proceeded at 37 °C for 30 min and the amount of the coloured product was measured as absorbance at 405 nm in a MicroELISA Auto Reader (Bio-Tel. Instruments, EL-310).

For each assay, a standard curve was prepared by use of commercial SBLOX dilutions ('standard free antigen') in PBS, to give a concentration range of 5 to 100 ng per well. The standard curve was plotted for each experiment (Fig. 1b) and used for comparison between assays and for the estimation of LOX content in experimental preparations.

For calculation of LOX quantity in the ACE, a curve of the means of three replications for each of ten dilutions of the ACE was plotted (Fig. 1a). Standard error for the mean of each triplicate did not exceed 9%. The adsorption of the dilution showing 50% inhibition of binding was defined and used to determine the amount of free antigen in the ACE based on the standard curve (Fig. 1b). The value was multiplied by the dilution factor and the result was expressed as µg LOX g^{-1} fresh weight. Experiments were repeated at least three times.

Analysis of epicatechin and antifungal compound

Epicatechin and the antifungal diene in avocado peel were extracted and determined by HPLC according to the methods described by Prusky *et al.* [*9, 11*]. Three separate extractions of antifungal diene and two of epicatechin were done at each sampling time. Experiments were repeated at least twice.

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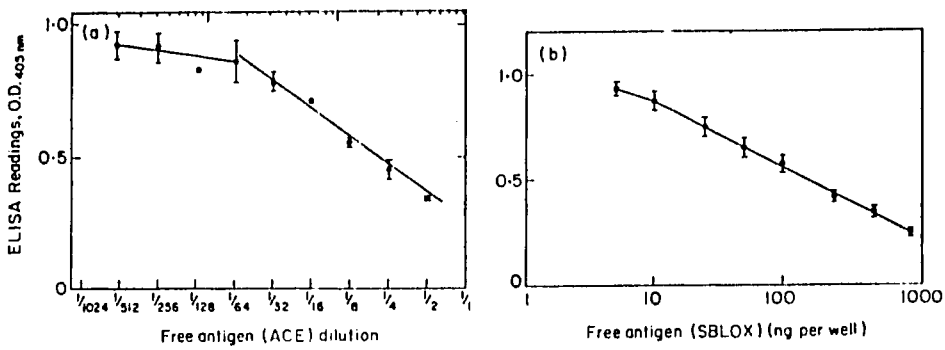

FIG. 1. Method for determination of amount of lipoxygenase protein in crude extracts of avocado peel: (a) ELISA readings at 405 nm for binding of avocado peel crude extract (ACE) dilutions to antibodies prepared toward purified avocado lipoxygenase; (b) ELISA readings at 405 nm for binding of soybean lipoxygenase (SBLOX) dilutions to antibodies prepared toward purified avocado lipoxygenase. The absorbance value of 50% inhibition of binding was obtained from a curve of the means of three replications of each of nine dilutions of the homogenized peel tissue (Fig. 1a) and this value was used to determine the amount of protein from a standard curve with soybean lipoxygenase (Fig. 1b). The standard error of the mean is indicated by bars.

RESULTS

Lipoxygenase amount and activity, antifungal diene and epicatechin in the peel of freshly harvested fruits

During the first hour after harvest, LOX activity in avocado peel increased from 20 μmol O_2 h^{-1} g^{-1} fresh weight to twice that value (Fig. 2a). Three hours later the activity decreased to that found in just-harvested fruits. Changes in the amounts of LOX in the same extracts, as measured by the ELISA, were not related to its activity. The concentration of the antifungal diene in the peel decreased during the first hour after harvest from 1700 to 200 μg g^{-1} fresh weight and increased again to about the initial value 3 h later (Fig. 2b). The epicatechin concentration varied between 145 and 155 μg g^{-1} fresh weight during the first 2 h after harvest and increased to 250 μg g^{-1} fresh weight 2 h later; then it decreased again to a low value of approx. 90 μg g^{-1} fresh weight, 6 h after harvest (Fig. 2b). Fruit firmness did not change significantly during this period and ranged between 10·2 and 9·8 kg.

Lipoxygenase amount and activity, antifungal diene compound and epicatechin concentration in the peel of ripening avocado fruits

Lipoxygenase activity varied during the first day after harvest, attaining twice its initial value at 24 h after harvest. The activity increased further on the fourth day after harvest, when it reached a maximum level. Lipoxygenase activity declined thereafter in completely soft fruits, 6 days after harvest (Fig. 3a). The enzyme content, as determined by ELISA, ranged from 10 to 30 μg g^{-1} fresh weight of peel, and was not directly related to the enzyme activity. Epicatechin concentration decreased during the first day after harvest, from 167 to 52 μg g^{-1} fresh weight when LOX activity started

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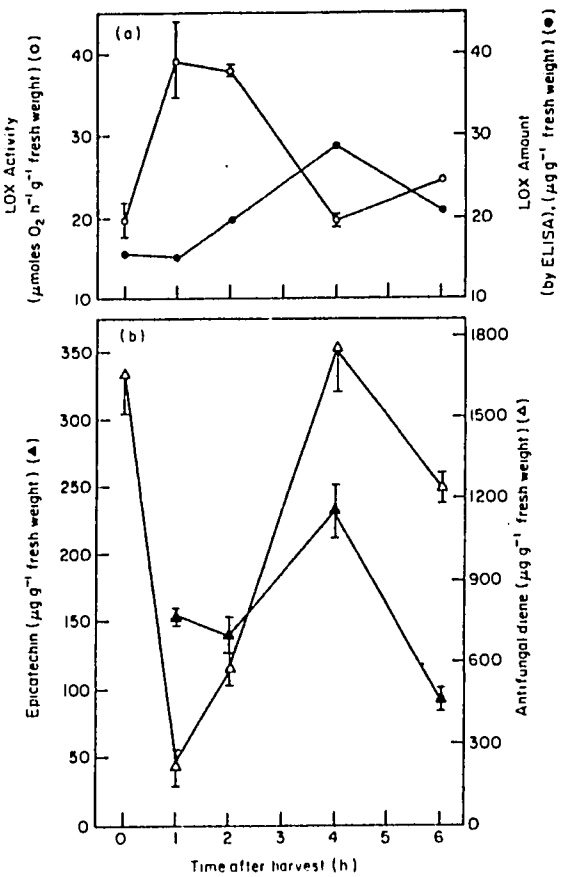

FIG. 2. (a) Lipoxygenase (LOX) activity and amount of lipoxygenase protein and (b) antifungal diene and epicatechin concentration in the peel of freshly harvested avocado fruits cv. Fuerte stored at 25 °C for 6 h. Peel tissue was homogenized in cold 5 mM sodium phosphate buffer, pH 7·2 containing 2% Triton X-100. LOX activity was determined immediately, in three replications of the filtered supernatant, by oxygen consumption in the presence of linoleate. Lipoxygenase protein was determined on the same extract by ELISA as described in materials and methods and Fig. 1. The average of three independent antifungal diene and two epicatechin extracts are reported. The standard error of the mean is indicated by bars.

to increase. On the second day, epicatechin concentration increased to its initial value in the peel of freshly harvested fruits. From the second day on, epicatechin concentration decreased to a minimum of 60 μg g^{-1} fresh weight concomitantly with the increase of LOX activity to its maximum (Fig. 3b). The increase in LOX activity during the first 4 days after harvest was accompanied by a continuous decrease of the antifungal compound.

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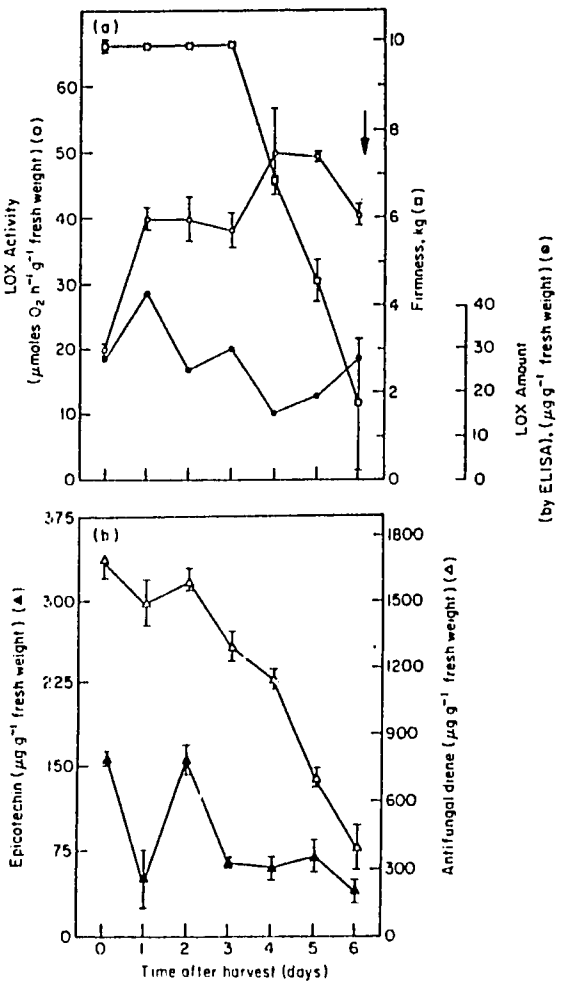

FIG. 3. (a) Fruit firmness, lipoxygenase (LOX) activity and amount of LOX protein and (b) antifungal diene and epicatechin in the peel of avocado fruits cv. Fuerte during storage at 25 °C for 6 days. Details as described in Fig. 2. Arrow indicates *C. gloeosporioides* symptom appearance in ripe fruits after harvest. Darkening of the peel causing lesions in excess of 5 mm diameter at the inoculation sites was considered as positive symptom appearance. Standard error of the mean is indicated by bars.

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DISCUSSION

It was suggested previously that LOX activity during fruit ripening is regulated by its endogenous inhibitor, epicatechin [*11*]. This was tested in the present study, where concentrations of epicatechin and diene as well as LOX activity were determined at each sampling in samples from the same avocado peel. An inverse relationship between LOX activity and epicatechin concentration was found during the first 24 h after harvest (Fig. 2) and during fruit softening (Fig. 3); LOX activity was high (50 μmoles O_2 h^{-1} g^{-1} fresh weight peel) when epicatechin concentration was low (60 μg g^{-1} fresh weight). The amount of LOX, as determined by ELISA, was found to range from 10 to 30 μg g^{-1} fresh weight, exhibiting no direct relation with the enzyme activity (Figs 2 and 3). These results suggested that the changes in LOX activity did not result from changes in the enzyme amount. The lack of relation between lipoxygenase protein amount and LOX activity was found also in soybean cotyledons [*6*], where the increase in protein content was concomitant with a decrease in its activity.

During the first hour after harvest, LOX activity increased rapidly; this was accompanied by a decline in the antifungal diene concentration by approx. 90% compared to unharvested fruit (Fig. 2b). Another decline in diene content occurred during fruit softening, beginning on the third day after harvest, coinciding with the second peak of enzyme activity (Fig. 3b). These results lend further support to the previous hypothesis concerning the possible involvement of LOX in the degradation of the antifungal diene [*10*].

The present data indicate that the variations in LOX activity, involved in the degradation of antifungal diene during avocado fruit ripening, are not due to changes in the enzyme protein content, but are closely related to changes of epicatechin. It was previously reported that: (1) epicatechin inhibited lipoxygenase *in vitro* [*5*]; (2) treatments with epicatechin and other antioxidants delayed the decrease of the antifungal diene [*7*], and (3) phenolic antioxidants are present in plant lipophylic regions [*4*, *12*]. Taken together, these findings suggest that epicatechin acts *in vivo* as a regulator of membrane bound lipoxygenase [*5*].

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EVALUATION OF ELICITOR ACTIVITY OF SEVERAL TREATMENTS

Induction of the antifungal diene in unripe avocado fruits:effect of inoculation with *Colletotrichum gloeosoporioides.*

By D. Prusky, L. Karni, I. Kobiler and R. A. Plumbley

The relationship between antifungal diene levels and fungal inhibition duiring quiescent infection of unripe avocado fruits by *Colletotrichum gloeosporioides.*

By D. Prusky, R.A. Plumbley and I. Kobiler.

Involvement of preformed antifungal compounds in the resistance of subtropical fruits to fungal decay.

By D. Prusky and N.T. Keen

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Physiological and Molecular Plant Pathology (1990) 37, 425–435

Induction of the antifungal diene in unripe avocado fruits: effect of inoculation with *Colletotrichum gloeosporioides*

D. Prusky, Leah Karni, Ilana Kobiler and R. A. Plumbley†

Department of Fruit and Vegetable Storage, ARO, The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

(*Accepted for publication November 1989*)

The concentration of the preformed antifungal compound 1-acetoxy-2-hydroxy-4-oxo-henicosa-12,15-diene in unharvested avocado fruit was almost doubled for a period of 3 days following inoculation by *Colletotrichum gloeosporioides*. The natural diene concentration in the peel and flesh of harvested fruits decreased between the first and third day after harvest but the initial level was regained faster the more mature the fruits were when harvested. A further decline in the concentration of the antifungal compound occurred during fruit ripening. If freshly harvested fruits were inoculated with *C. gloeosporioides*, the concentration of the diene was always enhanced 2–3 days later. Four days after inoculation of harvested fruits, the diene concentration in the peel and in the flesh of control fruit tissue was 490 and 650 μg g^{-1} fresh weight, respectively, compared with 1900 and 3260 μg g^{-1} fresh weight on the inoculated tissue. Mechanical wounding enhanced the concentration over a 24 h period only of the diene in the peel and the flesh. Fruit inoculation with the wound pathogens, *Diplodia natalensis* and *Penicillium digitatum* at harvest, did not increase the concentration of the diene. The diene concentration in freshly-harvested and peeled fruits was not enhanced by inoculation with *C. gloeosporioides* or treatment of the flesh with purified endopolygalacturonase of *C. gloeosporioides*.

It is suggested that the concentration of the preformed diene may be induced by the interaction between *C. gloeosporioides* and the fruit peel.

INTRODUCTION

Various causes have been considered as an explanation for quiescent infections by a pathogen in unripe fruits: insufficient enzyme production, nutritional requirements and the presence of antifungal compounds that inhibit pathogen development in unripe but not in ripening fruits [*28, 29*]. The involvement of antifungal compounds in quiescent infections of unripe fruits was suggested to be the result of either their induction or their presence as preformed compounds [*21*]. The capsicannol phytoalexins, 1 deoxycapsidiol and the related endesmadienol [*30*], have been associated with the formation of quiescent infections in *Capsicum annuum* fruits infected with *Glomerella cingulata* [*1*]. It has been suggested that these compounds also accumulate at the site of arrested lesions produced by *Botrytis cinerea*, but not in progressive lesions [*2*], and diminish considerably at the onset of rot development [*1*].

Fungal inhibition during quiescent infections of *Colletotrichum gloeosporioides* on unripe

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† Natural Resources Institute, Central Av., Chatham Maritime, Chatham ME4 4TB, U.K.

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avocado fruits was suggested to result from the presence of the preformed antifungal compound 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene [*22*]. This was considered to be the same compound as AVH found by Sivanathan & Adikaram [*26*] who also found three other less toxic compounds. All these compounds decrease to a subfungitoxic level, thus allowing the pathogen to recommence growth [*22, 24*]. Karni *et al.* [*13*] described a significant decline in the concentration of a diene in freshly harvested fruits; it is possible that this occurs as a result of harvesting stress. In the latter case, although the diene decreased to subfungitoxic levels, the period of low concentration is short and fungal development does not occur until fruit ripening 7–10 days later [*13*]. It appears that fungal quiescence or fruit resistance is dependent on the duration of, or modulation of the period of decline in diene concentration (25). Antifungal diene decrease is regulated by lipoxygenase activity which, in turn, is regulated by a decrease in the concentration of the endogenous antioxidant, epicatechin [*13, 23*].

This work is part of a project dealing with modulation of the concentration of a preformed diene which increases resistance to postharvest diseases. We report on the specific effect of inoculation with *C. gloeosporioides* on the induction of increased concentrations of the preformed diene in avocado fruits.

MATERIALS AND METHODS

General

Avocado fruits [*Persea americana* Miller var. *drymifolia* (Schlect and Cham.) Blake] of cv. Fuerte were obtained from an orchard at Ayanot, Israel. Fruit firmness (a parameter inversely related to ripening) was determined after harvest by recording the force (kg) required to penetrate the fruit skin and flesh with a 5-mm-diameter, 4-mm-long conic probe [*22*]. The average value of two determinations on each of five fruits is reported. Unripe fruits at two stages of maturation were obtained by harvesting at different times after fruit set.

Single-spore isolates of *C. gloeosporioides* and *Diplodia natalensis* from decayed avocado and *Penicillium digitatum* from decayed orange fruits, were used for fruit inoculation. Isolates were maintained by inoculation of either avocado or orange fruits every 10 days, or on potato dextrose agar at 25 °C.

One hour after harvest, peeled and unpeeled fruits were inoculated by immersing the longitudinal half of the fruit in a continuously stirred spore suspension (approx. 2×10^6 spores ml^{-1}) containing Triton-X 100 (50 µl in 100 ml) for 1 min. The stem end was prevented from touching the inoculum. Control halves were dipped in water. Fruits were incubated at 25 °C in a moist chamber (approx. 98% RH) for 24 h and then transferred to 20 °C at 90% RH. Symptoms of disease in peeled fruits were defined as the occurrence of macerated tissue to a depth of 3 mm at the inoculation point.

