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Physiology and Prevention of Latent Infections in Fruits

D. Prusky, N.T. Keen, B. Jacoby

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1. ABSTRACT

Antifungal compounds were found to be involved in the latency of Colletotrichum and Alternaria in unripe avocado and mango fruits, respectively. In avocado, where more extensive work has been done, a preformed compound was isolated from peel of unripe avocado fruits and identified as 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene. The compound inhibited vegetative growth of Colletotrichum gloeosporioides with an LD₅₀ of 450 ug/ml. Concentrations of the diene in peels of unripe fruits were as high as 1200 ug.g⁻¹ fresh weight of peel (1600 ug ml⁻¹), but these decreased during ripening to about 120 ug.g⁻¹ fresh weight of peel (160 ug ml⁻¹). Concentrations of the compound decreased differentially during ripening of two cultivars of avocado fruits that differed in rates of development of symptoms caused by C. gloeosporioides. The purified diene was oxidized by lipoxygenase extracted from avocado and by soybean lipoxygenase. The specific activity of lipoxygenase in peel extracts also increased by 80% during the climacteric stage, coincident with a rapid decrease of the diene in fruit peel, and before symptoms of C. gloeosporioides infections were observed. Infiltration of fungus-inoculated fruits with α -tocopherol acetate partially eliminated reduction of the diene compound during fruit ripening and fungus development in the peel was delayed. On the other hand, infiltration with 0.25 CaCl₂ prevented softening of the fruits, but did not affect the decrease of the diene compound or decay of the peel. The evidence supports the hypothesis that the preformed antifungal compound causes C. gloeosporioides to remain latent in unripe avocado peel and that subsequent active infections result from metabolism of the diene during ripening, probably mediated by lipoxygenase.

A method was developed for the assessment of latent infections of Alternaria in mango fruits. A high correlation was observed between the latent Alternaria infections at harvest time and the incidence of black spots that developed on the fruit during storage. This suggests the possibility of using preharvest assessment as the basis for a postharvest control program in Israel.

2. EXPERIMENTAL

2.1 LATENT INFECTION ASSESSMENT

2.1.1 Development of a method for pre-harvest assessment of latent Alternaria infections in mango fruits.

2.1.3 Assessment of latent infections for determining the need for post-harvest treatment in different regions of Israel.

Alternaria sp. is usually known to be affected significantly by dew and irrigation regimes, resulting in increased incidence of disease (3). Mango fruits are grown in Israel at different climatic regions. In order to determine the effect of the growth region on the incidence of latent infection of Alternaria alternata and the need of post-harvest treatments, assessment of latent infections was done on fruits from orchards in different climatic conditions.

Materials and Methods

Assessment of latent infections by Alternaria in naturally-infected mango fruits from trees at harvest was made according to the method described by Prusky et al. (1,2). Ten fruits from each orchard were sampled for latent infections assessment just before commercial harvesting. The orchards included in the survey were: 1. Southern desert: Samar, Yotvata, Ein-Gedi; 2. Southern Coastal Plain: Tzelim, Nir Itzjak, Kerem Shalom, Gvulot, Mivtachim; 3. Central Coastal Plain: Netzer Sireni, Maabarot, Mishmar Hasharon, Bnei Dror; 4. Southern Kinneret and Beit-Shean: Ramot, Ein-Gev, Hamadia, Reshafim, Nir David, Beit Hashita.

Results and Discussion

The relative latent infected area of fruits of the four different sample regions indicated (Table 1) that fruit being grown in the southern and central coastal plain are heavily infected by Alternaria. Fruit from the southern desert is almost free from latent disease, and fruit grown in

the inner valleys have a relative low level of latent disease. One main factor is common in the regions of high infected fruit - long dew periods. However, no significant differences concerning humidity are seen in the Beit Shean valley in relation to the coastal plain. Higher temperatures might affect the period with free water, possibly resulting in the lower incidence of the disease. The low latent disease incidence present in the southern desert region clearly indicated that dry conditions prevent Alternaria infections. The experiments have identified some of the regions of mango culture where decay problems will likely arise if no post-harvest treatments are applied to the fruits. Fruits of the southern Kinneret and Beit Shean, in addition to the southern deserts, showed a relatively infected area lower than 5 (2). Thus, post-harvest treatments are not needed for these regions. However, mango fruits from the southern and central coastal plain should be treated with fungicide dips to prevent post-harvest decay by Alternaria.

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Table 1

Occurrence of latent infections of Alternaria alternata in mango fruit cv. Haden at different regions in Israel.

<u>Region in Israel</u>	<u>Location of orchard</u>	<u>Latent infected area</u> (cm ²)
Southern Desert	Samar	0
	Yotvata	0.04
	Ein Gedi	0
Southern Coastal Plain	Tzeelim	36.52
	Nir Itzjak	27.29
	Kerem Shalom	26.65
	Gvulot	26.29
	Mivtachim	34.35
Central Coastal Plain	Netzer Sireni	17.08
	Maabarot	37.33
	Mishmar Hasharon	14.70
	Bnei Dror	23.24
Inner Valleys (Kinneret, Beit Shean)	Ramot	0.26
	Ein Gev	0.62
	Hamadia	0.64
	Reshafim	0.06
	Nir David	0.09
	Beit Hashita	0.37

2.2 MICROSCOPIC STUDIES OF LATENT INFECTIONS

2.2.1 Light and scanning electron microscopic observations of latent infections of Colletotrichum in avocado fruits.

Avocado anthracnose, which is caused by Colletotrichum gloeosporioides Penze, is the most important fruit-rotting disease of avocado in many countries. C. gloeosporioides infects unripe avocado fruits during the growing season through appressoria (1). However, these infections remain latent as non-germinated appressoria on the cuticle until the fruit ripens. In this work, light microscopic observations were used to describe the latent stage of Colletotrichum in avocado as infection hyphae and not appressoria as suggested in the past (1).

