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Involvement of Limonene Hydroperoxides Formed after Oil Gland Injury in the Induction of Defense Response against *Penicillium digitatum* in Lemon Fruit

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The effects of wounding oil glands of lemon [*Citrus limon* (L.) Burm.] fruit were investigated. Young mature-green lemons demonstrated significantly lower decay incidence than older yellow fruit when their oil glands were punctured in the presence of postharvest wound pathogen *Penicillium digitatum* Sacc. Contact with the released gland content on the green lemon surface reduced the viability of *P. digitatum* spores approximately twice. Wounding caused rapid production of limonene hydroperoxides that persisted for only a few minutes. The magnitude depended on the physiological maturity of the fruit; mature-green fruit produced much higher levels than did yellow lemons. Furthermore, wounding of the oil glands or injection of limonene hydroperoxides into the lemon peel elicited the production of the citrus fruit phytoalexins, scoparone and scopoletin, to levels known to be effective in reducing decay caused by *P. digitatum*. The mature-green fruit produced about twice as much of these phytoalexins as the older yellow fruit. This induced defensive elicitation of phytoalexin production, as well as the direct effects of these antifungal compounds, markedly inhibited the pathogen in mature-green fruits but was ineffective in older yellow ones.

KEYWORDS: Lemon; *Citrus limon*; *Penicillium digitatum*; scoparone; scopoletin; reactive oxygen species; catalase; limonene hydroperoxides

INTRODUCTION

One of the earlier responses of plant tissues to pathogen attack or treatment with elicitors is the oxidative burst that is characterized by a transient and rapid accumulation of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radical (*1–3*). ROS were reported to have a direct antimicrobial effect (*4, 5*) and are also considered to be involved in various defense responses such as oxidative cross-linking of cell wall proteins (*6*), reinforcement of plant cell walls by the production of lignin polymers (*7, 8*), the hypersensitive response (*9*), and phytoalexin production (*4*).

The peel of citrus fruit contains numerous oil glands that produce and accumulate essential oils. Citrus oils are composed of mainly limonene and other mono- and sesquiterpenes, with the addition of some phenolic compounds, for example, coumarins and furanocoumarins. Previous work has demonstrated that the content of oil glands may affect host−pathogen relationships in citrus fruit. Moreover, it was shown that the difference in disease susceptibility between mature-green and yellow lemons can be associated, at least partially, with factors localized in their oil glands (*10*). The major component of citrus essential oils, limonene, was demonstrated to be an enhancer of the development of *Penicillium digitatum* in yellow lemons (*11*). Monoterpene esters (geranyl acetate and neryl acetate) also stimulated pathogen growth in a wide range of concentrations (*10*), and the monoterpene aldehyde citral (a natural combination of the two isomers geranial and neral) exhibited significant antifungal activity (*10, 12*).

The involvement of the phytoalexins, scoparone and scopoletin (**Figure 1**), and various physical and chemical elicitors of the endogenous resistance mechanisms in citrus fruits has been reviewed (*12–14*). A novel biocidal formulation prepared from various essential oil components of lemon flavedo has been described (*15, 16*). An active new biocidal, antifungal compound was extracted from lemon peel having damaged oil glands. This active constituent was purified and identified as comprising four limonene hydroperoxides, the structures of which were reported

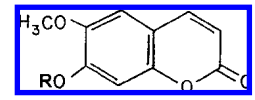

Figure 1. Chemical structures of the citrus phytoalexins scoparone (R = CH_3) and scopoletin (R = H).