Inoculation of whole unharvested fruits was carried out by painting them with the spore suspension using a cotton swab. After inoculation, the fruits were individually covered with a polyethylene bag for 18–24 h. No symptoms were observed in these fruits, since the fungus had become quiescent. Uninoculated fruits from the same tree were used as controls. Experiments were done at 270, 300 and 310 days after fruit set on fruit of approximately 250 g.

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For wound treatments, the longitudinal halves of the whole fruit were pricked with thumb tacks placed 0·4 × 0·4 cm apart on a piece of cardboard. The unwounded halves were used as controls.

Thirty to forty fruits were inoculated or wounded in each experiment, which was repeated at least twice.

Extraction and quantitative analysis of the diene

The diene in avocado peel was extracted according to the method described by Prusky *et al.* [*22*] with some changes to shorten the extraction process. In experiments with unharvested fruits, the fruits were peeled while on the tree. The peel was weighed and placed in ethanol for transport to the laboratory. Fruit peel (10 g) tissue was homogenized for 3 min in 95% ethanol (50 ml). After filtration, the filtrate was concentrated to 5 ml in a Rotavapor under vacuum at 40 °C and partitioned twice with dichloromethane. The organic layers were pooled, dried with anhydrous $MgSO_4$, filtered and dried under vacuum as before. The active compound in 5 ml hexane was further purified by flash chromatography of a 0·5 ml sample, on a 6 ml capacity Spe-ed Silica gel column (Laboratory Impex Ltd., U.K.). The 1·2 cm diameter column was filled with 1·45 g of 40–63 μm silica to a depth of 2·2 cm. The column was pre-equilibrated with 4 ml hexane. Inactive material was eluted using 8 ml hexane containing 25% dichloromethane followed by 6 ml of 100% dichloromethane. The active fraction was then eluted by washing the column twice with 8 ml of 25% ethyl acetate in dichloromethane and dried under nitrogen.

Determination of the diene was done by HPLC analysis of 50 μl samples of the active fraction in ethanol [*22*]. Calculations of the diene concentrations were based on comparison of HPLC peak areas with that obtained with a standard. Values from three different extractions were taken in each experiment, which was repeated at least twice.

Purification and treatments with endopolygalacturonase (PG) from C. gloeosporioides

PG from culture filtrate of *C. gloeosporioides* was purified as described by Prusky *et al.* [*21*]. The purification system involved chromatography of the concentrated culture filtrate on a phenyl sepharose column using decreasing concentrations of $(NH_4)_2SO_4$. The preparation used for fruit treatments contained two polygalacturonase species of 68 and 62 kD, based on SDS gel electrophoresis and thin layer gel electrofocusing that represent different degrees of glycosilation of a single protein species [*21*]. PG activity was quantified by measuring the rate of liberation of reducing termini from a 1% solution of polygalacturonic acid in 10 mM sodium acetate buffer at pH 6·0. Reducing groups were measured using the Nelson-Somogyi spectrophotometric assay [*18*]. One unit of enzyme activity was defined as that amount liberating 1 μ mol of reducing equivalents min^{-1}.

Maceration activity was determined by incubating five thin wedges (approximately 0·2 × 0·4 cm, and 0·5–0·6 cm thick), consisting of the exo- and mesocarp of avocado fruit, with 500 μl of enzyme (1 unit μl^{-1}) in 25 mM sodium acetate buffer, pH 5·0, for 7 h at 37 °C.

PG treatment was done by applying up to 0·5 ml of enzyme solution (1·25 units μl^{-1}) to freshly harvested and peeled fruits. Enzyme treatments were done at 0, 24 and 48 h

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after peeling. The effect of this treatment on the concentration of the diene was tested during the first 96 h after treatment.

RESULTS

Effect of inoculation with C. gloeosporioides *on diene increase in avocado fruits*

Unharvested fruits. Inoculation of unharvested fruits with *C. gloeosporioides* resulted in an increase of the diene concentration from 1300 to 1700 μg g^{-1} fresh weight in the peel and from 1050 to 2600 μg g^{-1} fresh weight in the flesh during the first 3 days after inoculation (Fig. 1). From the fourth day the diene concentration had decreased to

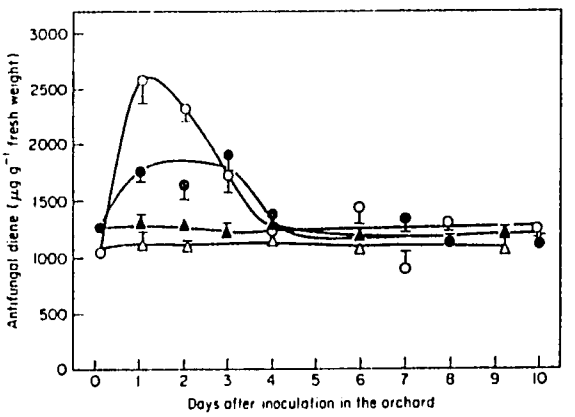

FIG. 1. Changes in the concentration of the diene in the peel and flesh of unharvested avocado fruits of cv. Fuerte-inoculated with *C. gloeosporioides*. ▲, peel control; ●, peel inoculated; △, flesh control; ○, flesh inoculated. Unharvested fruits on the tree were inoculated by painting the peel with a cotton swab dipped into a spore suspension of approx. 2×10^6 spores ml^{-1}. Fruits were then covered with plastic bags for 18 h. Uninoculated fruits on the same tree were used as controls. The mean of three separate extractions of the diene with SE is shown.

that found in unchallenged fruits. Diene concentration in uninoculated, unharvested fruits remained at approximately 1250 μg g^{-1} fresh weight throughout the 10 days of the experiment.

Harvested fruits. Inoculation of freshly harvested fruit approximately 240 days after fruit set, caused an increase in the diene concentration in the flesh and peel (Fig. 2 A and B). After an initial decrease of the diene concentration on both sides of the fruit, the concentration of the diene in the peel of the challenged side of the fruit increased more rapidly, and to a higher level, than in the unchallenged side of the same fruit (Fig. 2 B). In the flesh of the challenged side of the fruit, the diene concentration was significantly higher than that of the unchallenged side (Fig. 2 A). On the fourth day after harvest, the diene concentration had increased to 3260 μg g^{-1} fresh weight, while it was only 650 μg g^{-1} fresh weight on the control side of the fruit. The effect of inoculation was observed during the first 4 days after harvest. Five days after harvest the diene

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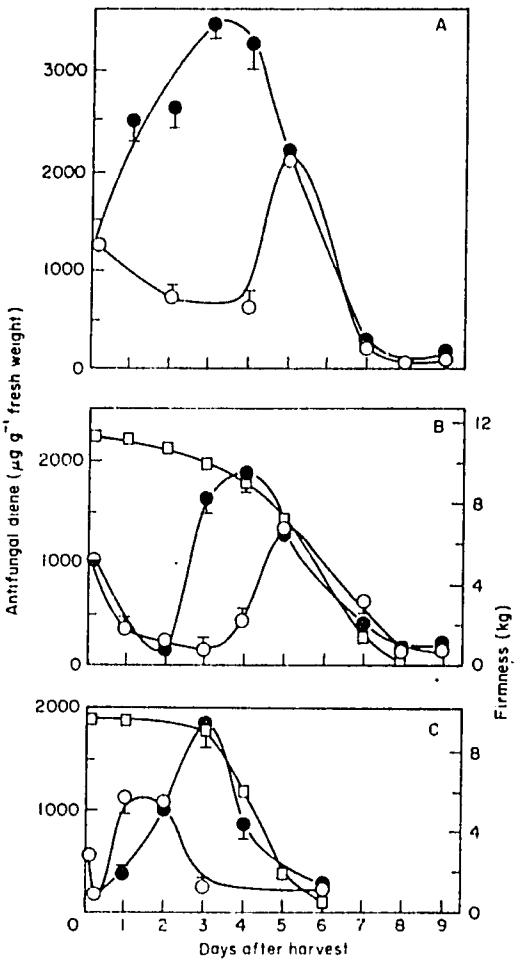

FIG. 2. Changes in concentration of the diene in freshly harvested avocado fruits of cv. Fuerte inoculated with *C. gloeosporioides* and stored at 20 °C. (A), flesh and (B), peel of avocado harvested 240 days after fruit set; (C), peel of avocado harvested 300 days after fruit set. ○, control half; ●, inoculated half; □, fruit firmness. Fruits were inoculated by immersing half of each fruit, at its longitudinal axis, in a spore suspension of *C. gloeosporioides*. The uninoculated halves were immersed in water and used as controls. Fruits were incubated at 25 °C for 24 h at high RH in plastic containers and then stored at 20 °C. The mean of three separate extractions of the diene with SE is shown.

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concentration was the same on both sides of the fruit, and continued to decrease in parallel until fruit softening.

More mature fruits, harvested 300 days after fruit set (Fig. 2 C) showed that the initial amount of the antifungal compound was smaller and the period of initial decline in diene concentration was shorter than for fruits harvested 240 days after fruit set (Fig. 2 B). In fruits of both harvesting periods however, inoculation with *C. gloeosporioides* enhanced the increase of the diene. Challenge inoculation with *C. gloeosporioides* 3 days after harvest on fruits of both harvesting periods did not enhance the effect on the diene concentration in the peel or flesh (results not shown).

Effect of inoculation with D. natalensis *and* P. digitatum

Inoculation of the peel of freshly harvested avocado fruits with *D. natalensis*, a wound pathogen of avocado fruits, produced no significant effect on the antifungal compound

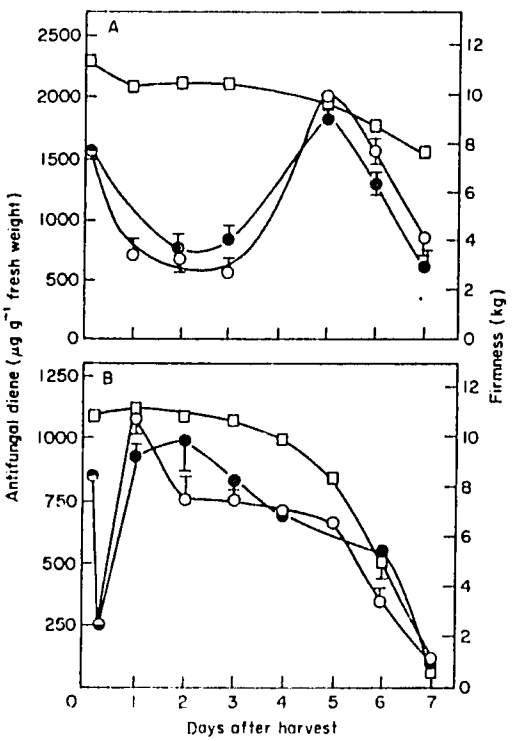

FIG. 3. Changes in concentration of the diene in the peel of freshly harvested avocado fruits of cv. Fuerte inoculated with (A) *D. natalensis* or with (B) *P. digitatum*, stored at 20 °C. Fruits were inoculated with a spore suspension of the fungus as described in Fig. 2. ○, control half; ●, inoculated half. The mean of three separated extractions of the diene with SE is shown. Experiment with *P. digitatum* were carried out on mature fruits late in the season.

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concentration when compared with the non-inoculated part of the fruit (Fig. 3 A). The antifungal diene concentration was unaffected by a challenge inoculation with *P. digitatum*, a non-pathogen of avocado (Fig. 3 B). Differential maturity of the fruits inoculated with *D. natalensis* and *P. digitatum* is the cause of the difference in the length of the initial drop in the concentration of the antifungal diene.

Effect of wounding on the concentration of the diene

Wounding of fruits enhanced the diene concentration in the peel and flesh during the first day after harvest; 3 h after wounding, the concentration of the antifungal compound in the flesh of the wounded side of the fruit was 2350 μg g^{-1} fresh weight compared to 780 μg g^{-1} fresh weight on the control side of the fruit (Fig. 4). The effect on the peel was less significant (Fig. 4). By the second day no effect on the antifungal compound concentration was observed in the flesh or in the peel of the treated fruits.

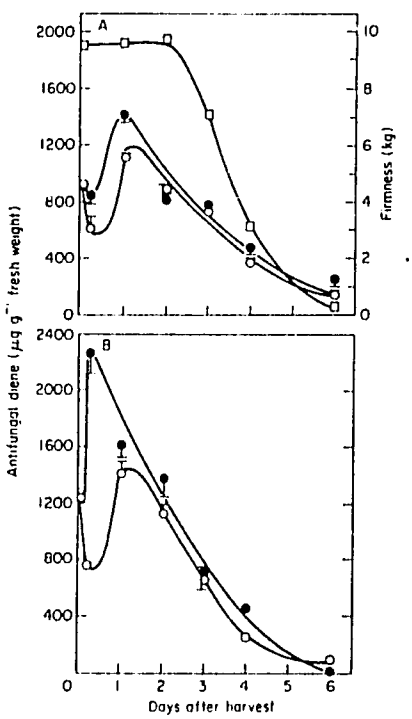

FIG. 4. Effect of wounding of freshly harvested avocado fruits of cv. Fuerte on the concentration of the diene when stored at 20 °C. (A), peel; (B), flesh. Fruit halves were wounded (●) as described in Materials and Methods. The unwounded side of the fruit (○) was used as a control. The mean of three separate extractions of the diene with SE is shown. □, fruit firmness.

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TABLE 1.
Concentration of the diene in the flesh of avocado fruits cv. Fuerte after treatment with purified endopolygalacturonase from C. gloeosporioides

Hours after enzyme treatment	Antifungal diene ($\mu g\ g^{-1}$ fresh weight)		
	Control	Enzyme-treated	SEM
2	2060	1870	160
6	1870	1890	100
22	2820	2480	159
47	1290	1420	108
72	2060	2090	100

Freshly harvested fruits were peeled and treated on the longitudinal half with 0·5 ml of purified enzyme solutions containing 1·25 units of activity μl^{-1} as described in Materials and Methods. Results represent the mean of four replications.

TABLE 2.
Concentration of the diene in the flesh of avocado fruits cv. Fuerte after challenge inoculation with C. gloeosporioides

Hours after inoculation	Hours after harvest and peeling					
	24 h			48 h		
	Control	Inoculated	SEM	Control	Inoculated	SEM
11	1420	1210	139	950	730	72
22	1120	1270	86	1270	1030	110
30	1320	1600	153	1670	1670	65
48	1420	1650	151	1230	1060	64

Freshly harvested fruits were peeled. Inoculation with a suspension of *C. gloeosporioides* (approx. 10^6 spores ml^{-1}) was done 24 and 48 h after harvest. Results represent the mean diene concentration in $\mu g\ g^{-1}$ fresh cut from three extractions.

Effect of PG or inoculation of peeled fruits by C. gloeosporioides *on diene induction*
PG was able to macerate avocado wedges at 37 °C during 7 h incubation. PG did not, however, affect diene concentration when applied to the flesh of freshly-harvested fruit kept at 25 °C (Table 1).

When freshly harvested fruits were peeled and inoculated with *C. gloeosporioides* after 0, 24, 48 or 72 h, the concentration of the antifungal compound was not enhanced compared to the uninoculated part of the fruit (Table 2, showing data for 24 and 48 h inoculations only).

DISCUSSION

A diene, the major antifungal compound in avocado fruit, was induced by challenge inoculation with *C. gloeosporioides* of unharvested and freshly harvested fruits. This is not

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the first report that a preformed antifungal compound can be induced by challenge inoculation. Chlorogenic and caffeic acids, scopolin, α-solanine and α-chaconine have been shown to accumulate in injured and infected tissue [*16*], but none of these preformed compounds was as directly involved in host resistance as the diene in avocado fruits [*20, 22, 23, 24, 25*]. In unharvested fruits the diene concentration was almost doubled in the peel and flesh for a period of 3 days after challenge inoculation by *C. gloeosporioides*. In freshly harvested fruits, after the initial drop in concentration of the diene resulting from harvesting stress [*13*], the concentration in the peel and in the flesh of the challenged part of the fruit increased to higher levels than controls. This suggests that the diene is not only a pre-infectional inhibitor [*28*] but the concentration could be enhanced by a biotic factor from *C. gloeosporioides*.

Fruits harvested at different ages after fruit set showed that the natural changes in the concentration of the antifungal compound in untreated fruits were dependent on fruit maturity. Fruits harvested at a more mature stage showed that the period until the antifungal diene regained the initial concentration following the decrease after harvest, was shortened. This behaviour is strikingly similar to the climacteric pattern of respiration for harvested avocado fruits before they ripen [*5*]. The length of the lag period until the rise in respiration (the preclimacteric period) [*5, 31*] and the diene changes are a reflection of the state of fruit maturity. Challenge inoculation of intact fruits at harvest always induced the antifungal compound 2–3 days after inoculation. If fruits were challenge inoculated 3 days after harvest, the concentration of the compound did not increase. Sivanathan & Adikaram [*26*] were unable to show an increase in the concentration of antifungal compounds following challenge inoculation of avocado fruits with *C. gloeosporioides*. This may have been because of the insensitivity of their detection method, the time of inoculation after harvest or the immaturity of the fruit. Their fruits were harvested 80–90 days after anthesis, as opposed to 240 days in our experiment; the fruits used by Sivanathan & Adikaram [*26*] were stated to be immature, but the degree of immaturity was not mentioned. Different varieties are known to require varying maturation times under the same conditions [*19*].