Materials and Methods

Avocado fruits (Persea americana) cv. Fuerte were used for inoculation of C. gloeosporioides and further microscopic observations. A single spore isolate of Colletotrichum from a decayed avocado fruit was used in all the experiments. Cultures were kept on PDA at 25°C. For fruit inoculation, conidia were harvested by adding 1-2 ml of sterile distilled water and gently rubbing the sporulating mycelial mat with a bent glass rod. The suspension of conidia was decanted into sterile distilled water containing 0.1% polyoxyethylene sorbitan monolaureate (Tween 20) as a wetting agent to prevent spore clumping and adjusted to 10^6 conidia per ml. Unharvested, non-wounded fruits in the orchard were inoculated by placing a drop of conidial suspension of the center of brown circle on the surface. After inoculation the fruit were covered carefully with polyethylene bags and incubated in this way for 3-4 days. Fixation and preparations of specimens

for light and scanning electron microscopy was done similarly to the methods used by Prusky in former work (4).

Results and Discussion

Conidia of Colletotrichum germinate and produce a globular rough appressoria of 20-30 um length, much bigger than the Colletotrichum conidia and easy to differentiate because of different shape (Figs. 1-3). One or more appressoria were observed to develop from one germinating hypha (Figs. 4-6). Light microscopic observations indicated the presence of ungerminated appressoria with a small infection peg in the cuticle of avocado fruits (Fig. 7). Most of the microscopic observations indicated that appressoria were able to germinate in unharvested fruit and develop freely in the cuticle (Figs. 8-12). However, no further development of the hyphae was observed when the cell wall was reached. This result suggests that the latent stage of Colletotrichum is not as an ungerminated appressoria as was described by Biniyamini and Schiffman-Nadel, but as a dormant subcuticular hyphae. In citrus, Brown (2) and in banana, Muirhead (3), have located sub-cuticular hyphae in green fruits but these did not resume activity during ripening. According to Muirhead, latency is complete depending on different factors affecting the appressoria. Our observations indicate inhibited sub-cuticular hyphae, possibly resulting from the presence of antifungal compounds. This work will be discussed in the next chapters.

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3. Muirhead, I.F. 1981. The role of appressorial in latent infection of banana fruits by Colletotrichum musae. Physiological Plant Pathology 15:331-340.
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Legends to Figures

Figs. 1-6 Scanning electron microscopy of appressoria of Colletotrichum gloeosporioides on avocado pears cv. Fuerte. Different examples of conidial (c) germination and production of appressoria (ap). The white line on the picture represent 10 um.

Figs. 7-12 Light microscopy observation of penetration of Colletotrichum gloeosporioides to avocado peel of cv. Fuerte pears.

- 7 Appressoria germination (ap) and hyphal peg production x 2000
- 8 Infection hyphae development on avocado cuticle (arrows) x 2500
- 9-12 Development of Colletotrichum on the cuticle up to cell wall of avocado fruits. Figs. 9, 11 and 12, x 2000. Fig. 10 x 2500.

2.3 CAUSES OF LATENCY OF COLLETOTRICHUM GLOEOSPORIOIDES ON UNRIPE AVOCADO FRUITS

Latent fungal infections on unripe fruits have been observed in many host-pathogen interactions. These may result from insufficient enzyme production to invade unripe fruits, nutritional requirements of the pathogen that are only supplied during fruit ripening or to induce and preform antifungal compounds present in unripe, but not ripening, fruits.

2.3.1 Lack of involvement of nutrients in the latency of Colletotrichum gloeosporioides in unripe avocado fruits.

The present work describes a method to prolong the latency period of C. gloeosporioides in harvested avocado fruits by inhibition of fruit ripening. The method was applied to investigate the possibility that latency is caused by the lack of available substrates.

Materials and Methods

Avocado Persea americana Miller var. drymifolia (Schlect. and Chamb.) cv. Reed fruits were picked in an orchard in Rehovot, Israel. Discs, 22 mm diameter and 7 mm thick, were randomly sampled from just harvested fruits. The discs were disinfected with 1% NaOCl for 2 min and further washed twice with sterilized water. Six discs were then placed, peel side up, on petri dishes. A 2% agar solution in 0.2 M potassium phosphate buffer, containing 0.025% synthomycetin and 10 ug/ml thiabendazole, was added up to half the height of the discs in all experiments.

A single spore isolate of C. gloeosporioides from an avocado fruit was used in all the experiments. The isolate was kept on potato dextrose agar (PDA) at 25°C and its continuing pathogenicity was tested by inocu-

lating soft avocado fruits once a month. The effects of various treatments on fungal growth on fruit discs or on PDA as substrate were tested as follows: petri dishes containing PDA were inoculated with small agar discs (5 mm diameter) taken from the edge of a colony containing both mycelia and spores, which had been inoculated 8 days earlier. Mycelial growth was determined by daily measurement of colony diameter. Fruit discs were inoculated by placing a 10 ul drop of a spore suspension (ca. 5.6×10^5 spores per ml) at the center of each avocado disc. High humidity, optimal for infection, was maintained in the petri dishes; darkening of the peel at the inoculation site, measured under a Zeiss binocular, indicated disease development.