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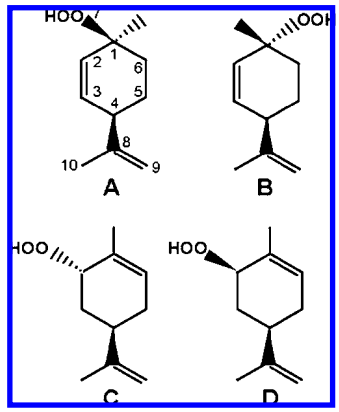

Figure 2. Chemical structures of the limonene hydroperoxides formed after oil gland injury of mature green lemon fruits (*15*): (**A**) (1*S*,4*R*)-*p*-mentha-2,8-diene 1-hydroperoxide; (**B**) (1*R*,4*R*)-*p*-mentha-2,8-diene 1-hydroperoxide; (**C**) (2*R*,4*R*)-*p*-mentha-6,8-diene 2-hydroperoxide; (**D**) (2*S*,4*R*)-*p*-mentha-6,8-diene 2-hydroperoxide.

as (1*S*,4*R*)-*p*-mentha-2,8-diene 1-hydroperoxide, (1*R*,4*R*)-*p*-mentha-2,8-diene 1-hydroperoxide, (2*R*,4*R*)-*p*-mentha-6,8-diene 2-hydroperoxide, and (2*S*,4*R*)-*p*-mentha-6,8-diene 2-hydroperoxide (*15*) (**Figure 2**). The production of various hydroperoxides by photosensitized oxidation of limonene was reported earlier (*17*), without any relevance to their antifungal activity. Furthermore, in agreement with this finding (*17*), it was found that limonene hydroperoxides could be generated artificially by treating limonene with UV or sunlight (*15, 16*).

Maturity effects on resistance of citrus fruits have been discussed (*18*), and it was demonstrated that the resistance of fruit against pathogens declines as the mature fruit ages. Scora et al. (*19*) investigated the effects of fruit maturity on the essential rind oil components in *Citrus sinensis*.

The objective of the present work was to study the nature of the inhibitory effects on *P. digitatum* induced by wounding the oil glands of mature-green lemons.

MATERIALS AND METHODS

Plant Material. Lemon fruit [*Citrus limon* (L.) Burm. cv. Eureka] served as a model for citrus fruits. The lemon offers an important advantage of enabling study of the effect of fruit maturity by considering the mature-green fruit as physiologically young and the yellow fruit as older. Both stages are commercially salable. Mature green was selected as young light green fruit, approximately 6–7 months after anthesis, when it becomes mature according to the maturity standards. The yellow fruit was selected at about 10–11 months after anthesis, although the fruit becomes yellow before this age, but this older fruit helped to emphasize the age effect. The lemons were obtained from one orchard in Palmahim, Israel. All experiments were done within 1–2 days after harvest; the fruits were surface sterilized by wiping with 70% ethanol and were air-dried.

Injury to Oil Glands and between Oil Glands and Decay Evaluation. Oil glands in lemon peel were injured to a depth of 1 mm with a sterile needle of 0.5 mm diameter. Injury between the oil glands was inflicted in the same way, with special care to avoid damage to the oil glands. In each sampling site 20 neighboring oil glands were injured in an area of 0.2−0.4 cm^2, or 20 injuries were made in the tissue between the oil glands in the same area.

Fruit decay susceptibility was evaluated in three inoculation experiments. Each treatment was applied in each of these experiments to 30 different fruits placed in three different crates. The inoculation was done using an isolate of *P. digitatum* taken from an infected lemon fruit and cultivated on potato dextrose agar (PDA) medium. Drops of *P. digitatum* spore suspension (25 μL, 10^3 spores/mL) were placed on the peel of mature-green and yellow lemons and left to air-dry. Direct contact between the content of the oil glands and *P. digitatum* spores was achieved by injuring 20 oil glands of each lemon in the spore-drop application area as described above. The control fruit, which had also received spores, were not injured. The crates were placed in 95% relative humidity at 20 °C to allow development of the inoculation, and the percentage of rotten fruit was determined.

Studying the Effect of the Oil Gland Contents on Fruit−Pathogen Interaction in Situ. Direct contact between the content of the oil glands and *P. digitatum* spores was achieved by injuring oil glands in the spore-drop application area as described above. After 15 min of spore contact with the oil gland contents, a 25 μL drop of sterile water was placed on the spore application site; the spores that were resuspended in water were collected from the fruit surface with a pipet and were spread on PDA in Petri dishes. The number of recovered and germinated spores was evaluated as colony forming units (CFUs) after 2 days of incubation at 28 °C, as averages of five replications (spore application sites), each site located on a separate fruit. The experiment was repeated three times and yielded reproducible results.