Enhanced concentrations of the diene persisted in challenge-inoculated fruits for 3–4 days, compared with only 1 day following mechanical wounding. Kuc [*15*] suggested that mechanical wounding generally does not lead to appreciable accumulation of phytoalexins and after sources of stress are removed, phytoalexin levels decline, unless a biotic elicitor causes the release of additional elicitors of host origin. It appears that a low level of persistent stress is required for the accumulation of high levels of the antifungal diene [*15*]. This is consistent with the observation that when infections of *C. gloeosporioides* become quiescent in unharvested fruits [*6*], levels of the antifungal diene decline to those found in uninfected fruits. More puzzling is the induction of the antifungal diene in the flesh of the fruit since hyphae of *C. gloeosporioides* remain quiescent for several days after inoculation [*6, 25*] and do not penetrate into the flesh. This result raises the possibility of signal generation and transduction which may lead to an increase of the antifungal diene at a distance from the point of infection. Challenge inoculation in avocado fruits by *Fusarium solani* enhanced ethylene production [*32*]. The involvement of ethylene in elicitation of defence reactions has been discussed previously [*7, 10, 11, 17*]. Chalutz [*10*] suggested that ethylene induced phenylalanine ammonia lyase, a key enzyme in secondary metabolism of plants, but this hormone has not been shown to be a signal substance [*11*] for diene induction.

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The antifungal diene was not induced if fruits were challenge inoculated with either *D. natalensis* – a wound pathogen of avocado, or with *P. digitatum* – a non-pathogen of avocado. The limited duration of the induction of the antifungal diene resulting from wounding suggests that the physical stress of peel penetration by *C. gloeosporioides* does not induce the 3-day-long induction period; an elicitor(s) of the fungus appears to be involved in the process [*12*]. PG from *Aspergillus niger* on unripe *Capsicum annuum*, resulted in increased capsidiol production [*30*]. The PG of *Rhizopus stolonifer* induced the biosynthetic synthesis of casbene in castor bean [*9*], but, at a similar concentration, the PG of *C. gloeosporioides* did not induce the antifungal compound in avocado flesh. Similarly, inoculation by *C. gloeosporioides* of peeled fruits did not induce the antifungal diene. The antifungal compound was induced only after penetration of intact fruits by the fungus. This suggests that although reception of the initial reaction probably occurs in the peel, a "signal" might activate the synthesis mechanism in the flesh. Induction of the diene may possibly result from the intimate interaction of the fruit peel and pathogen cell wall components [*3, 4, 8, 11*]; this will be the basis for future research.

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The relationship between antifungal diene levels and fungal inhibition during quiescent infection of unripe avocado fruits by *Colletotrichum gloeosporioides*

D. PRUSKY,* R. A. PLUMBLEY† *and* I. KOBILER*
**Department of Fruit and Vegetable Storage, Agricultural Research Organization, The Volcani Center, PO Box 6, Bet Dagan 50250, Israel*
†Natural Resources Institute, Central Avenue, Chatham Maritime, Chatham, UK

Quantitative changes in the antifungal compound, 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene, in freshly harvested avocado fruits during the initial stages of fungal development were investigated to determine the possible involvement of the compound in quiescent infections of *Colletotrichum gloeosporioides*. The concentration of the antifungal compound in the peel decreased to subfungitoxic concentrations 16 h after harvest. Fifty-six hours later the antifungal diene had increased to *c.* 3800 μg/g fresh weight. At this stage, germinated appressoria had penetrated the cuticle to the epidermal cells but no fungal development was observed until 7 days later when the concentration of the diene had decreased to 100–110 μg/g fresh weight. Following a dip treatment at 55°C for 5 or 10 min, the antifungal diene concentration decreased as in the controls, but it remained at subfungitoxic concentrations for a longer period enabling fungal development and early symptom expression.

The concentration of the diene in the flesh of freshly harvested fruit decreased to 120 μg/g fresh weight 24 h after harvest. Inoculation of peeled fruits with spores of *C. gloeosporioides* showed germination without appressoria formation and symptom expression occurred 24–48 h later. Symptom expression was delayed if fruits were inoculated after coating the flesh with epicuticular wax extracts or if the flesh was inoculated 3 days after harvest when the antifungal diene had regained a fungitoxic concentration. Disease symptoms were expressed in soft fruits containing subfungitoxic concentrations of the diene.

We conclude that the diene in unripe avocado fruits inhibits fungal development of germinated appressoria or conidia. The quiescent structure of *C. gloeosporioides* in unripe avocado fruit is a subcuticular hypha.

INTRODUCTION

Anthracnose caused by *Colletotrichum gloeosporioides* is the most important fruit-rotting disease of avocado. Binyamini & Schiffmann-Nadel (1972) have shown by fruit peel isolations that the fruit becomes infected at a very early stage during the growing season, although the fruits are free from visible disease symptoms until harvest. Lesions develop during fruit ripening and softening 7–15 days after harvest. The anatomical studies of Binyamini & Schiffmann-Nadel (1972) in hand-cut slices revealed that wax-embedded appressoria of *C. gloeosporioides* were the structures which persisted in unripe fruits. The viability of the early infections was determined by covering fruit with bags at different periods during growth in the orchard. Fruits covered early after fruit set showed a low incidence of infection, while fruits covered late after fruit set were exposed for a longer period to infection, and showed a high incidence of post-harvest disease (Binyamini & Schiffmann-Nadel, 1972).

Several hypotheses have been proposed to explain the formation of appressoria by *Colletotrichum* species: starvation (Parbery, 1981), surface hardness (Van Burgh, 1950; Parbery & Emmett, 1977), competition with saprophytic organisms (Blakeman & Parbery, 1977) or with antagonistic organisms (Lenne & Parbery, 1976), desiccation (Parbery & Emmett, 1977), inhibitory chemicals (Parbery & Blakeman, 1978) and the induction by epicuticular wax (Prusky & Saka, 1989). In some *Colletotrichum* species, such as *C. graminicola*, *C. lindemuthianum* and *C. trifolii*, host penetration occurs immediately after appressorium formation (Parbery, 1981). In other species, such as *C. gloeosporioides* and *C. musae*,

environmental conditions may be fungistatic to appressoria germination (Muirhead, 1981) and cause a prolonged period of dormancy. The eventual disappearance of an inhibitor in combination with the greater availability of nutrients may be associated with the breaking of quiescence.

The nature of the quiescent structure of *C. glocosporioides* depends upon the fruit in which the interaction was tested. In papaya (Stanghellini & Aragaki, 1966), citrus (Brown, 1975), banana (Muirhead & Deverall, 1981) and avocado (Binyamini & Schiffmann-Nadel, 1972), *C. glocosporioides* persists on the peel of the mature fruit as quiescent appressoria. In mango (Verhoeff, 1974), blueberry fruits (Daykin, 1984) and in some cases in citrus (Brown, 1975), the appressoria may germinate and produce infection hyphae that penetrate the cuticle and remain quiescent as hyphae.

The role of subcuticular hyphae of *C. musae* in green banana fruit was re-examined by Muirhead & Deverall (1981). In contrast to the findings of Simmons (1941) and Chakravarty (1957), it was observed that the subcuticular hyphae originating from hyaline appressoria of *C. musae* did not resume activity during ripening. The anthracnose lesions originated from dark thick-walled appressoria, which germinated at the onset of fruit ripening. Muirhead (1981) suggested that these appressoria, rather than subcuticular hyphae, were the key structures in quiescence of *C. musae*.

In an attempt to understand the mechanism of quiescence in unripe fruits, Prusky *et al.* (1982, 1983) isolated a preformed antifungal compound from unripe avocado fruits. This compound was present in the peel and flesh of the fruit (Prusky *et al.*, 1983) but its relation with the quiescent structure of *C. glocosporioides* in unripe fruits has not been studied. The aim of this research was to determine the relation between the inhibition of fungal development and the presence of the antifungal diene during the quiescent stage and at the onset of disease development of *C. glocosporioides* in freshly harvested and ripening avocado fruits.

MATERIALS AND METHODS

Mature avocado fruits (5–6 month after fruit set) (*Persea americana* var. *drymifolia* cv. Fuerte) were obtained from orchards near Rehovot, Israel. The experiments were started 30–60 min after fruit harvest. Firmness, an inverse parameter of ripening, was measured by recording the force (kg) required to penetrate the fruit skin and flesh (or directly into the flesh after peeling the fruit) with a conic probe 5 mm in diameter and 4 mm in length (Prusky *et al.*, 1983). The average of two determinations on each of five fruits was recorded. The fruits were harvested at firmness values of 10–12 kg and were considered ripe when firmness decreased to 1 kg.

A single-spore isolate of *C. glocosporioides* obtained from decayed avocado fruits was used for inoculation in all experiments. This isolate was maintained by inoculation of fruits every 10 days and also maintained on potato dextrose agar at 25°C (Prusky *et al.*, 1982). Spores were washed and collected in distilled water by centrifugation at 1500 *g* for 15 min. Fruits were inoculated by placing a 10-μl drop of a prewashed spore suspension (10^6 spores/ml) on the peel at three different spots along the longitudinal axis of each of five fruits. In some experiments fruits were peeled (1–2 mm thick) and then inoculated similarly on the flesh. Fruits were incubated at 20 or 25°C, depending on the experiment, in a moist chamber at *c.* 98% r.h. Symptoms were identified as darkened lesions larger than 5 mm in diameter.

Extraction of epicuticular wax from avocado peel and *in-vitro* bioassay

Fruit were dewaxed by dipping them for 1–2 min in hexane or ethyl acetate (Albrigo, 1972; Prusky & Saka, 1989). This extract was concentrated to dryness, weighed, and redissolved in hexane before use in the bioassay. The sequence of conidial germination of *C. glocosporioides* and appressorial formation and germination was determined on millipore discs covered with 4·8 mg avocado peel-wax extract. Epicuticular wax extracts dissolved in hexane were spotted on a 13-mm-diameter millipore filter (0·45 μm pore size, Millipore) and similar amounts of the solvent alone were placed on a control filter. After removal of hexane by drying, 25 μl of a suspension of washed spores of *C. glocosporioides* (10^6/ml) was placed on the dried wax or on controls. The filters were incubated in a moist chamber at 25°C. At different periods after incubation, the germination process was terminated with cotton blue in lactophenol. Activity of the epicuticular wax extracts was determined by comparing the percentage of conidial germination, appressorial formation and appressorial germination in the treatments and in the controls. Germinated conidia were defined as those with germ tubes longer than three times the length of the spores (58 ± 22

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μm). Appressorium formation was identified by its brown colour in the presence of cotton blue staining. Germinated appressoria had hyphal threads originating from the bottom of the dark structure, penetrating irregularly through the epicuticular wax deposit. A germ-tube length $\geq 180 \pm 45$ μm was considered as a germinated appressorium. One-hundred conidia were observed under the microscope on each of three millipore discs.

In-vivo bioassay of appressorial production

At different stages after harvest and inoculation, tangential 1-mm thick peel slices were removed with a razor blade and the fungus was fixed with cotton blue in lactophenol. Conidial germination and appressorial formation on the fruit surface were observed with a light microscope.

Assay of decay development on wax-coated fruits

The effect of epicuticular wax on decay development was determined by placing 10 μl of a hexane–wax suspension (4·8 mg wax/ml) on the flesh of freshly harvested, peeled fruits. The hexane evaporated rapidly without causing visual damage to the flesh. Drops (5 μl) of a suspension of *C. gloeosporioides* conidia ($0{\cdot}5 \times 10^6$/ml) were then placed onto the dried wax and symptom development was determined as before.

Microscopic observations

Fixation and dehydration of specimens for scanning-electron microscopy (SEM) and light-microscope observations were done in a series of ethanol and acetone solutions. For SEM observations, the dehydrated tissue was handled according to the method of Cohen (1974). Samples were observed in a JEOL JSM-35C scanning-electron microscope. For light-microscope observations, the dehydrated tissue was embedded in Spurr's low-viscosity medium (Spurr, 1969). Thin (5 μm) slices were collected in distilled water drops on glass slides and mounted in immersion oil. Several infection points were observed with a phase-contrast microscope.

Extraction and quantitative analysis of the antifungal compound

The antifungal diene in avocado peel and flesh was extracted according to the method described by Prusky *et al.* (1982) and determined by HPLC. Three different extractions were examined at each particular stage of the experiment. Chromatographs were made of 100-μl aliquots of a 1·0 ml concentrated extract from 30 g fresh weight of tissue. Calculations of concentrations of the diene were based on comparison of the HPLC peak areas of the compound with a standard. The average value of three replications is presented.

Heat treatment of fruits

Freshly harvested fruits were immersed in water at 55°C for 5 or 10 min and allowed to cool for 1 h prior to inoculation as previously described. Fruits immersed in water at room temperature for 10 min were used as controls.

RESULTS

Appressorial formation on the peel of avocado fruits

At 16 h after inoculation 97% of the conidia of *C. gloeosporioides* had germinated and most (97%) had formed appressoria immediately after germination on avocado peel (Fig. 1). The appressoria had germinated, penetrated the cuticle and reached the epidermal cells by 72 h after inoculation (Fig. 2). Disease symptoms were observed 10 days after inoculation, when the fruit became soft.

Effect of extracted avocado peel wax on appressorial formation and germination

Fifteen hours after incubation on wax-coated millipore filters, 99% of the conidia had germinated and 95% had formed appressoria (Fig. 3). Germinated appressoria were first observed after 24 h. Twenty per cent of the appressoria had germinated by 60 h after incubation and 28% by 104 h.

The relation between diene concentration and symptom expression of *Colletotrichum gloeosporioides* in intact and heat-treated fruits

The concentration of the antifungal diene in the peel of avocado fruits was found to be around 2800 μg/g fresh weight after harvest. Within 24 h the concentration had decreased rapidly to 190 μg/g fresh weight (Fig. 4). In unheated control fruit the levels rose rapidly after 24 h and had once again reached over 3000 μg/g fresh weight after 48 h (Fig. 4a). In heat-treated fruit the initial

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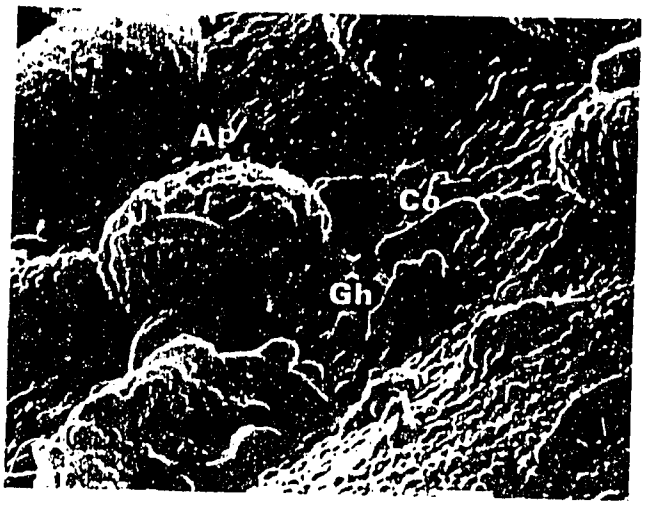

Fig. 1. Scanning-electron micrograph of conidium (Co) germinating hypha (Gh) of *Colletotrichum gloeosporioides* forming appressoria (Ap) on peel of avocado fruit cv. Fuerte (bar = 1 μm).

Fig. 2. Light micrograph of a germinated appressorium (Ap) of *Colletotrichum gloeosporioides* breaching cuticle (C) up to the epidermal cells 72 h after inoculation of freshly harvested mature fruit. Infection hypha (Ih) is denoted by arrows (× 3000).

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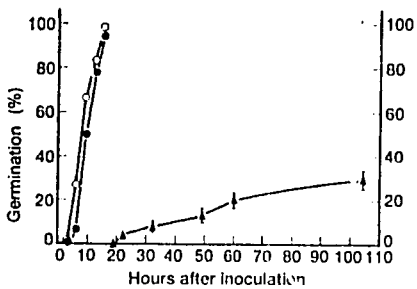

Fig. 3. Germination of conidia of *Colletotrichum gloeosporioides* and formation and germination of appressoria on avocado peel-wax extracts. Peel wax extract (4·8 mg) was placed on millipore discs and inoculated with a spore suspension. The discs were stained with cotton blue for observation after various periods of incubation. Standard deviations are denoted by bars. ○, germination of conidia; ●, germination of conidia with appressoria; ▲, germinated appressoria.

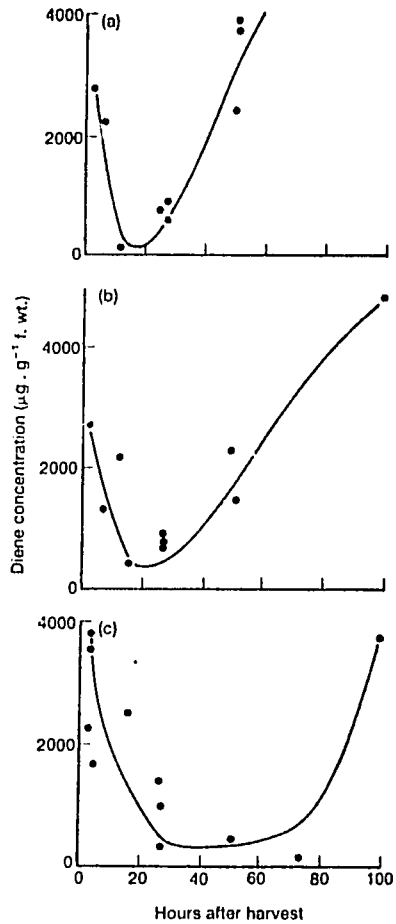

Fig. 4. Changes in the concentration of antifungal diene in avocado fruits cv. Fuerte at 20°C after a heat treatment at 55°C. (a) Untreated control, (b) 5-min heat treatment, (c) 10-min heat treatment.

decrease in concentration was similar to that of the controls but the lag period until re-establishment of the original high levels was delayed depending on the length of the time of treatment at 55°C (Fig. 4b,c). The original diene levels were not regained until 72 h after a 5-min treatment or 96 h after a 10-min treatment.