Softening of avocado discs during ripening was determined by recording the force (kg) required to penetrate the flesh with a conic probe - 5 mm diameter and 7 mm length. The average value from six determinations on discs from different petri dishes is reported. Experiments were repeated at least 4 times.

In order to investigate the effect of hypobaric pressures, petri dishes containing PDA cultures of the fungus, or infected and uninfected avocado discs were placed in 3-L glass, hermetically-closed containers which were evacuated continuously by means of a vacuum pump. A water-saturated air flow of 0.2 L per min was admitted to the containers through a Matheson Model 45 vacuum regulator which maintained the selected pressures. Avocado discs and fungal cultures held at atmospheric pressure (760 mmHg) were used as controls. The temperature was kept at 20°C in all experiments.

Results

The growth of C. gloeosporioides on PDA when measured 9 days after inoculation, was not significantly inhibited by decreasing the air pressure to 500 mmHg (Fig. 1). At lower pressures, down to 200 mmHg, growth was gradually reduced to 79% of the control (760 mmHg). A further decrease of pressure resulted in drastic inhibition of fungal growth, supposedly due to anoxia; at 50 mmHg it was reduced to 40% of the control. In contrast, C. gloeosporioides development on avocado discs, measured also 9 days after inoculation, was reduced to 35% of the control at 500 mmHg and total inhibition was obtained at pressures of 200 mmHg and less. Softening of avocado discs measured as firmness on the 7th day after harvest was also inhibited by hypobaric pressures. The inhibition of softening increased with decreasing air pressure (Fig. 1). The optimal pressure chosen was 150 mmHg because under this condition fungal growth on PDA showed little inhibition, while almost complete inhibition of fungal development was observed on avocado discs (Fig. 2).

The method was employed to assess the effect of adding available nutrients on susceptibility of avocado to C. gloeosporioides. Avocado discs were vacuum infiltrated with solutions containing essential minerals and either glucose 10 mM, sucrose 50 mM or 2% polygalacturonic acid. The infiltration was done by immersing the discs in the solutions and reducing the pressure to 45 mmHg for 90 sec. The discs absorbed 3.2 ± 0.1 g solution per 100 g fr. wt. After infiltration discs were disinfected and inoculated as described in Materials and Methods, incubated at 20°C for 6 h and then inoculated and not-infiltrated avocado discs were transferred to a hypobaric pressure of 150 mmHg. None of the nutrient treatments affected fungal development; they all behaved like the controls.

A slow stream of water saturated air at hypobaric pressures was employed to prolong the latency of C. gloeosporioides infection on avocado fruit discs. This treatment inhibited ripening of the discs, probably as a direct result of low oxygen pressure and because the ripening and senescence hormone, ethylene, escaped from the tissues, thus greatly retarding its action (2). At 150 mmHg fungal development on avocado discs was completely prevented, while fungal growth on PDA was affected only slightly. The inhibition of C. gloeosporioides development on avocado discs by reduced air pressures is thus probably a result of the delayed ripening process.

The method was used to test the hypothesis of nutritional limitations suggested as one of the possible explanations for latent infections (5). Results indicated that the lack of available nutrients is apparently not the cause of C. gloeosporioides latency in unripe avocado. This supports the hypothesis that a preformed antifungal compound present in unripe fruits, which decreased during ripening to subtoxic levels, confers resistance to C. gloeosporioides (3,4).

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Fig. 1 Effect of different air pressures on the development of C. gloeosporioides on discs of avocado fruits cv. Reed, and on potato dextrose agar 9 days after inoculation at 20°C. Firmness of uninfected discs was determined on the 7th day after the experiment started. o---o, diameter of infection on avocado discs; Δ---Δ, firmness; o---o, diameter of fungal growth on PDA. Firmness data is the average of 6 measurements of one single experiment.

Fig. 2 Effect of reduced atmospheric pressure of 150 mmHg on development of C. gloeosporioides on discs of avocado fruits cv. Reed, and on potato dextrose agar, and on firmness of uninfected avocado discs after 8 days at 20°C. Firmness data is the average of 6 measurements of one single experiment.

Fig 1- Prusky et al.

Fig 2- Prusky et al

2.3.2 Involvement of a preformed compound in the latency of Colletotrichum gloeosporioides as unripe avocado fruits.

2.4 CAUSES FOR THE LATENCY OF ALTERNARIA ALTERNATA IN MANGO FRUITS

2.4.1 Isolation and purification of antifungal compound possibly involved in the latency of Alternaria in unripe mango fruits

Alternaria alternata (Fr.) Keissler has been described (11) as the causal organism of the black spot disease in mango fruit Mangifera indica L. The fungus penetrates the fruit through lenticels. After infection in the orchard the hyphae remains latent intercellularly until fruit harvesting. After ripening, the fungus renews its development intercellularly resulting in browning of intercellular space and cell death.

Alternaria decay symptoms have been described for stored oranges and lemons (3), persimmons, blueberries (5,10), apples (4) and tomatoes (2). Latent fungal infections on unripe fruits have been observed in many host-pathogen interactions (19); these may result from insufficient enzyme production to invade unripe fruits (16), nutritional requirements of the pathogen that are only supplied during ripening (9) and preformed (9,17, 20) or induced (18) antifungal compounds present in unripe, but not ripening, fruits.