Preparation of Crude Extract and Detection of Antifungal Activity from Crude Extract and on TLC Plates. Extracts from the flavedo tissue of green and yellow lemons were prepared as previously described (*18, 20*). Detection of the antifungal activity was carried out on TLC plates as described widely, by a bioautography assay using *Cladosporium cladosporioides* as a test organism. *Cladosporium* is predominantly used in this bioassay because of its capacity to grow uniformly and to sporulate on the surface of a TLC plate, whereas using *P. digitatum* for this purpose presents technical problems (*18*). Peel strips 1 × 3 cm in size were cut from green lemons. Each such strip was injured by making superficial scratches with a spatula: three lengthwise and six laterally. The strips were washed for 10 s in a dish containing 5 mL of dichloromethane at several times after this injury (a few seconds and 5, 10, 20, 30, and 60 min), and the washed strips were discarded. The solvent was concentrated to a volume of 1 mL, and 50 μL samples from this were loaded on a TLC plate. In parallel, an extraction of such wounded strips was carried out for 2 h in dichloromethane. Then the tissue was separated from the solvent, the solvent was again concentrated to 1 mL, and a 50 μL aliquot was loaded onto a TLC plate. The plate was developed in dichloromethane/ethyl acetate at a ratio of 2:98 (v/v). After drying in air, a suspension of *C. cladosporioides* G.A. De Vries spores in a Czapek−Dox medium (10^6 spores/mL) was sprayed on the plate and incubated at 24 °C in high humidity for 2 days, to allow development of the fungus on the plate. White areas indicated inhibitory regions in the fungal growth, which showed the presence of antifungal materials.

Peroxides Measurements. The peroxides concentration was determined on the basis of peroxidase-catalyzed oxidation of the fluorescent compound scopoletin (*21*). The peroxides were measured as the decrease in fluorescence recorded by an automatic microplate fluorescence reader. The samples were excited at 360 nm, and the emission was measured at 460 nm. Quantification of the analysis was based on a calibration curve with hydrogen peroxide used as a standard.

Because it was found in preliminary experiments that peroxides generated by the injury of oil glands were degraded rapidly in vivo, supposdly by the fruit enzymes, the assay reaction was performed in situ on the fruit surface. Scopoletin and peroxidase were placed on the fruit surface in an aqueous solution, before the oil glands were injured, so that the wounding-generated peroxide could react with scopoletin at the moment of its production, before it was degraded. A 50 μL drop of double-distilled water (DDW) was placed on the fruit surface, and 10 μL of scopoletin solution (0.1 mM) and 5 μL of horseradish peroxidase (1 unit/μL) were added to it. In a first group of five fruits, 20 oil glands adjacent to one another were injured by inserting a needle through the water drop with its scopoletin and peroxidase contents. Five fruits in a second group were left uninjured. Five fruits of a third group received a 65 μL drop of DDW without scopoletin or peroxidase on their surface, and 20 adjacent oil glands were punctured by inserting the needle through this DDW drop. At various times after infliction of the injury (immediately and 1, 2, 5, and 10 min) a 50 μL sample of each drop was placed in a 96-well enzyme-linked immunosorbent assay plate. The wells in the microplate contained 50 μL of DDW. Each experiment was repeated three times.

The level of fluorescence from the samples taken from the fruit with intact oil glands was designated as 100%, that is, initial fluorescence of scopoletin before the glands were injured and generated peroxide. When oil glands were not injured, the scopoletin fluorescence did not decrease after 10 min, which shows that the decrease in fluorescence was due to injury of the glands.