No significant differences in fruit firmness were observed up to 96 h in untreated and heat-treated fruits; firmness ranged between 9·5 and 10·5 kg in all the treatments during this period. Appressoria formation was not affected by the pre-inoculation heat treatment and the percentage of formed appressoria was 94 ± 1 in heat-treated and non-treated fruits. Lesion development and symptom expression was dependent on treatment time and occurred earlier on treated fruits than on non-treated fruits (Fig. 5). The time required for symptom expression on control fruit was 7 days as compared with 3 and 2 days for a 5- or 10-min treatment, respectively.

The relation between diene concentration and symptom expression of *C. gloeosporioides* on avocado fruits peeled and coated with peel-wax extract

Inoculation of the flesh of freshly harvested peeled fruits resulted in symptoms 2 days later, when the fruit were still firm unripe (Fig. 6). Fungal conidia began to germinate during the period when concentrations decreased from 2010 to 120 µg/g fresh weight. No appressoria were formed and the germ tubes began to macerate the tissue when diene concentration was about 110 µg/g fresh weight. If the flesh of freshly harvested fruits was coated with an epicuticular wax extract and inoculated with *C. gloeosporioides*, symptoms developed 6–7 days after inoculation when the concentration of the antifungal diene was 120 µg/g fresh weight and fruits became soft (Fig. 6).

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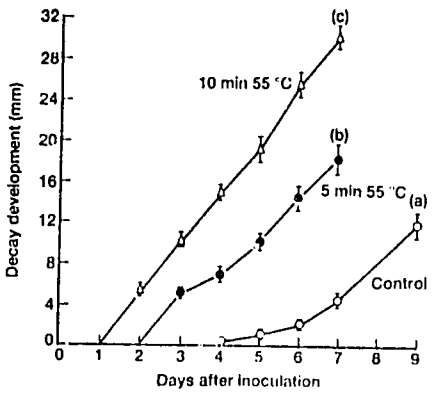

Fig. 5. Effect of heat treatment of avocado fruits cv. Fuerte on decay development by *Colletotrichum gloeosporioides*. Fruits were dip-treated at 55°C for 5 (b), and 10 min (c). Untreated controls (a) were dipped at 20°C. After treatment fruits were inoculated and incubated at 25°C and high humidity for 24 h and then transferred to 20°C until symptom expression.

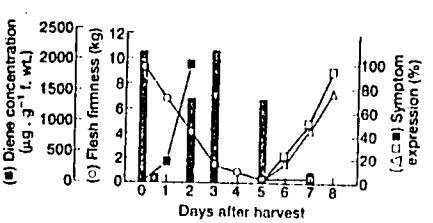

Fig. 6. Diene concentration, fruit firmness and symptom expression of *Colletotrichum gloeosporioides* in peeled avocado fruits cv. Fuerte stored at 25°C coated or uncoated with epicuticular wax. Uncoated fruits were either inoculated immediately (■ - - ■) or 3 days after harvest (□ - □). Epicuticular wax extract (10 µl) was spotted on the flesh of freshly harvested fruits, followed by conidial inoculation (△ - △). Inoculation was done with 5 µl drop suspension of 10^5 spores/ml.

Inoculation of the peeled fruit 3 days after harvest, when the concentration of the antifungal diene had regained its original fungitoxic concentration, showed symptom expression 3-4 days later, when the concentration of the antifungal diene had decreased to 120 µg/g fresh weight (Fig. 6).

DISCUSSION

Appressoria of *C. gloeosporioides* are produced in response to abiotic and biotic stimuli (Emmett & Parbery, 1975; Parbery, 1981). Prusky & Saka (1989), reported that appressoria formation of *C. gloeosporioides* is triggered by the epicuticular wax of avocado fruits, as increasing concentrations of wax raise the incidence of appressoria formation to 100%. This suggests that a stimulus of chemical origin present in avocado wax induces appressorium formation in *C. gloeosporioides*. The stimulation of appressorial formation by cuticular wax was reported for *Alternaria porri* (Akai *et al.*, 1967) and several rust fungi (Maheshwari *et al.*, 1967). Appressorial formation was not induced by the antifungal diene involved in fruit resistance (Prusky *et al.*, 1982) as the compound was not detectable in the epicuticular wax extracts and formation occurred equally well on ripening fruits showing decreasing levels of antifungal diene (Prusky & Saka, 1989).

Germination of appressoria started on extracted epicuticular wax, 24 h after inoculation. Almost 30% of the appressoria had germinated 104 h after inoculation. On avocado peel, 72 h after inoculation, appressoria had germinated and the hyphae had penetrated the fruit peel. No further hyphal growth was observed until the concentration of the antifungal compound decreased to subfungitoxic concentrations in ripe fruits (Prusky *et al.*, 1982, 1983). This suggests that the subcuticular hypha is the dormant structure of *C. gloeosporioides* in unripe avocado. This is not in accordance with the results of Binyamini & Schiffmann-Nadel (1972), who did not observe germinated appressoria in hand-cut sections of unripe avocado fruit peel.

In freshly harvested fruits, the concentration of the antifungal diene drops to subfungitoxic concentrations and then increases, after a lag period, to the initial concentration present in unharvested fruits (Prusky, 1988; Karni *et al.*, 1989). The process leading to the fast decrease of the antifungal diene was suggested to be similar to the process occuring in normal ripening fruits, where the breakdown of the antifungal diene is catalysed by lipoxygenase whose activity is regulated by the flavan 3-ol, epicatechin (Prusky *et al.*, 1988; Karni *et al.*, 1989). In freshly harvested fruits a fast increase in epicatechin was observed, accompanied by an increase in lipoxygenase activity and consequently a decrease in the concentration of the antifungal diene (Karni *et al.*, 1988).

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Two factors affected the development of *C. gloeosporioides* in the peel of freshly harvested fruits: the time required for the germinated appressoria to penetrate the epicuticular wax and become susceptible to the antifungal diene, and the period of time that the antifungal diene is present at subfungitoxic concentrations. In freshly harvested fruits this period is very short and the pathogen could not establish sufficient growth to overcome the subsequent increase in diene levels. In heat-treated fruits the period of subfungitoxic concentration was sufficient to allow further development of the germinated appressoria. A 5- or 10-min heat treatment prolonged the subfungitoxic period by 1 or 2 days, respectively. The re-establishment of fungitoxic concentrations of the antifungal diene did not contain lesion expansion once the fungus had become established, suggesting that fungitoxicity of the diene is dependent on the fungal biomass present.

In peeled fruits only one factor affects fungal development, fungitoxic concentrations of the diene. Germinated conidia did not form appressoria on fruit flesh (Prusky & Saka, 1989) and direct maceration occurred when subfungitoxic diene concentrations were present in the fruit. Disease symptoms were expressed within 2 days in firm-unripe fruits. Coating of peeled fruits with epicuticular wax enhanced appressoria formation and as a result fungal development, as growing hyphae, did not occur during the period when the diene was at a subfungitoxic concentration: the onset of fungal development was delayed until the antifungal diene decreased to sufficiently low levels. Inoculation of peeled fruits 3 days after harvest, at high diene levels, where no appressoria were formed, gave symptom expression when diene concentration decreased to subfungitoxic concentrations.

The present results suggest that the quiescence of *C. gloeosporioides* in unripe avocado fruits is the result of the presence of fungitoxic concentrations of the antifungal diene on the subcuticular hyphae of the germinated appressoria.

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Dov Prusky
The Volcani Center, Bet Dagan, Israel

Noel T. Keen
University of California, Riverside

Involvement of Preformed Antifungal Compounds in the Resistance of Subtropical Fruits to Fungal Decay

Fungal pathogens must perform precise functions and overcome several barriers before they are able to initiate disease in plants. First, the pathogen must locate and adhere to suscept tissue and then initiate infection (10,12,14). The first plant barriers encountered are generally the cuticle and cell wall, which may be breached by enzymatic (6) or physical (12) assault, or the pathogen may infect through wounds (27). Contact with underlying plant tissues presents the invading pathogen with a different set of barriers, most notably preformed antibiotic compounds and/or morphologic barriers and phytoalexins induced by the plant (13,25,26).

Pathogens that infect fruits are confronted by several problems not normally facing pathogens of vegetative plant tissues. Fruits are generally protected by differentiated integumentary structures, and their physiology changes markedly during development, particularly when ripening occurs. Pathogens frequently infect unripe fruits but cause relatively minor damage until ripening, when they may cause extensive decay. Such quiescent infections have been observed in tropical (15), subtropical (8), and deciduous fruits (9). The resultant decays have great economic importance, since they reduce the shelf life of fruits during storage and transport (27). These disease problems have been further exacerbated by the development of pathogen resistance to fungicides and the withdrawal of pesticides on environmental grounds. Consequently, there is considerable interest in determining mechanisms accounting for the natural resistance of unripe fruits to fungal pathogens and extending its effectiveness to fruits after harvest. One of the possible mechanisms that may account for such differential resistance during ripening is the level of preformed antifungal compounds.

Dr. Keen's address is: Department of Plant Pathology, University of California, Riverside, CA 92521-0122.

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Activity of Preformed Antifungal Compounds

Critical tests of the role of preformed compounds in disease resistance are difficult, primarily because of problems in accurately assessing the quantities of inhibitory compounds that may contact an invading pathogen, uncertainty as to their in vivo biological activity, and difficulty in relating changes in their concentrations to resistance (27). The most compelling evidence for a functional role of preformed antifungal compounds in disease resistance has been produced by using genetically related pathogen strains that differ in their ability to tolerate or degrade a plant antifungal compound or barrier (2,4,5). On the other hand, simple correlations of changes in the level of a preformed compound with resistance may be serendipitous and must therefore be interpreted cautiously. However, if mechanisms accounting for the in vivo concentration of a preformed inhibitory compound are determined, then it may be possible to exogenously modulate these systems and observe whether an altered level of the inhibitory compound and disease resistance are in fact linked. For example, Holowczak et al (11) used exogenously supplied DL-phenylalanine to alter the levels of phenolic compounds and resistance of apple fruits to the scab disease. In addition to providing a critical test of whether a putative preformed resistance mechanism is physiologically important, this approach may provide treatments that can have practical uses in disease control.

In this paper we discuss the role of preformed inhibitors in fungal decays of avocado (*Persea americana* Miller) and mango (*Mangifera indica* L.). We emphasize the interaction of avocado fruit with *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (Fig. 1), in which external modulators have been used to elucidate the nature of a preformed fungal resistance mechanism.

Preformed Antifungal Compounds in Mango Fruits

C. gloeosporioides attacks unripe mango fruits in the orchard. Germinating spores produce appressoria, but the resultant subcuticular hyphae do not develop further until fruit ripen (7). A mixture of antifungal compounds consisting of 5-12-*cis*-heptadecenyl resorcinol and 5-pentadecenyl resorcinol (Fig. 2) was found at fungitoxic concentrations in the peel but not in the flesh of unripe mango fruits (7,8). The antifungal 5-alkylated resorcinols occurred in unripe fruit of several mango cultivars at concentrations ranging between 154 and 232 $\mu g \cdot ml^{-1}$ fresh weight but declined to levels between 74 and 125 $\mu g \cdot ml^{-1}$ in ripe fruits when decay developed (Fig. 3). Inhibition of germ tube elongation indicated an ED_{50} of approximately 120 $\mu g \cdot ml^{-1}$, a concentration similar to that present in fruits when symptoms begin to develop.

Several lines of evidence suggested that the preformed antifungal compounds might be involved in the resistance of mango fruits to fungal development: 1) The concentration of 5-substituted resorcinols occurred at fungitoxic levels in the peel of unripe fruits of eight mango cultivars tested and decreased to nontoxic levels in uninoculated fruits at the same time that decay appeared in inoculated fruits; 2) the concentration of the 5-substituted resorcinols decreased faster during ripening in disease-susceptible cultivars than in resistant cultivars; 3) delayed reduction in levels of the antifungal compounds was related to delayed decay development; and 4) the flesh of unripe fruits contained subfungitoxic concentrations of the antifungal compounds and was susceptible to fungal attack. These observations suggested, but did not conclusively prove, that the resistance of unripe mango fruits results from fungitoxic concentrations of the antifungal 5-substituted resorcinols present in the peel acting on subcuticular hyphae from germinated appressoria. However, addi-

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tional experiments are required to more thoroughly evaluate the role of the preformed resorcinols in resistance.

Resistance of Avocado Fruits to *C. gloeosporioides*

The resistance of unripe avocado fruits to attack by *C. gloeosporioides* was shown several years ago to be correlated with the presence of high concentrations of preformed antifungal chemicals (19). Extracts from the peel of freshly harvested fruits inhibited fungal growth 78% as compared with 7% for extracts from ripe fruits showing disease symptoms. The major antifungal compound was shown to be 1-acetoxy-2-hydroxy-4-oxoheneicosa-12,15-diene (Fig. 4). This compound inhibited in vitro germ tube elongation with an ED_{50} of 450 $\mu g \cdot ml^{-1}$. Concentrations of the diene in peels of unripe fruits were as high as 1,200 $\mu g \cdot g^{-1}$ fresh weight of peel (approximately 1,600 $\mu g \cdot ml^{-1}$) but decreased 10-fold during ripening (Fig. 5). A second antifungal compound was subsequently purified from unripe avocado fruits and identified as 1-acetoxy-2,4-dihydroxy-*n*-heptadeca-16-ene (Fig. 4) (20). This monoene was less fungitoxic than the diene, and its concentration in the peel of unripe fruits varied between 500 and 650 $\mu g \cdot g^{-1}$ fresh weight (approximately 900 $\mu g \cdot ml^{-1}$) and decreased to 40 $\mu g \cdot g^{-1}$ fresh weight (approximately 60 $\mu g \cdot ml^{-1}$) when decay symptoms were conspicuous. It therefore appeared that the diene accounted for most of the antifungal activity while the monoene had a smaller effect (20). Cultivars that were more susceptible to decay also showed a faster decrease in both compounds as the fruit matured.

The concentration of the antifungal diene in avocado fruits is affected by harvesting as well as by ripening. Freshly harvested fruits showed a rapid, almost 10-fold decrease to subfungitoxic levels 1–2 days after harvest (Fig. 5) (23). Levels subsequently recovered to toxic concentrations. The durations of the decrease and recovery periods were related to fruit maturity, with more mature fruits having a shorter recovery period. The cause of this decrease in diene concentration is not clear but seems to be related to stress occurring during fruit harvest. Nevertheless, fungal development does not occur during this period and is observed only when fruits become ripe (Fig. 6).

Two factors affecting development of *C. gloeosporioides* in the peel of freshly harvested fruits (23) are the time required for germinated appressoria to penetrate the epicuticular wax and the period of time in which the antifungal diene is present at subfungitoxic concentrations. In freshly harvested fruits, the period of decline of the antifungal diene is very short and the pathogen is unable to establish sufficient growth before the subsequent increase in diene levels. Fruits treated with hot water at 55 C, however, showed a delay in recovery of the antifungal diene (23) (Fig. 7). This treatment resulted in a longer period of subfungitoxic diene concentration, and the fungus caused decay in these unripe fruits. All of these observations are consistent with, but do not prove, the view that resistance to fungus development depends on a threshold concentration of the antifungal diene that is present in unripe, but not ripe, fruits.

Fig. 1. Symptoms of decay caused by *Colletotrichum gloeosporioides* on a ripe avocado fruit.

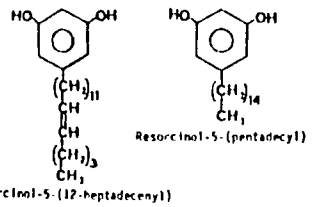

Fig. 2. Chemical structure of the 5-substituted resorcinols isolated from mango fruits.

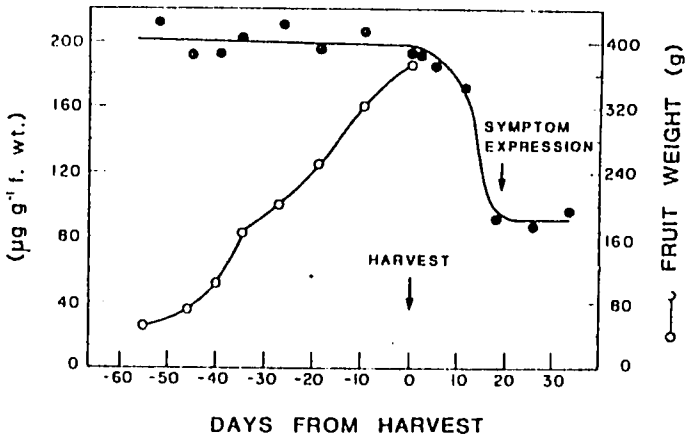

Fig. 3. Changes in the level of 5-substituted resorcinols during fruit growth and after harvesting in ripening mango cv. Hayden fruits.