The aim of our research was to determine if any antifungal compounds were present in unripe mango fruits which may have some role in the latency of A. alternata in mango fruits.

Materials and Methods

Fruit Material and Extraction Procedure

Fruit material was obtained from trees of Mangifera indica L. cultivars Tommy atkins, and Haden, all over Israel. Fruits were harvested at a green-mature stage, 300-350 g weight, without any colour development; stage

that in commercial conditions the fruits are picked. Fruit peel tissue 1-2 mm thick was homogenized in 95% ethanol (150 ml per 50 g fresh wt. tissues) with a Sorval Omnimixer for 5 min preventing heating of the vase. The extract was filtered and concentrated at 40 in vacuo (x3).

The concentrate was partitioned twice with equal volumes of dichloromethane. The organic layers were pooled together, dried with anhydrous MgSO_4 , concentrated to dryness in vacuo, dissolved in ml of 100% ethanol and used as a crude extract.

Isolation and Purification

The biologically active compound was isolated from the crude extract by flash chromatography on a column of silica gel 60h (15-40 μm , Merck) in a 8.5 cm diameter Buchner funnel with a fritted disk. The silica gel was poured into the funnel to a depth of 6 cm packed and run in vacuo. The column was pre-equilibrated with hexane. The crude extract (50 g fr. wt. tissue) dissolved in hexane, was applied to the column and different fractions were eluted by: 100% hexane, 25% dichloromethane in hexane, 50% dichloromethane in hexane, 100% dichloromethane, 12.5% ethyl acetate in dichloromethane, 25% ethyl acetate in dichloromethane. The active compound was eluted with 25% ethyl acetate in dichloromethane, dried in vacuo and dissolved in 1 ml of 100% ethanol.

The compound was further purified by high pressure liquid chromatography (hplc) on a Varian model 5000 liquid chromatograph fitted with a 25x1 cm Merck, 10 RP-18 column, eluted with 90% methanol in water. The biologically active peak was collected, dried in vacuo and re-chromatographed in the same system. The hplc column was monitored with a variable wavelength U.V. detector (model U.V.-50) and the appropriate peak was detected at 275 nm.

Quantitative determination and identification of the antifungal compound

The highly purified compound obtained from the reversed phase hplc column was examined by NMR, U.V. and mass spectrometry. For quantitation of the antifungal compound, 10 μ l aliquots of 1.0 ml of concentrated flash chromatographed material of crude extract obtained from 50 g fr. wt. of mango peels was chromatographed on the reversed phase hplc column as described above. Known amount of purified antifungal compound were used as standards in separate runs.

As an external standard, aliquots of crude extracts were run before and after column chromatography and compared to separate runs of the purified compound. Efficiency of the column was 95-100%.

Calculations of concentrations of the antifungal compound were based on the peak heights of the compound. The highly purified compound obtained from reversed phase column was examined by NMR and U.V. spectroscopy. Absorption spectrum were taken in 100% ethanol for the flash chromatographed material. Calculation of concentration of antifungal compound were done as well by measuring the absorbance of the semi-purified compound at 275 nm compared to known amounts of the purified antifungal compound.

Detection and bioassay of the antifungal activity

Crude extracts were applied to thin layer chromatography (tlc) plates (0.375 mm, Silica gel GF 254, Merck) were subsequently developed with hexans:ethyl acetate:methanol (60:40:1 v/v/v). The detection of antifungal substances at different R_f values on tlc plates was done in two ways: 1) by the method of tlc bioassay using Cladosporium cucumerinum and A. alternata as assay organism, and 2) by scrapping various zones of the silica gel from the plate, extracted with ethylacetate, concentrated in

vacuo and dissolved in 100% ethanol (20 $\mu\text{l g}^{-1}$ fr. wt. tissue initially extracted). For the bioassay of the possible inhibitory fractions - a glass slide bioassay was used (13). Fifty μl of crude fruit extracts, partially or fully purified, dissolved in ethanol 95% were spotted on 13-mm diameter Millipore filters (0.45 μm pore size, Millipore Co.) and further placed on glass slides. After removal of the ethanol by drying, 8 μl of a suspension of spores of A. alternata (10^6 ml^{-1}) in ethanol containing 0.05% polysorbate 20 (Tween-20) and 0.0015% dimethyl sulfoxide (DMSO) were placed on the disc and the slides were incubated in a moist chamber (petri dishes) for about 8 h at 25°C . Activity of mango compound was determined by comparing the percent of inhibited germ tubes, with the control without crude extracts or purified mango compound. Germ tube elongation was considered to be inhibited when length was less than two-fold of the average spore length. A single-spore isolate of A. alternata from decayed mango fruit was used in all experiments. The isolate was kept on potato-dextrose agar at 25°C and pathogenicity was tested by inoculating mango fruits.

Dose response curve

A suspension of spores of A. alternata was prepared in a solution containing 0.05% polysorbate (Tween-20) and 0.0015% dimethylsulfoxide (DMSO). Different amounts of the highly purified antifungal compound dissolved in 100% ethanol was included into the spore suspension to give the needed final concentration at 5% ethanol. Spore suspension without antifungal compound was used as control. Ten μl of suspension of spores from every concentration were spotted on 13-mm diameter Millipore filters, placed on glass slides and incubated in a moist chamber (petri dishes) for about 8 h. Percent of inhibited germ tubes and percent of germination was compared

to germ tube elongation and percent of germination in the control.