The level of fluorescence from the samples from the fruit with injured oil glands under drops of pure DDW was regarded as background fluorescence caused by the presence of fluorescent compounds other than scopoletin in the oil gland content, and it was deducted from the fluorescence of the samples from the fruit with injured glands. In preliminary experiments the level of 460 nm emission from samples of the oil glands in mature-green and yellow lemons had been measured over a broad excitation wavelength range of 400–540 nm and was found to be very well correlated with the emission spectrum of scopoletin at the same wavelengths. This correlation confirmed that the fluorescence of the oil gland content could be treated as background. No effect of peroxidase on the background fluorescence from injured oil glands was observed in the preliminary experiments.

The nature of the peroxide formed was checked by adding 1 or 10 units of the enzyme catalase to the 50 μL of water medium. The catalase would break down all of the H_2O_2 in the vicinity but not the organic peroxides.

Phytoalexin Measurements. Scoparone and scopoletin were measured according to previously published methods (*12, 20*). Peel disks were extracted with dichloromethane at a ratio of 1:10 (w/v) for 1 day and homogenized in an Ultra Turrax TPI8-10 (Janke & Kunkel KG) for 1 min at full speed. The homogenate was filtered in vacuo through Whatman no. 1 filter paper. The residue was washed twice with solvent, and the filtrate was concentrated to a volume of 2 mL. A 50 μL sample was pipetted onto a TLC aluminum plate covered with silica gel (Merck), with a toluene/ethyl acetate (4:1, v/v) solution as the carrier. The developed sheets were air-dried and exposed to UV light (366 nm). Individual spots were collected after comparison with authentic scoparone standards on the plate. The scoparone spot was eluted with methanol, and quantitative analysis of the scoparone content was performed on an FL 600 microplate fluorescence reader by measuring the relative fluorescence intensities for excitation at 355 nm and emission at 460 nm. The analysis was standardized against an authentic scoparone sample.

Effects of Various Materials on Phytoalexins Production. In vivo effects of several treatments on the phytoalexin content of lemon fruit flavedo were measured. Induction of scoparone and scopoletin production in mature-green lemons was studied by injecting water, citral, dichloromethane crude extract, 4% limonene hydroperoxides in 25% aqueous ethanol solution, and sun-treated limonene into the albedo or by injuring oil glands in mature-green lemons. These materials were injected into the albedo in amounts of 5 μL just below the flavedo tissue of 10 fruit for each treatment. Each treatment was applied in three different experiments.

The sun-treated limonene was prepared as follows. Pure redistilled limonene was placed in a Petri dish and left for 3 h outside a window of the laboratory at noon when the outside temperatures were 23–27 °C. During this time 5% of the limonene was converted to other products, much of which were shown by a HPLC to be limonene hydroperoxides. The crude extract was prepared as follows. Lemon fruit flavedo (exocarp) was taken and extracted overnight in dichloromethane and then exposed to sunlight for about 18 h until the color of the dichloromethane extract turned brown. The flavedo was blended and filtered through a layer of Whatman paper. The extracted solution was evaporated to remove the dichloromethane, and the dense liquor was further separated by chromatography in a silica 60 column, with dichloromethane as a carrier. Two fractions of dichloromethane, one green and the other colorless, were obtained after chromatography. The crude active extract was isolated by evaporation of the green dichloromethane fraction (*15*). The consistency of obtaining a stable crude extract was satisfactory.

Limonene hydroperoxides were synthesized by the heterogeneous catalytic method. Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) was used as a catalyst; 300 mL of concentrated hydrogen peroxide as an oxidation agent was added to the solvent, which was 700 mL of ethanol. The reaction conditions to get the desired products were atmospheric pressure of 50 °C temperature and a 5–6 h reaction time with continuous stirring. The conversion of limonene and the yield of the reaction were nearly 100%. Because the synthesis of limonene hydroperoxides is an exothermic reaction, its handling requires proper care.