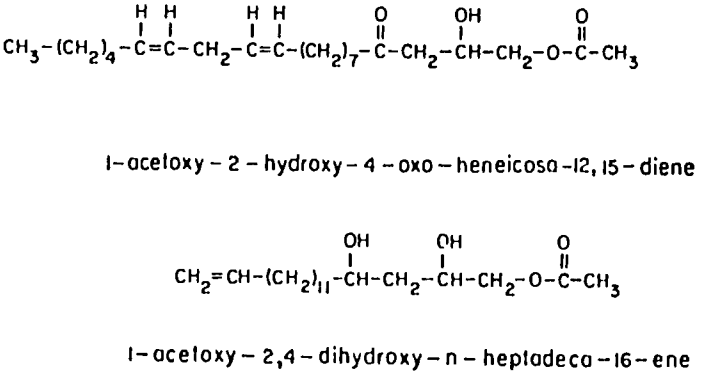

Fig. 4. Chemical structures of the antifungal diene and monoene compounds isolated from avocado fruits.

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Induction of Avocado Preformed Antifungal Compounds

The possibility that preformed biocides can be induced to higher levels in fruits after fungal infection has not been widely investigated. In avocado fruit, however, concentrations of the antifungal diene increased in response to challenge inoculation with *C. gloeosporioides* on unharvested or freshly harvested fruits (17). In both cases, the level of the antifungal compound in freshly harvested fruits was enhanced for 2-3 days but fungal resistance was not affected. Certain abiotic treatments also increased the concentration of the antifungal diene (24). For example, freshly harvested fruits exposed to 30% CO_2 showed an initial increase in the diene level immediately upon removal from the high CO_2 atmosphere (Fig. 8). The concentration of the diene then decreased before a second increase that correlated with delayed development of fungal decay. Exposure of avocado fruits to 11 or 16% CO_2 enhanced the initial increase in diene concentration but not the second. The 11 and 16% CO_2 treatments did not affect decay development, suggesting the importance of the second increase in diene level for fruit resistance. These experiments are of interest because they show that the avocado diene compound has some of the characteristics of classical phytoalexins.

Concentration of the Compounds and Decay Resistance

Because disease susceptibility in avocado fruits was closely related to decreased concentrations of the preformed antifungal compounds, the mechanism controlling this reduction may predispose fruits to disease. The antifungal diene (Fig. 4) contains a *cis,cis*-1,4-penta diene system and serves as a substrate for oxidation by lipoxygenase (18). Several experiments (22) indicated that lipoxygenase activity in avocado fruits was related to degradation of the diene: 1) The apparent specific activity of the enzyme increased by 80% during fruit ripening; 2) partially purified avocado lipoxygenase oxidized the antifungal diene in vitro; 3) treatments with α-tocopherol acetate, an antioxidant that nonspecifically inhibits lipoxygenase, delayed the decrease of the antifungal diene as well as the appearance of disease symptoms; and 4) a specific inhibitor of lipoxygenase, 5,8,11,14-eicosatetraynoic acid, inhibited decay development by *C. gloeosporioides*.

Lipoxygenase activity in ripening avocado fruit was affected by an endogenous inhibitor, epicatechin, present in avocado peel (21). This flavan-3-ol competitively inhibited lipoxygenase activity. Its concentration decreased from 500 $\mu g \cdot g^{-1}$ fresh weight of peel in unripe fruits to 8 $\mu g \cdot g^{-1}$ fresh weight in ripe, symptom-expressing fruits. The concentration of epicatechin in fruits of a highly susceptible cultivar decreased in parallel with decreasing fruit firmness (Fig. 9), and symptom expression occurred when the epicatechin concentration was at its lowest level (21). In a resistant cultivar, however, the initial concentration of epicatechin was much higher and considerable epicatechin was still present in the peel of soft, ripe fruits. Decay resistance was therefore related to the period required for complete decrease of epicatechin levels in softening fruits. These results suggested that lipoxygenase activity and degradation of the antifungal diene might be regulated by epicatechin acting as a trap for free radicals (Fig. 10). This mechanism could, in principle, be exogenously modulated in various ways to test whether corresponding changes occurred in development of anthracnose disease in ripe avocado fruits.

Modulation of Diene Levels and Practical Disease Control

As with the experiments involving mango fruits, the considerable correlative evidence did not conclusively link altered concentrations of the avocado diene with decay resistance. We therefore searched for treatments that would modulate alterations in diene levels in ripening fruits and observed whether they led to concomitant changes in decay resistance.

Levels of the antifungal diene in avocado fruit tissue could be increased by exogenously supplying inhibitors of lipoxygenase (16,22). For example, a commercial formulation of butylated hydroxy anisole, Xedaphen-20, sprayed on fruits in a packinghouse during the

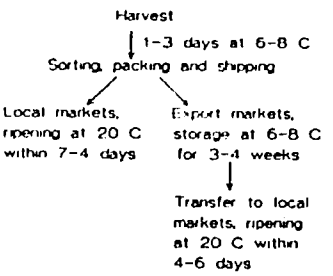

Fig. 6. The commercial processes and time frame of harvest, storage, and ripening of avocado fruits.

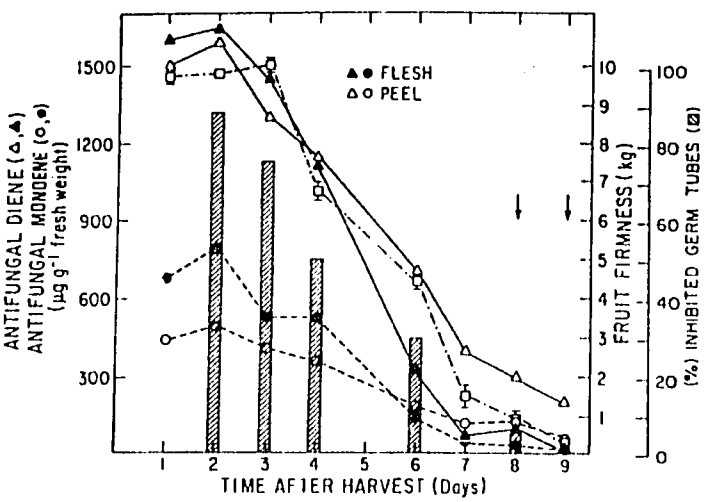

Fig. 5. Antifungal diene and monoene levels in the flesh and peel, effects on germ tube elongation (bars), and fruit firmness (open squares) in freshly harvested avocado cv. Fuerte fruits maintained at 20 C. Arrows indicate the appearance of decay symptoms.

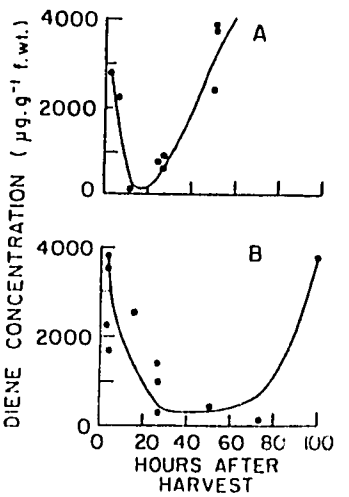

Fig. 7. Changes in the concentration of the antifungal diene in avocado cv. Fuerte fruits at 20 C after heat treatment at 55 C: (A) Untreated control and (B) 10-min heat treatment.

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first day after harvest, provided significant decay control (Fig. 11). Inhibition of lipoxygenase activity in these fruits was accompanied by a delayed decrease of the antifungal diene compound and reduced fungal colonization.

Freshly harvested fruits exposed to an atmosphere of 30% CO_2 also contained higher concentrations of the antifungal diene and showed reduced decay (Fig. 12) (24). However, several factors were critical to enhancing the levels of the preformed compound and achieving a significant delay of symptom development: 1) Concentrations of CO_2 below 30% and application for less than 24 hr were less effective, 2) fruits required treatment as soon after harvest as possible, and 3) more mature fruits were less capable of reacting to the CO_2 treatment.

In addition to providing independent evidence suggesting a role for the antifungal diene level in decay resistance, the results with Xedaphen-20 and high CO_2 atmosphere indicate that these treatments may be useful in postharvest decay control. While it is not yet clear from pilot scale experiments whether either treatment will in fact provide economical and effective postharvest disease control, they clearly constitute feasible and environmentally sound approaches.

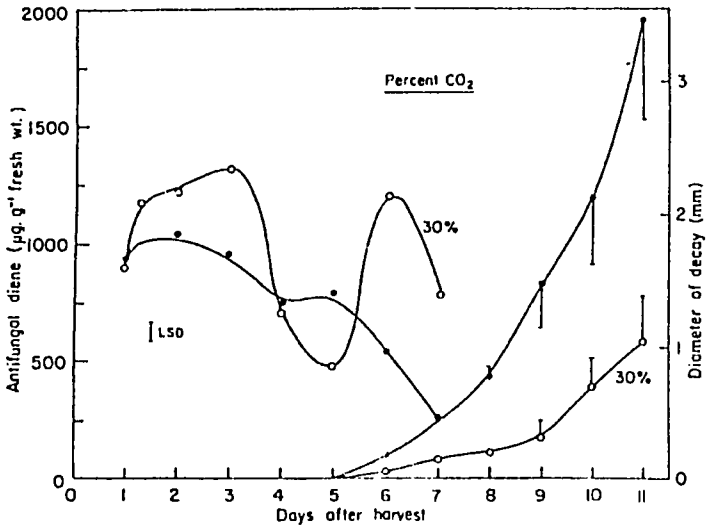

Fig. 8. Diene levels (curves at left) and decay development by *Colletotrichum gloeosporioides* (curves at right) in avocado cv. Fuerte fruit peel exposed to ambient air (●) or 30% CO_2 (○) at 20 C. LSD = 0.05.

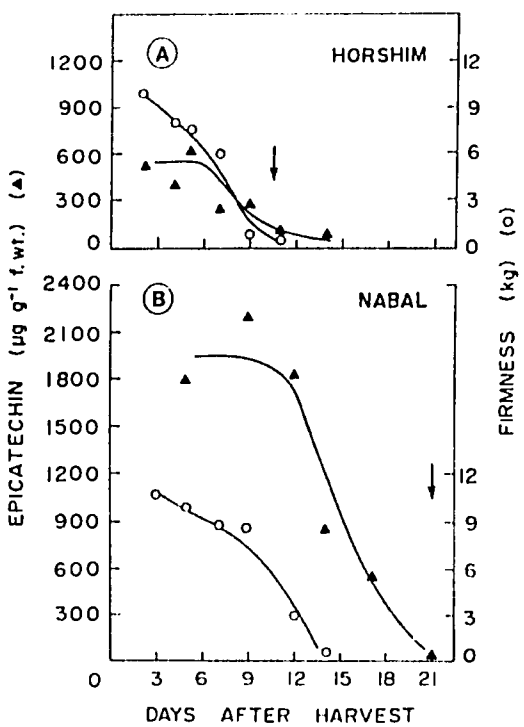

Fig. 9. Epicatechin concentration (▲) and firmness (○) of fruits of two avocado cultivars at various days after harvest. Arrows indicate the appearance of decay symptoms caused by *Colletotrichum gloeosporioides*.

Potential Toxicity of Natural Compounds in Food Products

One of the important considerations involved in attempts to enhance disease resistance by increasing concentrations of natural antifungal compounds is their potential toxicity to animal and human consumers. No information is available concerning potential toxicity of the avocado antifungal diene and monoene compounds, but several reports exist on toxic preformed compounds in other plants (3). In potatoes, α-solanine and α-chaconine are plasma cholinesterase inhibitors in humans and teratogens in animals. Because of the toxicity of these compounds, the potato cultivar Lenape was withdrawn from the market. Celery, parsley, and parsnip contain the linear furanocoumarin phytoalexins psoralen, bergapten, and xanthotoxin, which can cause photosensitization and also are photomutagenic and photocarcinogenic. Sweet potato roots produce phytoalexins that are toxic to vertebrates. Thus, preformed or induced plant antifungal compounds can be highly toxic, which may temper enthusiasm for elevating their levels in edible foodstuffs.

An optimal situation would be the induction of high levels of antifungal compounds for a limited period while fruits are at risk of pathogen attack, with decline of these levels before the produce is consumed. There is evidence that this occurs in some cases. In immature peppers inoculated with *Colletotrichum cap-*

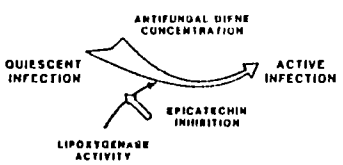

Fig. 10. Apparent relationship between relative diene concentration (as thickness of arrow), lipoxygenase activity, and epicatechin levels during avocado fruit ripening and susceptibility to anthracnose decay.

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sici, the level of the phytoalexin capsicannol increased, but the compound was absent in ripening fruit at the onset of lesion expansion (1). In avocado, we have shown that the level of preformed compounds can be enhanced during fruit ripening but decreases markedly in ripe fruits. Although additional experimentation is needed, it appears that antifungal diene levels in avocado fruits can be effectively modulated to control disease with minimal risk of toxicity during human consumption.

Conclusions

By means of comparative biochemical approaches, we elucidated putative defense mechanisms controlling postharvest disease in unripe avocado and mango fruits. Levels of the avocado diene compound and the 5-substituted resorcinols were high in unripe, disease-resistant fruit but decreased during fruit ripening, a time when latent fungal infections cause proliferating lesions that make fruit unmarketable. Such correlative evidence, however, does not prove a role for the diene compounds in resistance. To provide more critical tests, we employed various exogenous treatments that modulated the normal disappearance of the diene during the ripening of avocado fruits and observed whether the treatments concomitantly affected the development of fungal decay. The most effective measures were antioxidant and high carbon dioxide treatments, which inhibited decreases of the avocado antifungal diene compound during ripening or induced the accumulation of higher levels. The fact that modulation of diene levels by these agents led to the predicted changes in fruit resistance constitutes important evidence that the diene is a physiologically important agent effecting resistance to *C. gloeosporioides*.

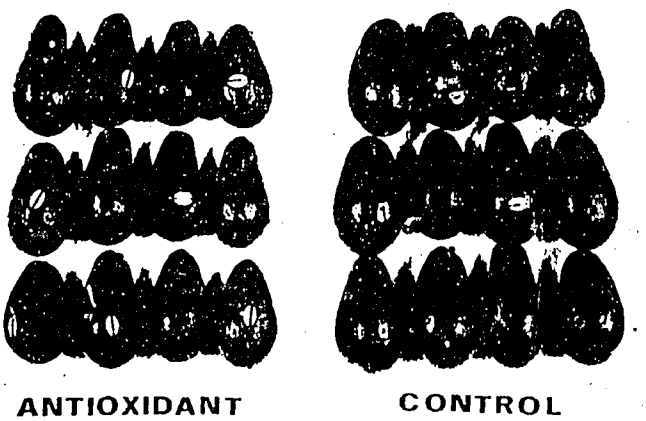

Fig. 11. Effect of antioxidant on decay caused by *Colletotrichum gloeosporioides* on avocado cv. Fuerte fruits.

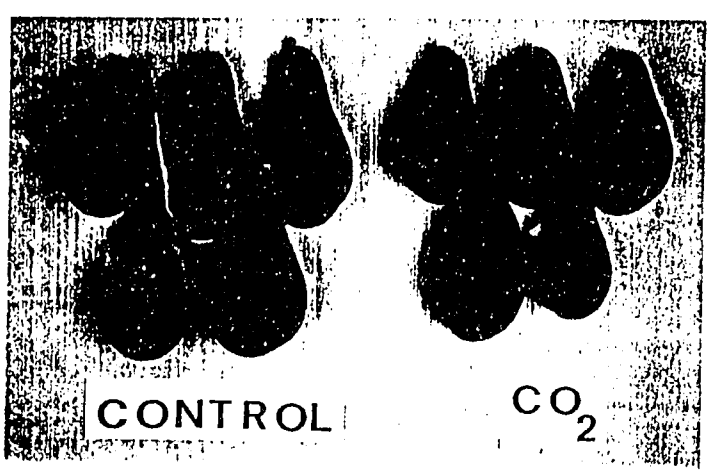

Fig. 12. Effect of 30% CO_2 on decay caused by *Colletotrichum gloeosporioides* on avocado cv. Fuerte fruits.

Treatments such as the antioxidants that affect levels of preformed antifungal compounds may be useful for practical disease control. For example, we are currently investigating the possibility of applying antioxidants or 30% carbon dioxide treatment of avocado fruits after harvest to control anthracnose disease during fruit storage and shipment. Increasing environmental pressure for reduced pesticide usage encourages experimentation to determine if levels of preformed antifungal agents in fruits of other plants can be similarly modulated to provide postharvest disease control.

Acknowledgments

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Dov Prusky

Dr. Prusky is a research plant pathologist in the Department of Fruit and Vegetable Storage, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. His current research interest is the biochemical and molecular basis of resistance of subtropical fruits to postharvest diseases. He received his B.S. degree in 1971, M.S. degree in 1973, and Ph.D. degree in 1977, all from the Hebrew University of Jerusalem, Israel.

Noel T. Keen

Dr. Keen is a professor in the Department of Plant Pathology at the University of California, Riverside. Current research interests involve the biochemical basis of microbial pathogenicity (particularly in soft-rotting erwiniae), the biochemical basis of gene-for-gene specificity, and the molecular basis of disease resistance in plants. He received his B.S. degree in 1963 and his M.S. degree in 1965 from Iowa State University, Ames, and his Ph.D. degree in 1968 from the University of Wisconsin, Madison.