In-vivo bioassay of antifungal activity

Unripe (1 day after harvest) and ripe (34 days after harvest) mango fruits cultivar Tommy Atkins were used for the in vivo bioassay. From an ideal square (1x1 cm), three slides were sliced (1 mm depth) and part of the fruit peel was gently cut (1 mm thick) leaving the square peel attached in one side to the fruit. Eight μ l of a spore suspension of A. alternata (10^6 sps.ml⁻¹) in 5% ethanol containing 0.05% polysorbate 20 (Tween-20) and 0.0015% dimethylsulfoxide (DMSO) were placed on 13-mm diameter Millipore filters, which were placed carefully afterwards spore-side up in close contact with the mango peel and sealed by the peel with cello tape. The fruits were kept under room temperature for about 8-10 h. Termination of fungal growth was done by fixation of the fungus on the Millipore disc with cotton blue in lactophenol. Percent of inhibited germ tubes was compared between fruits at different ripening stages in relation to germ tube elongation of conidia germinating on a moistened disc.

Fruit inoculation

Fruit were inoculated with a suspension of spores of A. alternata (5×10^5 ml⁻¹) in sterilized-distilled water, which was prepared from one week old culture of A. alternata obtained from decayed mango fruit. Inoculation was done by pricking fruits with a needle, 2 mm depth, in six sites per fruit and then placing 8 μ l spores suspension on every wounded zone. After inoculation the fruits were held at 25°C in a moist chamber for 24 h, and then stored at 20°C. Eight μ l drops of sterile distilled water placed in pricked sites on the fruit were used as a control. Disease expression of the infected site was considered when the black symptoms

develop over 2 mm diameter observed under a Zeiss binocular microscope. If not specifically described, appearance of symptoms in mango fruits was considered when 50% of the infected sites showed disease expression.

Hypobaric pressure, ethylene and calcium chloride treatments of freshly harvested fruits

Mango fruits cv. Tommy Atkins were inoculated as described above and 24 h later several treatments that affect ripening of the fruit were applied. For hypobaric treatment, mango fruits were placed in 3-L glass containers at 20°C where the air was continuously evacuated by means of a vacuum pump. A water saturated air flow of 0.2 L per min was admitted to the containers through a Matheson Model 45-vacuum regulator which maintained the selected pressure at 150 mmHg. Fruit was stored under these conditions for 7 days and then transferred to atmospheric pressure for the remainder of the experiment. Mango fruits, held continuously at atmospheric pressure, 760 mmHg, were used as controls. Ethylene treatment was applied to fruits placed during 72 h at 25°C in a polyethylene tent where a continuous stream of ethylene at 100 $\mu\text{l/ml}$ was introduced. Fruits were then transferred to 20°C during the rest of the experiment. For CaCl_2 treatments fruits were vacuum infiltrated after totally immersing them in a solution of 0.35 M CaCl_2 . The average amount of solution infiltrated was 1 g.100 g⁻¹ of fruit. The fruits were inoculated after infiltration and kept in a moist chamber for 24 h at 20°C. Freshly harvested mango picked at a mature green stage became fully ripe with total colour development within 12 days when stored at 20°C. The effect of the former treatments on fruit ripening was determined by recording symptom expression on the disease in a binocular, as described before. Parallely, 50 g of peel from representative fruits at different stages of symptoms expression were

sampled and the mango antifungal compound was extracted and quantitated as described above.

Effect of hypobaric pressure on in vitro development of A. alternata was tested after inoculating potato dextrose agar (PDA) plates, with 5 mm diameter discs sampled from the edge of one-week-old colony of A. alternata and placing the plates with the treated fruits. Petri dishes seeded with the fungus held at atmospheric pressure were used as controls.

Results

Changes in susceptibility of mango fruits after ripening to Alternaria development

Inoculation of cv. Tommy Atkins mango fruits at increasing periods after harvest resulted in more rapid symptom expression. The incubation period was reduced from 19, 14, 12 to 10 days when fruits were inoculated 1, 4, 8 and 12 days after harvest, respectively. Twelve days after harvest at 20°C fruits were fully coloured and ripe. Inoculation of fruits at 18 days after harvest showed a similar, short incubation period of 2-4 days (Fig. 1). Susceptibility to Alternaria development was also assessed in a in vivo test where inhibition of elongation of germinated conidia in close contact with fruit peel was checked. Unripe mango fruits caused 87.3% inhibition of germ tube elongation of germinated Alternaria spores, while fully coloured, ripe fruits showed 29% inhibition (Table I).

Isolation and purification of a biological active compound

Developed tlc plates with crude extracts from ripe fruits of cvs. Tommy Atkins and Haden and sprayed with either Calldosporium sp. or Alternaria showed biological activity at R_f 0.46. The antifungal compound (s) could

also be detected as a brown or blue spot that developed after the plates were sprayed with 50% H_2SO_4 or 3.5% phosphomolybdic acid and heated to 110°C for 10 min and 20 min respectively. The active fraction obtained by flash chromatography was further purified by hplc on a Lichrosorb RP-18 column and it appeared after 27 min (Fig. 2) at a flow rate of $3 \text{ ml} \cdot \text{min}^{-1}$.

Effect of the antifungal compound on *A. alternata*

The ED_{50} of the purified compound for inhibition of germ tube elongation was $100 \text{ ug} \cdot \text{ml}^{-1}$. Spore germination was less sensitive than germ tube elongation. At concentrations ranging between 50 and $300 \text{ ug} \cdot \text{ml}^{-1}$, inhibition of spore germination ranged between 11 and 28%, respectively.