Applying Sun-Exposed Limonene to Kumquat Fruit. Freshly harvested kumquat fruits were subjected to postharvest treatment by dipping them in aqueous solution of 5000 μL/L of sun-exposed limonene in 25% ethanol + 5000 μL/L of Tween 20. The sun-exposed limonene was prepared as described above. Decay was evaluated in three different experiments to compare fruit that were treated with sun-exposed limonene dissolved in 25% ethanol, with 25% ethanol without limonene, and not treated (control fruit). Each treatment was applied to five crates of kumquat, each containing 100 fruit, in each of the three experiments.

Statistical Analysis. Experiments were repeated three times, each with about five separate replicates. The results were subjected to Duncan's multiple-range test. The decay measurements were also subjected to arcsin transformation because its evaluation was based on counting.

RESULTS

Effect of Injury of Oil Glands on *P. digitatum* Development on Lemon Fruit. Injury by puncturing the oil glands with a needle in the presence of *P. digitatum* spores caused the development of green mold decay in lemon fruit, but the decay percentage depended strongly and significantly on the maturity of the fruit, as indicated by its color. When *P. digitatum* spores were placed on the fruit surface and the oil glands in the area were injured, 92–100% of the yellow fruit developed decay after 4 days. The decay incidence on mature-green fruit subjected to the same procedure was significantly ($P = 0.01$) lower, varying in different experiments between 22 and 35%. If the spore suspension was applied 1 h after the release of the oil gland content, then there was no difference in decay between green and yellow lemons. No fruit decay was observed when *P. digitatum* spores were placed on a lemon surface without oil gland wounding.

To examine the involvement of the oil glands in the interaction between lemon fruit and *P. digitatum*, the direct effects of the oil gland contents on the viability of the pathogen were investigated. Conidial spores of *P. digitatum* were exposed to the essential oil in situ on the fruit surface by injuring the glands and subsequently collecting the spores from the fruit surface and placing them on PDA medium. Without gland puncturing the spore recovery rate from the fruit surface was between 23 and 24 CFU/plate, that is, 92–94% of the initially applied spores. After puncturing, the average spore recovery rates from yellow and green fruit were 13 and 7 CFU/plate, respectively. It is difficult to know what proportion of this spore loss was due to viability reduction caused by the released content of the oil glands and what was due to changes in the peel surface, because any spores that entered the ruptured oil glands most probably could not be easily collected. However, the finding that spore recovery from the wounded green fruit was about half that from the wounded yellow ones was statistically significant ($P = 0.05$) according to Duncan's multiple-range test. This difference in recovery was due to viability loss because the surfaces of the green and yellow fruits were similar.

Effect of Injury of Lemon Peel on the Production of Antifungal Materials. Following the injuries to the flavedo tissue of green lemons, new antifungal materials appeared on the peel surface (**Figure 3**). When these materials were placed on a TLC plate, their strong antifungal activity was exhibited as white areas in which the *Cladosporium* growth was inhibited. Furthermore, the size of the inhibited areas indicated that the

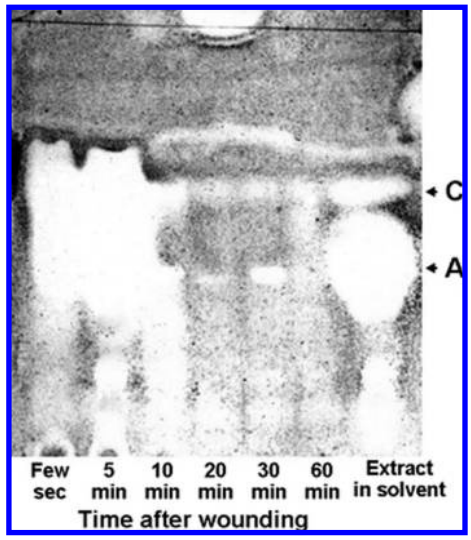

Figure 3. Detection of short-lived antifungal activity on TLC plates. The white areas indicate regions of fungal growth inhibition on the plate, showing the presence of antifungal materials. C, citral; A, new antifungal compounds.