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PURIFICATION OF NEW ANTIFUNGAL COMPOUNDS IN AVOCADO

Biological activity of four antifungal compounds in immature avocado

By S. Sivanathan and N.K.B. Adikaram

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By N.K.B. Adikaram, D.F. Erwing, A.M. Karunaratne and E.M.K.Wijeratne

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Nimal Adikaram
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Department of Botany, University of Peradeniya, Sri Lanka

Biological Activity of Four Antifungal Compounds in Immature Avocado

S. Sivanathan and N. K. B. Adikaram

Authors' address: Department of Botany, University of Peradeniya, Peradeniya, Sri Lanka.

With 6 figures

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Abstract

Anthracnose caused by *Colletotrichum gloeosporioides* in ripe avocados originates as latent infections in the immature fruits. Concentrated ether extract of the peel of apparently healthy, immature avocados when bioassayed on thin layer chromatographic plates with conidia of either *Cladosporium cladosporioides* or *C. gloeosporioides* produced four inhibition areas at Rf 0.30, 0.32, 0.70 and 0.75 (these were denoted as AvIV, AvIII, AvII and AvI respectively). A hot chloroform extract was partitioned on a silica gel column and the four antifungal compounds were separated. Spectroscopic data revealed that one of these compounds, AvII, was similar to cis-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene and another (AvIV) was a long chain saturated compound comprising hydroxyl group(s) having molecular weight of 268. Toxicity of AvII to *C. gloeosporioides* was 2 times that of AvIV and 6.5 and 7.5 times that of AvIII and AvI respectively. The amounts of these four antifungal compounds increased gradually during fruit development and reached their maxima at harvest. The concentration of AvI, AvII, AvIII and AvIV was 1300, 920, 1050 and 780 μg/g fresh peel respectively in the fruit at harvesting maturity. The amount of AvII and AvIV decreased to 53 and 64 μg/g fresh peel respectively in the fruit at the ripe stage at which neither AvI nor AvIII was detected. This took place in coincidence with the onset of progressive lesion development by the fungus.

Zusammenfassung

Biologische Aktivität von vier antipilzlichen Substanzen in unreifen Avocados

Eine Anthraknose, die durch *Colletotrichum gloeosporioides* verursacht ist, geht in reifen Avocadofrüchten von latenten Infektionen der unreifen Früchte aus. Konzentrierter Atherextrakt der Schale aus scheinbar gesunden, unreifen Avocadofrüchten, der nach dünnschichtchromatographischer Trennung mit Konidien von entweder *Cladosporium cladosporioides* oder *C. gloeosporioides* entwikkelt wurde, zeigte vier Hemmzonen mit Rf-Werten 0,30, 0,32, 0,70 und 0,75 (die als AvIV, AvIII, AvII bzw. AvI bezeichnet wurden). Nach einer heißen Chloroformextraktion wurde der Extrakt an einer Silicagelsäule fraktioniert, und die vier fungiziden Verbindungen getrennt. Spektroskopische Analyse ergab, daß die Verbindung AvII eine Ähnlichkeit mit cis-1-acetoxy-2-hydroxy-4-oxo-

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heneicosa-12,15-dien besaß und AvIV eine langkettige, saturierte Verbindung, die aus Hydroxylgruppe(n) mit einem Molekulargewicht von 268, ist. Die Toxität von AvII gegenüber *C. gloeosporioides* wurde als 2-, 6,5- bzw. 7,5mal höher als AvIV, AvIII bzw. AvI ermittelt. Der Gehalt der fungitoxischen Verbindung stieg mit der Fruchtentwicklung langsam an, um zur Zeit der Ernte ihr Maximum erreicht zu haben. Bei der Ernte betrug die Konzentration von AvI, AvII, AvIII bzw. AvIV in frischen Schalen 1300, 920, 1050 bzw. 780 µg/g. In der Zeit bis zur Reife verringerte sich der Gehalt an AvII und AvIV in frischen Schalen auf 53 bzw. 64 µg/g; zu diesem Zeitpunkt konnte ein AvI- bzw. AvIII Gehalt nicht festgestellt werden. Mit der Abnahme der fungitoxischen Verbindungen wurde eine Läsionenentwicklung durch den plötzlichen Befall beobachtet.

Anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. is recognized as the most important disease of avocado. Young fruits are usually free from visible symptoms of anthracnose before harvest and characteristic decay lesions develop during fruit ripening after harvest showing that this disease originates as latent infections in the immature avocado fruit (BINYAMINI and SCHIFFMANN-NADEL 1972).

In an attempt to explain the basis of latency in avocado by *C. gloeosporioides*, PRUSKY *et al.* (1982) isolated a preformed antifungal compound from the peel of unripe avocado fruits which they identified as 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene. This compound had previously been isolated from avocado leaves (CHANG *et al.* 1975). It inhibited *in vitro* vegetative growth of *C. gloeosporioides* and totally inhibited spore germination at 790 µg/ml. Concentration of this compound was high in the immature fruit tissue and declined during ripening to non-toxic levels in coincidence with the renewed development of previously latent infections.

Further work by PRUSKY *et al.* (1983) revealed that the purified diene was oxidized *in vitro* by lipoxygenase extracted from avocado peel and soybean. The specific acitivity of lipoxygenase in peel extracts also increased by 80 % during the climacteric stage, coincident with the rapid decrease of the diene in fruit peel, and before symptoms of *C. gloeosporioides* infection were expressed. They also demonstrated that butylated hydroxy anisole and α-tocopherol acetate inhibited oxidation of linoleic acid and of the avocado diene by lipoxygenase from avocado and soybean. PRUSKY *et al.* (1985) subsequently isolated a natural inhibitor of avocado lipoxygenase from peel of unripe avocado fruit and identified it as epicatechin. It inhibited avocado lipoxygenase with a Ki of 0.64 µm. The concentration of epicatechin in unripe fruits was 514 µg/g fresh weight of peel, this decreased during ripening to 8 µg/g fresh weight, before symptoms of *C. gloeosporioides* infection were expressed.

In this paper we report the presence of three more preformed antifungal compounds in the immature avocado fruit peel and their role in the development of latent infections in the immature avocado fruits by *C. gloeosporioides*.

Materials and Methods

Fungus

C. gloeosporioides was isolated from an anthracnose lesion on a naturally infected ripe avocado fruit and maintained on potato dextrose agar at room temperature. Pathogenicity of the isolate was maintained by inoculation and reisolation from ripe fruits at intervals of not more than two months.

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Fruits

Fruits of *Persea americana* cv. "Green" were obtained mostly from trees grown in the University area. They were allowed to remain on the trees for 80 to 90 days after anthesis before being harvested for use in the experiments and these were described as immature fruits. In some experiments, fruits of the same cultivar bought in the market (Kandy and Peradeniya) were also used. Fruits free from any kind of damage were selected at the laboratory and used in the experiments.

Extraction of antifungal compounds from immature avocado fruit peel

For the extraction of antifungal compounds, the outer skin (about 1—2 mm thick) was cut from immature avocado fruits and stored in the deep freezer (- 20 °C) for 1—2 days. Deep freezing did not affect the concentration of antifungal compounds. Solvent extraction was done by two different methods as follows:

Method I:

Peel tissue (25 g) was homogenized twice with two 50 ml portions of diethylether for 5 min in a homogenizer operated at its highest speed. The extracts were combined, filtered through Whatman No. 1 filter paper and evaporated *in vacuo* at 40 °C. The residue was dissolved in 10 ml ethanol and used in the assay for antifungal compounds.

Method II:

Immature avocado peel tissue (25 g) was extracted in 95 % ethanol and the extract was partitioned with dichloromethane. The residue obtained after evaporation of the dichloromethane layer was taken up in 10 ml ethanol (PRUSKY *et al.* 1982).

Detection of antifungal compounds

Spore germination assay:

Portions of the two extracts (1 ml) obtained from method I and method II were streaked and chromatographed separately on two Thin Layer Chromatographic (TLC) plates (20 × 20 cm; Kieselgel 254 PF60; MERCK; 0.5 mm thick) and the compounds eluted at different Rf regions were tested for the germination of conidia of *C. glocosporioides* as described by PRUSKY *et al.* (1982).

TLC bioassay:

The two extracts obtained as above were tested for the presence of antifungal compounds using TLC bioassay (KEARMAN and STANFORD 1968). Aliquots (100 *µl*) of the extracts were spotted onto TLC plates (20 × 20 cm; Kieselgel 254 PF60; MERCK; 0.5 mm thick). The plates were developed in chloroform : methanol (90 : 10, v/v) and air-dried overnight. Sporulating cultures of *C. cladosporioides* were flooded with Czapek-Dox nutrient solution and with the help of a spatula the mycelia were scraped. The mycelial suspension was passed through glass wool to remove hyphal fragments and the filtrate containing the conidia was collected. The suspension of conidia was carefully sprayed onto the developed chromatograms and the plates were incubated in a moist chamber for 2—3 days at room temperature. The presence of compounds toxic to *C. cladosporioides* was indicated by the lack of aerial mycelium in that zone. Another set of developed chromatograms was similarly sprayed with a suspension of conidia of *C. glocosporioides* in Czapek-Dox nutrient medium. The plates were incubated in moist chambers as described above. Here the plates were exposed to iodine vapour in order to visualize inhibition areas.

Antifungal activity of the immature fruit inoculated with *C. glocosporioides*

A set of 20 fruits was inoculated with conidia (5×10^5 ml^{-1}) of *C. glocosporioides* and another set was treated with drops of sterile distilled water. Suspensions of conidia were prepared as described by ADIKARAM *et al.* (1982). Discs of tissue (6 mm diameter and 2 mm thick) were cut from the inoculated regions of the fruits daily until 5 days after inoculation using a cork borer. Similar discs of tissue were cut from the control fruits beneath the water drops. The cut tissues were immediately placed in a deep freezer at -18 °C until the solvent extraction was performed.

Each set of cut tissue (2.5 g) placed in the deep freezer was separately extracted using method I as described above. The residue remaining after evaporation of the solvent was taken up in 2.5 ml of

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ethanol and 100 μl aliquots subjected to TLC bioassay as described above. Extra precautions were taken to spot all the extracts on the TLC plates over areas of 0.5 cm diameter. The diameters of the resultant inhibition areas were measured and recorded.

Isolation and partial purification of antifungal compounds

Above experiments indicated the presence of four antifungal compounds at Rf 0.30, 0.32, 0.70 and 0.75 (these were denoted as AvIV, AvIII, AvII and AvI respectively) and further work was carried out to separate them. Preliminary experiments showed that freeze drying of tissues facilitated solvent extraction and that hot chloroform extracted all the antifungal compounds in the tissue efficiently. Fresh avocado peel tissue (400 g) was first freeze dried overnight at −60 °C and 90 g of freeze dried material was extracted with 500 ml chloroform in a Soxhlet apparatus for 3 days at 50–55 °C. The chloroform extract was filtered through ceilite to remove suspended particles, dried over anhydrous $MgSO_4$ and evaporated at 45 °C.

Column chromatographic separation of antifungal compounds

Crude extract (5.25 g) as obtained above was chromatographed in a column (length 20 cm; diameter 2 cm) packed with 100 g of active neutral Kieselgel 254 PF60 (MERCK) using solvent combinations of increasing polarity. The crude extract was first dissolved in a minimum volume of chloroform and a slurry was then prepared with silica gel. This was applied to the column pre-equilibriated with petroleum ether. Fractions were collected using the following solvent systems: i) petroleum ether, ii) petroleum ether : dichloromethane (50 : 50, v/v), iii) dichloromethane, iv) dichloromethane : methanol (99 : 1, v/v), v) dichloromethane : methanol (98 : 2, v/v), vi) dichloromethane : methanol (97 : 3, v/v), vii) dichloromethane : methanol (96 : 4, v/v), viii) dichloromethane : methanol (95 : 5, v/v), ix) dichloromethane : methanol (90 : 10, v/v) and x) dichloromethane : methanol (50 : 50, v/v). Approximately 1000 ml of each solvent combination was passed through the column and 50 ml fractions were collected. A iquots of each fraction eluted were spotted on small TLC plates (7.5 × 2.5 cm) with the help of capillary tubes. After air-drying, the plates were developed in chloroform : methanol (9 : 1, v/v). These were then sprayed with anisaldehyde reagent and heated to about 110 °C for 1 min. The fractions having similar TLC behaviour were combined and the combined fractions were evaporated to dryness and the residues were subjected to TLC-*Cladosporium* bioassay for antifungal compounds.

Purification of antifungal compounds AvII and AvIV from fractions 82—160

The two antifungal compounds AvII and AvIV were further purified using two separate pressure columns. One column (A), was pre-equlibriated with solvent combination v and the other (B) with solvent combination vii. The compounds in the fractions 82 to 126 were eluted with solvent combination v and vi using the column A and the compounds in the fractions 127 to 160 were eluted with solvent combination vii and viii using the column B. Approximately 500 ml of each solvent system was passed through each column and 25 ml fractions were collected.

The eluted fractions having similar TLC behaviour (with anisaldehyde reagent) were combined and the combined fractions were evaporated to dryness. The residues were tested for antifungal activity on TLC plates using *C. cladosporioides*.

Chemical analysis of the antifungal compounds AvII and AvIV

Chemical analysis of the two purified compounds was done at the Department of Chemistry, University of Peradeniya, Sri Lanka, using IR, mass and H^1NMR spectroscopy. Some chemical data were also obtained by sending a sample of AvIV to the Department of Organic Chemistry, Queen's University of Belfast, U.K. Physical properties of these compounds such as solubility and melting point were also determined.

Toxicity of the antifungal compounds to *C. glocosporioides*

A series of solutions containing 2.50, 2.25, 2.00, 1.75, 1.50, 1.25, 1.00, 0.75, 0.50 and 0.25 mg/ml ethanol of each antifungal compound was prepared. Aliquots (20 μl) of each were spotted on TLC

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plates, the area of each spot being restricted to 1 cm diameter. Care was taken to keep the area of the spot corresponding to each dilution constant. After air-drying overnight, the plates were sprayed with a suspension of conidia of *C. gloeosporioides* in Czapek-Dox nutrient medium and incubated in moist chambers for 2—3 days at 26 ± 2 °C. The areas of inhibition of fungal growth caused by varying amounts of the four compounds were determined by tracing them on to a graph paper with 1 mm^2 squares. Dose-response curves were drawn by plotting the amount of antifungal compound applied against the resultant area of inhibition. The amount of each compound required to bring about inhibition of growth over a 1 cm^2 was calculated. These curves were also used to estimate the approximate amounts of the four antifungal compounds present in tissue extracts made in subsequent experiments following TLC-bioassay under the same conditions.

Vertical distribution of antifungal compounds

Cylinders of tissue of 6 mm diameter were cut through the peel and the pulp of immature fruit to about 8 mm depth, using a cork borer. These were then sliced into smaller discs of 2 mm thickness and the discs corresponding to different depths from the surface of the fruit were bulked separately. Two g of tissue from each layer were extracted 3 times in three 25 ml volumes of diethylether. The ether extracts were combined, dried over anhydrous $MgSO_4$ and evaporated to dryness at 45 °C. The residue was dissolved in ethanol (1 ml) and 100 μl aliquots were bioassayed on TLC plates using *C. gloeosporioides*. The plates were examined for inhibition zones, the diameters of which were measured.

Antifungal activity of the fruit at different stages of maturity

Fruits at different stages of maturity ranging from 15 to 90 days after anthesis were obtained from a single tree and the fruits free from damage of any kind were selected and divided into 6 sets, each containing 8 fruits, according to their maturity. Discs of tissue (2 g), 6 mm diameter and 2 mm thickness, were cut out from the peel of each set of fruits using a cork borer. Another set of fruits which was at harvesting maturity was allowed to ripen and discs of tissue were cut at two day intervals until they reached the fully ripened stage. The cut tissues were subsequently extracted in diethylether as described above. The residues remaining after evaporating the solvent were taken up in 1 ml portions of ethanol. Aliquots (100 μl) were applied to TLC plates and bioassayed using *C. gloeosporioides*. The plates were then examined for inhibition zones the diameters of which were measured. Concentration of each antifungal compound in the peel at harvest and during fruit ripening was determined by using a dose-response curve obtained as described previously.

All the experiments were repeated at least twice.

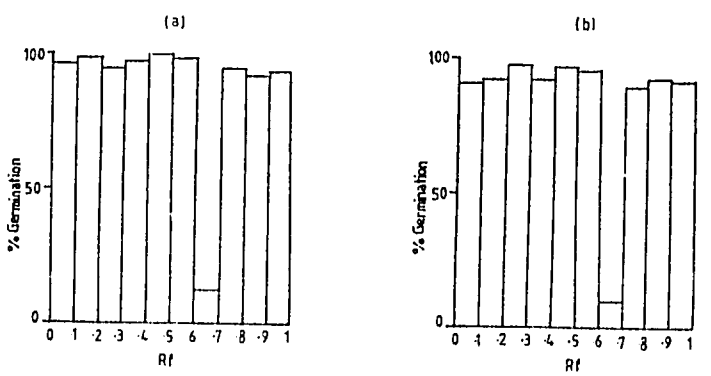

Fig. 1. Percentage germination of conidia of *C. gloeosporioides* in components eluted at different Rf regions from chromatograms prepared with 95 % ethanol (a) and ether (b) extracts of immature avocado fruit peel

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Fig. 2. Photograph of a TLC plate spotted with ether extract from immature avocado fruit peel tissue and sprayed with a suspension of conidia of *C. cladosporioides*, showing four inhibition zones (chloroform : methanol, 8 : 2, v/v)

Results

Both the ether and the ethanol extracts of the immature fruit peel were tested for antifungal activity by spore germination assay and in both extracts the compound(s) eluted from silica gel at Rf 0.6—0.7 inhibited germination of conidia by about 90 % (Fig. 1) whereas all the other compounds eluted at the other Rf regions were stimulatory or had no effect on germination (Fig. 1).