Occurrence of the antifungal compound in mango peels at different stages of ripening and its possible relation to symptom expression

The concentration of the antifungal compound calculated by UV absorbance varied during 1-12 days after harvest between 262 and $286 \text{ ug} \cdot \text{g}^{-1}$ fr. wt. tissue of peel in unripe cultivar Tommy Atkins fruits. At 22 days after harvest, the concentration decreased to $160 \text{ ug} \cdot \text{g}^{-1}$ fr. wt. and 31 days after harvest the concentration of the compound was $32.7 \text{ ug} \cdot \text{g}^{-1}$ fr. wt. (Fig. 4).

Concentrations of the antifungal compound calculated by hplc analysis showed that the concentration of the antifungal compound at 1-12 days after harvest varied between 195.3 and $160.8 \text{ ug} \cdot \text{g}^{-1}$ fr. wt. of tissue of peel. Seventeen days after harvest, the concentration decreased to 112.0 ug and 31 days after harvest the concentration of the antifungal compound was $101.2 \text{ ug} \cdot \text{g}^{-1}$ fr. wt. (Fig. 5). Symptoms of disease were evident at 18-19 days after harvest. The biological activity of the partially purified extracts decreased from 90% in freshly harvested fruits to 47% inhibition

of hyphal elongation at 17 days after harvest and further decreased to 19% when the partially purified extract was tested 31 days after harvest. These results indicated a relation between the concentration of the antifungal compound, symptom expression and the inhibitory activity of the partially purified extracts by flash chromatography.

The relation between the concentration of the antifungal compound and the differential susceptibility of mango cultivars

The compound was extracted from peels of mango fruits, cultivars Tommy Atkins and Haden, at different stages after harvest. The preparations from cultivar Haden yielded identical hplc retention time to those obtained from cultivar Tommy Atkins. The R_f value and the uv spectrum were identical to the compound obtained from Tommy Atkins.

The concentration of the antifungal compound had decreased when decay symptoms were fully developed in cultivars Tommy Atkins (T.A.) and Haden (Table 2). First symptoms were seen in cultivar T.A. 18 days after harvest and full symptom development checked 28 days after harvest. The concentration of the antifungal compound decreased from 203.8 to 97.4 $\mu\text{g g}^{-1}$ fr. wt. Biological activity of the extracts decreased during the same period from 87 to 33% elongation of hyphal growth. In cultivar Haden first symptoms developed 25 days after harvest and full decay symptoms were observed 31 days after harvest. In this case a delay in the start of decrease of the antifungal compound is observed until 17 days after harvest and from then on a significant decrease of the compound from 222 to 110 $\mu\text{g g}^{-1}$ fr. wt. was observed when 100% of the inoculated sites showed decay symptoms. Biological activity of the extracts obtained from cv. Haden decreased during 31 days from 92 to 35% inhibition of germ tube elongation (Table 2).

Effect of ethylene, hypobaric pressure and CaCl_2 treatments on the occurrence of the antifungal compound in mango fruits

The concentration of antifungal compound decreased in ethylene-treated fruits of cv. Tomato Atkins, from $195\text{-}108\text{ ug g}^{-1}$ fr. wt., during 12 days.

While the compound decreased in normal ripening fruits from $195\text{-}101\text{ ug g}^{-1}$ fr. wt. during 31 days (Table 3). The appearance of decay symptoms and the decrease of biological activity of the partial purified extracts was enhanced in ethylene-treated fruits in relation to the normal ripening fruits.

Hypobaric pressure treated fruits showed the antifungal compound decreased from 195 to 107 ug g^{-1} fr. wt. 28 days after harvest (Table 4). Seventeen days after harvest the fruits showed decay development in 8% of the infection sites and peel extracts inhibited by 65% germ tube elongation. No significant difference in fungal growth of A. alternata was detected at 150 and 760 mmHg. In normal ripening fruits the antifungal compound decreased from $195\text{-}112\text{ ug g}^{-1}$ fr. wt. during 17 days. First infected sites were already present 12 days after harvesting accompanied with a faster decrease in inhibition of germ tube elongation. Delayed softening of the fruits resulting from the CaCl_2 treatment (Table 4) did not affect significantly the decay development of infected sites by Alternaria and the decreased antifungal compound that were similar to those of the control fruits.

Occurrence of antifungal compound in the fruit peel of various mango cultivars

The compound was extracted from peels of unripe mango fruits of cultivars Tommy Atkins, Haden and Erwin, and these extracts yielded identical hplc retention times. The U.V. spectra of the extracted compounds were

also identical, indicating that the same compound was present in the tested cultivars. The concentration of the compound observed in peels of unripe fruits were Tommy Atkins - 195.3, Haden - 187.8, and Erwin - 140.8 $\mu\text{g.g}^{-1}$ fr. wt. fruit tissues.

Discussion

Alternaria alternata infects mango fruits early in the season and enters a latent or quiescent state soon after the initial stages of infection are completed (11). After harvest, the fruits gradually lose their resistance as they ripen and the pathogen renews growth, giving rise to an active decay of black spots that spray all over the fruit surface. A. alternata is today the principal agent of post-harvest decay in Israeli mangoes (11).

In our research, trying to explain the latency of A. alternata in mango fruits, we isolated a preformed antifungal compound which has a possible role in regulation of A. alternata latency in mango. The antifungal compound was isolated from peels of unripe mango fruits. Preliminary results for the identifications of the antifungal compound indicates the presence of a single preformed compound, with a possible structure of fatty acids attached to a carbon chain. Although the final identification is not completed, chemical data indicated the presence of fatty acid esters after methanol-hydrolysis and gas chromatographic determination. Fatty acids and their derivatives have been demonstrated to be fungitoxic (8,14, 15) and recently a derivative of linoleic acid was reported as a preformed antifungal compound having a possible role in the latency of the fungus Colletotrichum gloeosporioides in avocado fruits (13).

Germ tube elongation was the most sensitive stage that affected Alternaria by the antifungal compound; with high concentration spore germination was totally prevented.

A few pieces of evidence for the involvement of an antifungal compound in the resistance of unripe and susceptibility of ripe mango fruits to the attack of *Alternaria* are presented:

- (i) A significant increase in susceptibility to *Alternaria* attack is observed in mango fruits after harvest (Fig. 1).
- (ii) *In vivo* test of germination of *Alternaria* conidia exhibited inhibition of germ tube elongation three times more in the peel of unripe than in ripe fruits (Table I).
- (iii) The *in vitro* activity of the purified antifungal compound against *A. alternata* showed an ED_{50} of 112 ug.ml^{-1} for inhibition of germ tube elongation.
- (iv) Quantitative determinations showed that the concentrations of the antifungal compound decreased from 200 ug g^{-1} fr. wt. in unripe fruits to 97 ug g^{-1} fr. wt. in ripe fruits, accompanied with a significant decrease in inhibition of germ tube elongation (Fig. 2).
- (v) Fruits cultivar Tommy Atkins exhibited disease symptoms more rapidly after harvest than Haden, and concentrations of the antifungal compound decreased more rapidly during ripening of the former (Table 2).
- (vi) Enhanced decay of infected sites by *Alternaria* in ethylene-treated fruits happens together with a faster decrease in antifungal compound (Table 3).
- (vii) Delay of decay of infected sites by *Alternaria* in hypobaric pressure treatment happens parallelly with the delay of the antifungal compound decrease (Table 4).
- (viii) Delay softening of the fruits resulting from CaCl_2 treatment did not affect the appearance of decay and the decrease of the antifungal compound (Table 4).

(ix) All the tested cvs. possessed high amounts of the antifungal compound in the peel of fruits.

All data reported in this paper provide much evidence that could support, but does not prove, the hypothesis that the antifungal compound found in mango peels confers resistance to A. alternata in unripe mango fruits and its breakdown during ripening leads to renewed development of the fungus that then causes black spots on the fruit surface.

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Table 1 - Effect of ripe and unripe peel of mango fruit cv. Tommy Atkins of the inhibition of germ tube elongation of germinated Alternaria alternata spores.

	Unripe fruit day after harvest	Ripe fruit 34 days after harvest
Inhibited elongation (%)	87.3	29.0

Control spores germinated on Millipore discs in petri dishes showed no inhibition of germ tube elongation.

Table 2 - Occurrence of the antifungal compound, biological activity of the crude extracts and symptom expression of Alternaria alternata in cultivars Tommy Atkins and Haden at different stages after harvest

Days after harvest	----- Tommy Atkins -----				----- Haden -----			
	Antifungal compound ug.g ⁻¹ fr. wt.	Inhibited germ tube elongation(%)	Appearance of symptoms (% infected sites)		Antifungal compound ug.g ⁻¹ fr. wt.	Inhibited germ tube elongation (%)	Appearance of symptoms (% infected sites)	
1	203.8 ^a	87	0 ^b		187.8	92	0	
8	183.8	75	0		174.0	82	0	
12	95.8	43	14 ^b		209.0	68	0	
17	103.4	39	51		222.2	56	3	
22	110.4	30	87.5		119.0	39	47	
28	97.4	33	100		88.0	29	89	
31	94.8	21	100		110.0	35	100	

^aConcentration of the compound was determined by hplc.

^bBlack spot of the peel over 2.0 mm in diameter was considered as an infected site.

Table 3 - Occurrence of the antifungal compound, biological activity of the crude extracts and symptom expression of *Alternaria alternata* in cultivar Tommy Atkins mango fruits during ethylene-induced ripening.

Days after harvest	Normal Ripening -----			Ethylene treated (induced ripening) ^a ---		
	Antifungal compound ^b ug.g ⁻¹ fr. wt.	Inhibited germ tube elongation (%)	Appearance ^c of symptoms (% infected sites)	Antifungal compound ug.g ⁻¹ fr. wt.	Inhibited germ tube elongation (%)	Appearance of symptoms (% infected sites)
1	195.3	90.0	0	195.3	90.0	0
12	160.8	55.0	16	108.7	59.0	73.3
17	112.0	47.0	49	-	-	100.0
31	101.0	19.0	100	-	-	100.0

^aHarvested fruits were treated with 100 u.L⁻¹ ethylene at 20°C for 3 days and then transferred to 20°C.

^bAntifungal compound was determined by hplc.

^cBlack spot of the peel over 2.0 mm in diameter was considered as an infected site.

Table 4 - Occurrence of the antifungal compound, biological activity of the crude extracts and symptom expression of Alternaria alternata in cultivar Tommy Atkins mango fruits stored under hypobaric pressure of 150 mmHg or infiltrated with 0.35 M CaCl₂.