Thin Layer Chromatograms prepared with the ether extract of unripe avocado peel tissue and oversprayed with *C. cladosporioides* produced four

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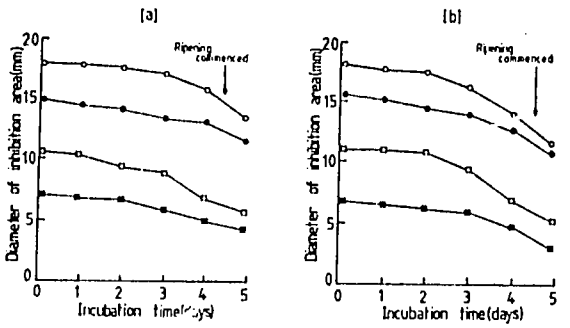

Fig. 3. (a) Antifungal activity of ether extracts of avocado fruit peel removed after varied incubation times following inoculation with *C. gloeosporioides* (expressed as the diameter of inhibition areas produced on TLC plates), AvI -■-, AvII -○-, AvIII -□-, AvIV -●-. (b) Antifungal activity of ether extracts of uninoculated avocado fruit peel at varied incubation times (expressed as the diameter of inhibition areas produced on TLC plates), AvI -■-, AvII -○-, AvIII -□-, AvIV -●-

inhibition areas at Rf 0.30, 0.32, 0.70 and 0.75 (Fig. 2). In the plates which were oversprayed with *C. gloeosporioides* there were much larger inhibition areas at these Rf values than those found in *C. cladosporioides* sprayed plates. In both, the inhibition areas at Rf 0.30 and 0.70 were much larger and clearer than those found at Rf 0.32 and 0.75. The toxic components which caused inhibition at Rf 0.75, 0.70, 0.32 and 0.30 were assigned symbols AvI, AvII, AvIII and AvIV respectively.

Chromatograms prepared with extracts from fruit peel taken 24, 48 and 72 h after inoculation with *C. gloeosporioides* showed inhibition areas at Rf 0.30, 0.32, 0.70 and 0.75 as in those chromatograms which were prepared with extracts taken from corresponding healthy fruit peel (Fig. 3 a, b). The size of the inhibition area at each Rf value was similar in the two sets of chromatograms. Inoculated regions of the fruit started to turn brown gradually 48—72 h after inoculation and these browned areas developed into progressive lesions following another 3—4 days of incubation. Extracts of the tissues cut from the fruits, both inoculated and control, on the 4th, 5th and 6th day following inoculation, when bioassayed showed a gradual decline of the size of these inhibition areas.

TLC-bioassay of the fractions eluted from the column indicated that the compounds eluted with solvent system v (dichloromethane : methanol, 98 : 2, v/v), vi (dichloromethane : methanol, 97 : 3, v/v), vii (dichloromethane : methanol, 96 : 4, v/v) and viii (dichloromethane : methanol, 95 : 5, v/v) contained antifungal activity. The active components eluted with solvent systems v and vi were similar in Rf values to those denoted by the symbols AvI and AvII respectively and the active components eluted with solvent system vii and viii were similar to those of AvIII and AvIV. Fractions eluted with solvent combination v and vi (1.76 g) and

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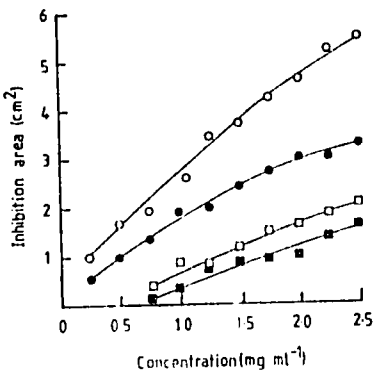

Fig. 4. Areas of inhibition of *C. glocosporioides* caused by varying amounts of the four antifungal compounds, AvI -■-, AvII -○-, AvIII -□-, and AvIV -●-

also with vii and viii (1.77 g) were combined separately and passed through two separate pressure columns for further purification. Following this 1.13 mg and 1.41 mg of purified AvII and AvIV respectively were obtained. Pure materials of AvI and AvIII could not be obtained even after the second column chromatographic separation and the partially purified materials obtained from this were not sufficient to proceed towards further column chromatographic separation.

Purified compound AvII was a light yellow semi-solid at room temperature, insoluble in water but completely soluble in dichloromethane, chloroform, ethanol and methanol, m.p. 54—58 °C; IR spectrum showed absorption bands at ϑ max (KBr) 3650-3050, 2860, 2790, 1725, 1625, 1450, 1360, 1245, 1105, 1060, 1025 and 895 cm^{-1}; it showed ^{1}H NMR (60 MHz) signals at δ 7.67, 5.63, 5.07, 4.90, 4.13, 3.31, 2.13 and 1.33. The purified compound AvIV was a white semi-solid at room temperature, insoluble in water but completely soluble in dichloromethane, chloroform, ethanol and methanol, m.p. 51—55 °C; its mass spectrum showed significant mass fragments at *m/z* (relative abundance) 268 (3.6), 239 (1), 237 (2), 219 (0.9), 194 (1.6), 149 (1.2), 135 (1.9), 121 (2.8), 109 (3.5), 95 (7.4), 87 (100); the IR spectrum of AvIV showed absorption bands at ϑ max (KBr) 3675-3025, 2925, 2875, 2470, 1115, 905 and 845 cm^{-1}; ^{1}H NMR (60 MHz) signals appeared at δ 3.93, 3.60, 1.83, 1.70 and 1.30.

The areas of inhibition of fungal growth caused on the TLC plates by different amounts of the four antifungal compounds are given in Fig. 4. Using these dose response curves the amount of each compound required to bring about inhibition in an area of 1 cm^2 was calculated. Much smaller amounts of AvII and AvIV were required to bring about inhibition in 1 cm^2 area than AvI and AvIII. The concentration of AvII required for 1 cm^2 inhibition area was smaller than that of AvIV. The amounts of AvI and AvIII required for the same inhibition area were almost similar (Table 1).

The size of the inhibition areas corresponding to the four antifungal compounds produced on TLC plates by the tissue extract increased with increase in

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Table 1
The amounts of antifungal compounds required to cause 1 cm² inhibition

Compound	Concentration, mg/ml
AvI	1.875
AvII	0.250
AvIII	1.625
AvIV	0.525

the depth at which the tissues were cut from the fruit peel and pulp. This increase was noticed up to a depth of 6 mm and thereafter the size of the inhibition areas decreased (Fig. 5).

Extracts of the peel tissue taken from fruits at different developmental stages i.e., from 15 days after anthesis up to ripening, produced four inhibition areas on TLC plates. The size of the inhibition areas produced by the extracts taken from the fruit peel tissue 15 days after anthesis was the smallest among all (Fig. 6) and, up to harvest, this increased gradually when the age of the fruits, from which extracts were taken, increased. The highest antifungal activity was found in the tissues taken from fruits at harvesting maturity. Fruits which were at harvesting maturity were obtained and allowed to ripen; tissues cut from these at two-day intervals till they became fully ripe were bioassayed. A gradual decrease in the size of the inhibition areas corresponding to all four antifungal compounds was

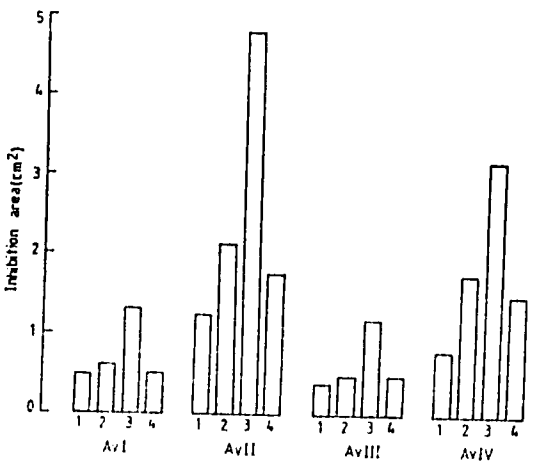

Fig. 5. Vertical distribution of antifungal compounds AvI, AvII, AvIII and AvIV in the immature fruit (1 = 0–2 mm; 2 = 2–4 mm; 3 = 4–6 mm; 4 = 6–8 mm thick)

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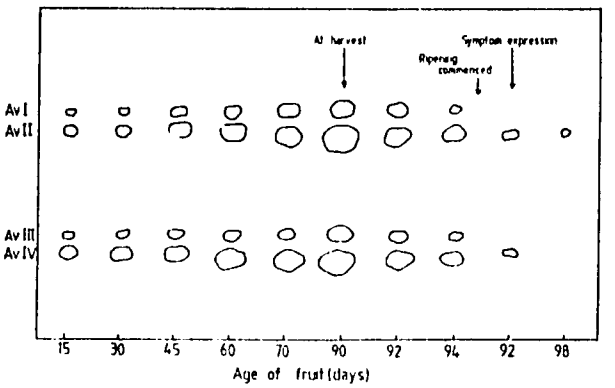

Fig. 6. Sketch of a thin layer chromatogram prepared with ether extracts of avocado fruit peel at different maturity stages, sprayed with *C. gloeosporioides*

noticed during fruit ripening (Fig. 6) and only a trace or no antifungal activity was detected in tissues taken from fully ripened fruit (Table 2). Symptoms of anthracnose disease are usually noticeable at this stage of fruit ripening.

Table 2
Concentration of antifungal compounds in the fruit peel at harvest and symptom expression

Compound	Concentration, μg/gm fresh weight at harvest	at symptom expression
AvI	1300	none
AvII	920	52
AvIII	1050	none
AvIV	780	64

Discussion

PRUSKY *et al.* (1982), who investigated the antifungal activity of the unripe avocado fruit in relation to the development of latent infections by *C. gloeosporioides*, isolated one preformed antifungal compound, 1-acetoxy 2-hydroxy-4-oxo-henicosa-12,15-diene (diene) from the fruit peel. They concluded that this compound was solely responsible for the establishment of latent infections in the unripe fruit of avocado by this fungus.

A similar study, carried out in this laboratory to identify the factors that govern latency in unripe avocados using more sensitive bio-assay techniques, revealed that there are at least four preformed antifungal compounds in the immature avocado fruit peel (these were denoted by AvI, AvII, AvIII and AvIV).

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The method that PRUSKY *et al.* employed to detect the antifungal activity of the tissue extracts was spore germination assay and it is possible that this method may have failed to identify the toxic effect of the other three compounds. Their experiment was repeated in this laboratory using the procedure adopted by them and it was found that the fungitoxicity of only one compound eluted at Rf 0.6—0.7 (diene) could be detected by spore germination assay. Our later experiments revealed that the other three compounds were only sparingly soluble in water and that it was not possible to detect their presence by slide germination assay technique. Solubility of the test material is a pre-requisite of this assay. There is also a slight possibility that the avocado varieties used by PRUSKY *et al.* in their experiment did not contain these three compounds. Experiments which were conducted to examine if challenge inoculation of fruit by the fungus results in an increase of these antifungal compounds gave negative results.

The IR spectrum of the antifungal compound AvII indicated the presence of hydroxyl (ϑ max 3650-3050) and ester carbonyl (ϑ max 1725) groups and C-H stretching (ϑ max 2860 and 2790 cm^{-1}). The ^1H NMR spectrum indicated the presence of an acetate group (δ 2.13) and olefinic protons (δ 5.63—4.90) in the compound. The spectral data of AvII compared well with those of the diene described by PRUSKY *et al.* (1982) and the compound was thus identified as cis-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene. This compound was previously reported by CHANG *et al.* (1975).

The IR and ^1H NMR spectral data of AvIV indicated the presence of hydroxyl group(s) (ϑ max 3675-3025 cm^{-1}) and a saturated long chain (δ 1.30). The presence of hydroxymethine and hydroxymethylene protons in the molecule was inferred from the multiplets at δ 3.93 and 3.60. The IR spectrum also indicated the absence of a carboxyl group in the compound. The mass fragments at *m/z* 268, 239 and 237 may be assigned to M^+, $M-C_2H_5$ and $M-CH_2OH$. This suggested that AvIV could be a saturated long-chain compound with hydroxyl group(s), which is not among those reported previoulsy from avocado plants (KASHMAN *et al.* 1969, NEEMAN *et al.* 1970). However, the structure of AvIV could not be deduced from the present data. Attempts made to purify the compounds AvI and AvIII were not successful and therefore their chemical structures could not be studied further.

Relative toxicity of the four compounds to *C. gloeosporioides* was estimated by determining the amount required to bring about an inhibition area of 1 cm^2 on the TLC plates. Antifungal compounds AvII (diene) and AvIV were found to be more toxic to *C. gloeosporioides* than AvI and AvIII. The highest toxicity was found with the compound AvII (diene) which is about 2 times as toxic as AvIV and about 6.5 and 7.5 times as toxic as AvIII and AvI respectively.

The concentration of the four antifungal compounds which was low in the very young fruit increased gradually with fruit development and reached a maximum at harvest. A decrease in the concentration of all four compounds was noticed during fruit ripening and only traces of AvII (diene) and AvIV were detected in fully ripened fruits, at which time anthracnose lesions were prominent. Similar observations were made by PRUSKY *et al.* (1982) with respect to the antifungal diene, where the concentration of the diene, as high as 1200 $\mu g/g$ fr. wt.

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in the peel of unripe fruit, decreased to 120 μg/g fr. wt. during ripening. The decrease apparently varied with the cultivar. In the cultivar that was used in our experiments, the amount of AvII (diene) and AvIV in the fruit peel was about 920 and 780 μg/g fr. wt. respectively at harvest. These amounts decreased to about 52 and 64 μg/g fr. wt. respectively in the ripe fruit, at the time of visible symptom development. Compounds AvI and AvIII were found to be in high concentrations, 1300 and 1050 μg/g fr. wt., at the time of harvest and the presence of these compounds could not be detected in the ripe fruit.

PRUSKY *et al.* (1982) found that the concentration of the diene by itself was adequate to prevent the growth of the pathogen in immature fruit, i.e. a solution containing 790 μg/g caused total inhibition of germination of the pathogen. However, the situation here appears to be different in that the resistance of the fruit tissue is evidently due to all four antifungal compounds rather than a single compound. Whether or not the fungitoxicity of these compounds is additive has not yet been determined.

Considering the situation in the ripe fruit at the time of anthracnose lesion development, the peel tissue contains only 52 and 64 μg/g fresh weight of AvII and AvIV respectively and no detectable amounts of AvI and AvIII and these amounts are not sufficient to cause total or even partial inhibition of the fungus. This may be the factor that allows the pathogen to reactivate and develop progressively in the ripe fruit tissue.

The concentration of these compounds was higher in the underlying 2—6 mm deep layers of the fruit (this includes both peel and pulp tissue) than in the outer 2 mm layer of the fruit. The fact that the fungus in the anthracnose lesions is normally restricted to the peel suggests that the higher antifungal activity in the deeper tissues prevents the colonization of the fungus at deeper levels.

In conclusion these results further strengthen the hypothesis put forward by PRUSKY *et al.*, that the antifungal activity of the peel is the factor responsible for the development of latent infections in the unripe avocado fruit by *C. gloeosporioides*. It has also been shown that the latency in unripe avocado was not due to nutrient differences between the unripe and ripe fruit (PRUSKY *et al.* 1984) nor to the inadequate enzyme potential of the fungus (SIVANATHAN and ADIKARAM 1989).

The resistance of certain other fruits to fungal diseases has been attributed to preformed antifungal compounds, for example, ALLISON (1952) reported that the presence of a preformed fungitoxic compound, solanine, found in fungitoxic concentrations in tomato fruit, prevented the development of *Colletotrichum phomoides*, and another preformed antifungal compound, tomatine, present in high concentrations in the peel of green tomato, was inhibitory to mycelial growth of *Botrytis cinerea* (VERHOEFF and LIEM 1975).

This work was supported by grants from NARESA, Sri Lanka and IFS, Sweden. The authors wish to thank Dr B. M. R. BANDARA and W. M. WIMALASIRI for their help in the chemical analysis and Professor M. D. DASSANAYAKE for critical reading of the manuscript.

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ANTIFUNGAL COMPOUNDS FROM IMMATURE AVOCADO FRUIT PEEL

N. K. B. ADIKARAM,* D. F. EWING,† A. M. KARUNARATNE and E. M. K. WIJERATNE‡

Department of Botany; ‡Department of Chemistry, University of Peradeniya, Peradeniya, Sri Lanka; †School of Chemistry, University of Hull, Hull HU6 7RX, U.K.

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Key Word Index—*Persea americana*; Lauraceae; avocado; fruit peel; antifungal activity; 1,2,4-trihydroxyheptadec-16-yne; 1,2,4-trihydroxyheptadec-16-ene; 1-acetoxy-2,4-dihydroxyheptadec-16-yne; safynol.

Abstract—Three antifungal compounds isolated from the peel of immature Avocado fruit have been identified as 1,2,4-trihydroxyheptadec-16-yne, 1,2,4-trihydroxyheptadec-16-ene and 1-acetoxy-2,4-dihydroxyheptadec-16-yne, previously detected in extracts from avocado seeds.