Days after harvest	----- Normal Ripening -----			-Hypobaric Pressure 150 mmHg ^c -----			----- CaCl ₂ 0.35 M -----		
	Antifungal compound ^a	Inhibited germ tube elongation (%)	Appearance of symptoms infected sites	Antifungal compound	Inhibited germ tube elongation (%)	Appearance of symptoms infected sites	Antifungal compound	Inhibited germ tube elongation (%)	Appearance of symptoms infected sites
1	195.3	90.0	0	195.3	90.0	0	195.3	90.0	0
12	160.8	55.0	16.0	-	-	0	-	-	0
17	112.0	47.0	49.0	162.0	65.0	8.0	121.4	63.0	40.0
28	117.0	32.0	100.0	107.8	44.0	75.0	118.4	48.0	83.0

^aAntifungal compound (ug g⁻¹ fr. wt.) was determined by hplc.

^bBlack spot of the peel 2.0 mm diameter was considered as an infection site.

^cHypobaric pressure at 150 mmHg was applied 24 h after inoculation of the fruits. Fruits were maintained in these conditions during 7 days at 20°C and then transferred to atmospheric conditions for the rest of the experiment. Fungal diameter after 7days of growth on PDA was 6.5 ± 0.3 at 150 mmHg, and 7.7 ± 0.4 cm at 760 mmHg.

FIGURES

- Fig. 1. - Inoculation of cultivar Tommy Atkins mango fruits at increasing periods after harvest.
- Fig. 2. - Fractionation of partially-purified antifungal compound by high pressure liquid chromatography using Licrosorb 10 μ m column (1 x 25 cm) eluted with 90% methanol in water. A v.v. detector was used with maximum emission 275 nm and a chart speed of 0.5 cm. min⁻¹ (alternation 4 x). The peak present at retention time 27 min. showed the biological activity.
- Fig. 3. - Concentration of the antifungal compound in mango fruits cv. Tommy Takins calculated according to peak height by U.V. absorption at 275 nm at different stages after harvest.
- Fig. 4. - Concentration of the antifungal compound ( ) and antifungal activity ( ) in crude extracts from peels of mango cultivar Tommy Atkins at different stages after harvest. Antifungal compound concentration was calculated by hplc. The arrow denotes the first visible symptoms of decay.

Figure - 1

Fig. - 2

Fig. - 3

Fig. -4

3. EVALUATION OF RESEARCH ACHIEVEMENT

The objective of the research, as stated in the proposal, was "to investigate the processes responsible for the resistance of unripe fruits to fungal infections and the changes leading to the susceptibility of ripe fruits". Today we can say that in the latency of Colletotrichum gloeosporioides and Alternata in avocado and mango, respectively, antifungal compounds are involved. The possibility that nutrients available in ripening fruits might be involved in the resistance of unripe fruits was denied in Colletotrichum-avocado system. In both fruits studied, the decrease of the antifungal compound correlates with symptom expression of the disease and different treatments like ethylene, CaCl_2 , hypobaric pressure affecting differentially fruit ripening, and support the hypothesis that reduction in the amount of antifungal compounds causes development of the fungus. In the Colletotrichum-avocado system, where the antifungal compound was already identified as 1-acetoxy-2-hydroxy-4-oxo 12,15 heneicosa-diene, it was found as well the enzymatical system that regulates the breakdown of this compound. This enzyme seems to be lipoxygenase and the inhibition of its activity by a scavenger inhibitor has resulted in the partial control of the disease. From a practical point of view, this research has developed a rapid and effective method for the assessment of latent infections with already present practical use in Israel for the determination of the need of post-harvest treatments in different regions of the country.

4. DESCRIPTION AND RESULTS OF COOPERATION

The following list of contributions to this research were made:

Section:

2.1, 2.1.2 were executed by D. Prusky with the help of Y. Fuchs,

G. Zauberman and U. Yanko in Israel.

2.1.3, 2.2.1, 2.2.2 were executed by D. Prusky in Israel.

2.3.1 was done by D. Prusky, A. Ascarelli and B. Jacoby in Israel.

2.3.2, 2.3.3 were performed in UCR by N.T. Keen, D. Prusky, J.J. Sims,

S. Midland, E. Eaks.

2.4 is part of a Ph.D. thesis being done in D. Prusky's lab. by

Samir Droby, together with B. Jacoby and E. Gloter.

Dov Prusky has spent a year working in Dr. Keen's lab. during the first year of the research that resulted in a very fruitful cooperation and productive work. Dr. Keen visited Dr. Prusky's lab. during the third year of cooperation and Dr. Prusky visited Dr. Keen for the second time also during the third year of research, to summarise the results of their cooperation.

5. BENEFITS TO AGRICULTURE

The basic study on the avocado *Colletotrichum*-system has created a new outlook on a non-conventional way of disease control, in general and in avocado in particular. Present results indicate that conventional food additives, like α -tocopherol, butylated hydroxy anisole and butylated hydroxy toluene, might, after proper infiltration, delay symptom expression by inhibition of lipoxygenase and prevention of antifungal diene breakdown. The development of a method of assessment of latent infections of Alternaria in mango, the determination of the maximal level of latent infections over that fungicide treatments are needed, has resulted in a firm basis for the use of this method by growers for the determination of the need of post-harvest treatments of mango fruits. In the last harvesting season (summer, 1983) this method has saved the growers from the regional Southern Golan Heights and Beit Shean Valley from dipping their fruits in post-harvest fungicides developed also during this research.

6. LIST OF PUBLISHED MANUSCRIPTS

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A method for pre-harvest assessment of latent infections
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