INTRODUCTION

Avocado anthracnose, caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. is a major disease factor contributing to post-harvest rotting in avocado fruit. Unripe fruit show no evidence of incipient decay lesions but the decay process develops rapidly during ripening, indicating the presence of latent infection [1]. This characteristic behaviour has been attributed to the presence of a significant concentration of antifungal compounds in the peel tissue in the early stages of growth [2, 3]. These compounds suppress the vegetative growth of *C. gloeosporioides* but during ripening this natural antifungal activity is rapidly lost, presumably due to chemical or enzymatic degradation of the active species [4]. Detailed study of extracts of avocado peel tissue have shown that at least four antifungal fractions were present [3] but only one compound was fully characterised [2, 3]. This compound was a long chain alkadiene with a dihydroxyketone moiety at one end, isolated in the form of a monoacetate (1).

$$Me(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COCH_2CHOHCH_2OCOMe$$

1

These antifungal species in unripe avocado have been reexamined and we now report the characterisation of a further three active constituents of peel extract.

RESULTS AND DISCUSSION

Freeze-dried avocado peel was powdered and extracted with dichloromethane and this extract chromatographed on silica gel to afford four fractions with antifungal activity (as determined by our bioassay method [3, 5]). These four fractions had R_f values (ethyl acetate) of 0.78, 0.60, 0.54, and 0.42, similar to those reported previously [3] and are labelled AV(1), AV(2), AV(3) and AV(4) respectively. Fraction AV(4) was further purified by flash chromatography on silica gel, gel permeation chromatography on Sephadex LH 20 and finally preparative scale HPLC on a reverse phase column. This procedure produced two antifungal compounds AV(4a) and AV(4c) in the ratio 1:4. Species AV(4b) was only a trace component. A similar purification of fraction AV(3) afforded only one active compound AV(3a). These three compounds were white or off-white crystalline materials and were examined by ^{1}H NMR spectroscopy.

The presence of characteristic spectral features indicated that both compounds AV(4a) and AV(4c) were unbranched long chain aliphatic compounds containing a primary and two secondary methoxyl groups. By analogy with 1 the presence of the $-CHOHCH_2CHOHCH_2OH$ moiety was inferred. The essential difference between AV(4a) and AV(4c) was the presence of a terminal vinyl group ($CH_2{=}CH-$) in the latter and a corresponding alkynyl group ($CH{\equiv}C-$) in the former. Compound AV(3a) appeared to be the monoacetate of AV(4a). These structural assignments were confirmed by ^{1}H–^{1}H correlation spectroscopy and, in the case of AV(3a) (at *ca* 98%, the purest of the three compounds), by the ^{13}C and DEPT spectra. Thus AV(4a) was assigned the structure of 1,2,4-trihydroxyheptadec-16-yne (2), AV(4c) is 1,2,4-trihydroxyheptadec-16-ene (3) and AV(3a) is 1-acetoxy-2,4-dihydroxyheptadec-16-yne (4). The full NMR characterisation of 2–4 is given in Table 1. At this point, a literature search revealed that compounds of this type had been discovered by Kashman *et al.* [6] over 20 years ago in extracts of avocado seed or dried avocado fruit. The antifungal activity was unsuspected but the structures appeared to be firmly established.

$$CH_2{=}CH(CH_2)_{11}CHOHCH_2CHOHCH_2OH$$

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*Author to whom correspondence should be addressed.

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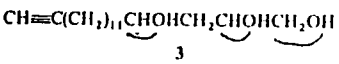

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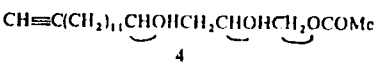

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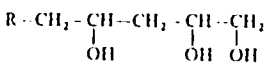
To confirm the chain length in compounds **2**–**4** the EI mass spectra were examined. The expected $[M]^+$ ion was not observed for **2** nor was the ion $[M-CH_2OH-H_2O]^+$ which is reported [6] to have an intensity of 63%. Furthermore the ion with *m/z* 209 $[M-CH_2CHOHCH_2OH]^+$ had an intensity of 3% compared to 62% reported by Kashman *et al.* [6]. Compound **3** showed similar results, the ion with highest mass being that at *m/z* 211 (4%). The acetate **4** had a 2% ion at *m/z* 209 $[M-CH_2CHOHCH_2OAc]^+$. Both these species are reported [6] to have EI mass spectra with a high intensity peak for the loss of the three carbon fragment from the hydroxylated end of the molecule. Our results were unchanged by variation in the electron acceleration voltage and fully reproduced on a different instrument. We are unable to account for the difference between our EI mass spectral results and those of Kashman *et al.* [6]. The CI mass spectra (with NH_3 as reactant gas) gave the expected results in all cases with peaks for $[M+H]^+$ and $[M+NH_4]^+$ and evidence for successive loss of three molecules of water.

We have previously established the presence of the dihydroxyketone (**1**) in avocado peel and demonstrated that it is the most potent of the fungitoxic fractions which were isolated [3]. Although the other active fractions obtained from peel extract were all less fungitoxic they had a similar activity vs concentration profile, showed a similar pattern of development in the peel tissue of ripening fruit and had a parallel behaviour in their distribution with respect to depth in the fruit with a maximum concentration at *ca* 5 mm. These strong similarities in general behaviour of all the fungitoxic fractions suggested a similar chemical structure and this has now been confirmed by the present results.

The most significant structural feature is the trihydroxy fragment which could be present in a precursor to all active compounds. If this structural feature is taken to be a five carbon fragment then the whole series of compounds can be represented as **5** or derived species.



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Thus the oxygenated fragment can occur as a triol, a triol acetate or a keto-diol acetate and the hydrocarbon residue corresponding to C_{12}-1-ene, C_{12}-1-yne, or C_{16}-6,9-diene. Their distribution in avocado plants is summarised in Table 2. The biochemistry of the antifungal activity of these compounds is unknown. The keto acetate **1** is the most active species [3] but compounds **2**–**4** have intrinsic activity and are not simply precursors or degradation products of a form similar to **1**. The significance of the length of the hydrocarbon chain or the type and degree of unsaturation has not yet been established but it is notable that the structural features of these avocado antifungal compounds are similar to those of safynol (**6**) and dehydrosafynol (**7**), phytoalexins from safflower (*Carthamus tinctorius*), which have a potent toxic effect on many fungi [8]. The oxygenated fragment in **6** differs from that in **2**–**4** only by the loss of one molecule of water.

Table 1. ^1H and ^{13}C NMR data for hydrocarbons **2**–**4***

Position	^1H 2	^1H 3	^1H 4	^{13}C 4
1	3.65 (3.5, 11.0)	3.64 *m*	4.14 *m*	68.66 *t*
1'	3.49 (6.5, 1.0)	3.48 *m*	4.00 (7.7, 12.2)	
2	3.98 *m*	3.97 *m*	4.10 *m*	72.63 *d*
3	1.58 *dd*	1.57 *dd*	1.6†	39.11 *t*
4	3.91 *m*	3.90 *m*	3.89 *m*	70.92 *d*
5	1.6	1.6	1.6†	38.23 *t*
6-13	1.26-1.45	1.26-1.45	1.26-1.45	25.39, 28.84, 29.19 29.57, 29.64
14	1.5	1.5	1.5†	
15	2.18 (2.2, 7.0)	2.04 *dt*	2.19 (2.2, 7.1)	18.49 *t*
16	5.82 *m*			68.13 *s*
17	1.94 (2.2)	4.99 (1.5, 18.0)	1.94 (2.2)	84.92 *t*‡
17'		4.33 (1.5, 7.0)		
OH-1, OH-2, OH-3	2.75 *br*	3.00 *br*	3.40 2.65	
CO				171.31 *s*
Me			2.11 *s*	21.00 *q*

*^1H NMR: 270.17 MHz, $CDCl_3$, TMS as internal standard; ^{13}C NMR: 67.94 MHz, $CDCl_3$, solvent at 77.1 ppm, implied multiplicities from DEPT spectrum.

†Established by a COSY spectrum.

‡Identified by the absence of a signal in DEPT spectrum set for $J = 130$ Hz.

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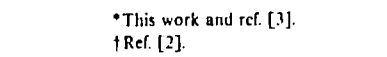
Table 2. Occurrence of fungitoxic derivatives of compound 5 in the avocado plant

	Triol		Triol-1-acetate		Keto-diol-1-acetate		
	C_{12}ene	C_{12}yne	C_{12}ene	C_{12}yne	C_{12}ene	C_{12}yne	C_{16}diene
Peel	*	*		*			*†
Seeds	‡	‡	‡	‡	‡	‡	
Leaves							§

*This work and ref. [3].
†Ref. [2].
‡Ref. [6].
§Ref. [7].

Extension of these structural features into the area of preinfective activity in avocado is significant.



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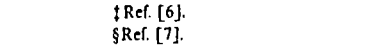

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EXPERIMENTAL

NMR spectra were obtained using standard conditions (32 K acquisition giving data point resolution of 0.24 Hz for ^{1}H and 1.2 Hz for ^{13}C spectra respectively). DEPT and COSY spectra were also obtained with standard conditions. Mass spectra were obtained on a Finnigan 1020 instrument (EI) and on a VG12-250 instrument (EI and CI).

Isolation of antifungal compounds. Immature fruit of *Persea americana* cv 'Green' were obtained as described previously [3]. The outer skin (1–2 mm thick) was peeled, bulked, freeze-dried and powdered. This material (479 g) was extracted with hexane, CH_2Cl_2 and MeOH under reflux conditions. Both the hexane extract (23.1 g) and the CH_2Cl_2 extract (25.2 g) gave dark green semi-solids which displayed antifungal activity. The latter, more active, extract was fractionated by CC (silica gel 250 g, Merck art 7734 ASTM). Sequential elution with hexane, EtOAc and MeOH gave 80 frs which were reduced to 12 by pooling on the basis of TLC behaviour. These were tested for antifungal activity (Table 3). The fraction labels AV(1)–AV(4) correspond to the four different zones of antifungal activity which were distinguished by our TLC assay (see below). Part of the AV(4) material (fr. 9, 2.0 g, active spot R_f 0.42), was further purified by flash chromatography (silica gel, 230–400 mesh, 30 g) with $CHCl_3$–MeOH (97:3) as eluant. Two frs were obtained and the less polar, more active one was passed through a Sephadex LH20 column ($CHCl_3$–MeOH, 3:7) to remove pigments. A solid was collected and subjected to prep HPLC on a μBondapack C_{18} column eluted with MeOH–H_2O (7:3) at a flow rate 4 ml min^{-1} using refractive index detection). Four species were detected: AV(4a), (14%, capacity factor, k' 3.5), AV(4b) (1%, k' 9.3), AV(4c) (68%, k' 11.7), AV(4d), (17%, k' 24). Only AV(4a) and AV(4c) showed antifungal activity.

Part of the AV(3) material (fr. 7, 1.6 g, active spot R_f 0.54) was further purified by flash chromatography (column as above, $CHCl_3$ as eluant). Four frs were obtained all showing some antifungal activity. The most polar fr. was separated by preparative HPLC (as above) into two components AV(3a) (62%, k' 9.6), and AV(3b) (38%, k' 23.1) of which only the former had antifungal activity.

Table 3. Distribution of antifungal activity in chromatographic fractions

Fraction number	Antifungal activity	R_f
1	—	—
2	—	—
3	+	0.78 AV(1)
4	+	0.60 AV(2)
5	—	—
6	+	0.54 AV(3)
7	+	0.54 AV(3)
8	+	0.42 AV(4)
9	+	0.42 AV(4)
10	+	0.42 AV(4)
11	+	0.42 AV(4)
12	—	—

Detection of antifungal activity. Antifungal activity was monitored throughout the purification process by a TLC bioassay [5]. Thus, aliquots of the extracts were spotted on to TLC plates (Kieselgel 254 PF60 Merck, 0.5 mm thick), the plates developed with EtOAc and air-dried overnight. The plates were then sprayed with a suspension of conidia of *Cladosporium cladosporioides* and incubated in a moist chamber for 2–3 days at 28°. The presence of compounds toxic to the fungus was indicated by a zone characterised by a lack of aerial mycelium.

1,2,4-*Trihydroxyheptadec-16-yne* (2). Mp 71–72°, EI/MS 70 eV *m/z* (rel. int.) (below *m/z* 121 only major peaks are given): 253 [M − CH_2OH]$^+$ (2) 235 [M − CH_2OH − H_2O]$^+$ (2), 209 [M − $CH_2CHOHCH_2OH$]$^+$ (4), 149 (4), 135 (7), 123 (3), 121 (8), 109 (15), 105 (22), 95 (31), 87 (100), 81 (38), 69 (38), 67 (43), 55 (59), CI/MS (NH_3) *m/z* (rel. int., only peaks above *m/z* 230): 302 [M + NH_4]$^+$ (25), 285 [M + H]$^+$ (45), 267 [M + H − H_2O]$^+$ (9), 249 [M + H − $2H_2O$]$^+$ (7), 231 [M + H − $3H_2O$]$^+$ (2).

1,2,4-*Trihydroxyheptadec-16-ene* (3). Mp 64–65°, $[\alpha]_D^{21}$ −3.5° ($CHCl_3$; c 2.0); EI/MS 70 eV *m/z* (rel. int.) (below *m/z* 121 only major peaks are given): 255 [M − CH_2OH]$^+$ (2), 237 [M − CH_2OH − H_2O]$^+$ (3), 219 (4), 211 [M − $CH_2CHOHCH_2OH$]$^+$ (5), 149 (2), 135 (3), 123 (5), 121 (4), 109 (16), 105 (16), 95 (35), 87 (100), 81 (33), 69 (52), 67 (39), 55 (78); CI/MS (NH_3) *m/z* (rel. int., only peaks above *m/z* 230): 304 [M + NH_4]$^+$ (25), 287 [M + H]$^+$ (33), 269 [M + H − H_2O]$^+$ (17), 251 [M + H − $2H_2O$]$^+$ (3), 233 [M + H − $3H_2O$]$^+$ (3).

1-*Acetoxy-2,4-dihydroxyheptadec-16-yne* (4). Mp 69–70°, $[\alpha]_D^{21}$ −7.0° ($CHCl_3$; c 1.0); EI/MS 70 eV *m/z* (rel. int.)

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(below *m/z* 121 only major peaks are given): 253 $[M-CH_2OAc]^+$ (1), 235 $[M-CH_2OAc-H_2O]^+$ (2), 209 $[M-CH_2CHOHCH_2OAc]^+$ (2), 147 (25), 135 (6), 129 (9), 121 (8), 117 (9), 109 (12), 100 (32), 95 (28), 87 (100), 81 (37), 69 (38), 67 (38), 55 (49); CI/MS (NH_3) *m/z* (rel. int., only peaks above *m/z* 230): 344 $[M+NH_4]^+$ (23), 327 $[M+H]^+$ (63), 309 $[M+H-H_2O]^+$ (63), 285 $[M+H-MeCO]^+$ (5), $[M+H-MeCO_2H]^+$ (5), 249 $[M+H-2H_2O]^+$ (5), 231 $[M+H-3H_2O]^+$ (3).

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6. IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

By means of comparative biochemical approaches, we have elucidated putative defense mechanims controlling postharvest disease in unripe avocado fruits. Levels of the avocado diene and monoene were high in unripe, disase-resistance fruit but decreased during fruit ripening, a time when latent fungal infections cause proliferating lesions that make fruit unmarketable. Such correlative evidence, however, does not prove a role for the diene compound in resistance. To provide more critical test we employed various exogenous treatments that modulated the normal disapearance of the diene during the ripening of avocado fruits and observed wheter the treatment concomitantly affected the development of fungal decay. The most effective measures were antioxidant and high carbon dioxide treatments, which inhibited decreases of the avocado antifungal dicne compound during ripening or induced accumulation of higher levels. The fact that modulation of diene levels by these agents led to the predicted changes in fruit resistance constitutes important evidence that the diene is a physiologically important agent affecting resistance to *C. gloeosoporioides.*

Treatments such as antioxidants affect levels of preformed antifungal compounds may be used for practical disease control. As described in one of the ms, this treatment is currently beeing tested in

Israel for the prevention of postharvest diseases during storage and shipment. Increasing environmental pressure for reduced pesticide usage encourages experimentation to determine if levels of preformed antifungal agents in fruits of other plants can be similarly modulated to prevent postharvest disease.

This project has developed a new way for the control of postharvest disease that should further be tested in several developing countries. Dr.Adikaran scientific capabilities have broaden since he has done very important finding on the chemistry, analysis of new antifungal compounds in avocado. Further more he has been exposed to new aspects of the biochemistry of resistance of unripe fruits and new aspects in the same direction are being studied in his lab at present time.

7. PROJECT ACTIVITIES

The scientists of this project have met twice as proposed by the project activities. Furthermore the close friendship developed by the two scientist have resulted in several other meeting during international conferences like in International Plant Pathology Meeting in Kyoto, Japan and in Montreal, Canada. During Dr. Adikaran visit in Israel, he was able to check our laboratory methods and the use of the antioxidants.

The list of published ms of this proposal is very significant and represent the basis for the study of resistance of unripe fruits to postharvest diseases.

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8. PROJECT PRODUCTIVITY

The project has achieved most of his goal. The project has described new antifungal compounds, the are involved in the resistance to postharvest diseases. It has been described the possible regulation or modulation of the natural resistance and we have resulted in a commercial treatment that should be further tested in order to be included in a packinghouse treatment.

9. FUTURE WORK

The project has resulted in the development of a new idea, the use of treatment with high concentration of CO2 as a possible stress for the induction of fruit resistance and it is presently being studied.