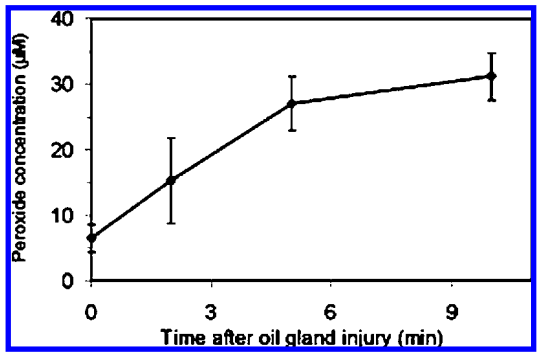

Figure 4. Peroxides accumulation in response to injury of oil glands in mature green lemons. The bars indicate a confidence interval of 5 replications ($P = 0.05$).

effect of this new antifungal material was much larger than that of citral, which had been shown to be the major antifungal material in lemon fruit (*18*). However, in the present study this new material disappeared after about 10 min (**Figure 3**).

Effect of Injury to the Oil Glands on the Production of Peroxides. Injuring the oil glands induced a very rapid production of peroxides in the peel of mature green lemons (**Figure 4**). This production started immediately after the injury was inflicted and reached a concentration of 34.8 μM after 10 min. In the peel of yellow lemons only a low concentration of peroxides (17.1–17.7 μM) was induced after the same time by injury of the oil glands (**Figure 5**). This difference between the green and yellow fruits was highly significant ($P = 0.01$).

Injuring green lemon peel between the oil glands, to avoid the release of the essential oil, did not induce the production of peroxides after 5 min, or any that was induced was at a level that could not be measured. Untreated peel produced no peroxides.

The nature of the peroxide was checked by adding the enzyme catalase to the reaction medium. The concentration of peroxides were the same whether or not catalase was present in the reaction medium (**Figure 5**), which indicates that the materials generated were organic peroxides and not H_2O_2. Addition of 0.1% of antioxidants such as butylated hydroxyanisole (BHA) greatly reduced, and addition of butylated hydroxytoluene (BHT)

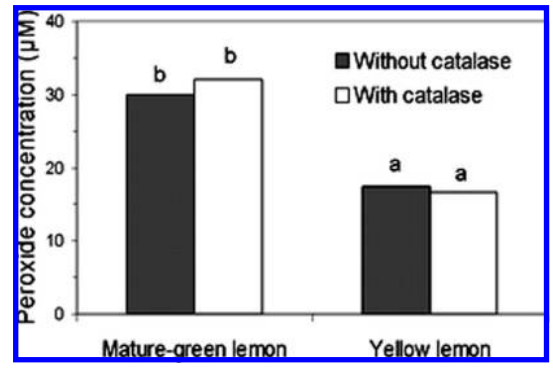

Figure 5. Effects of oil glands injury, catalase application, and maturity on the accumulation of peroxides in lemon fruits. The absence of an effect of catalase shows that the peroxide formed is not hydrogen peroxide, but an organic peroxide. Different letters above columns indicate statistically significant differences in Duncan's multiple-range test ($P = 0.01$).

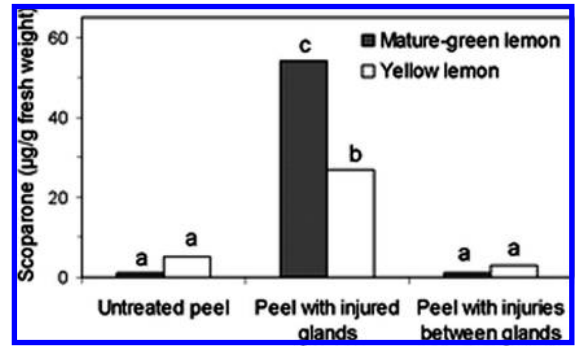

Figure 6. Induction of scoparone accumulation by the released content of oil glands in lemon fruit. Different letters above columns indicate statistically significant differences according to Duncan's multiple-range test ($P = 0.01$).

completely prevented, the production of the new antifungal material when these antioxidants were added to a homogenate of discs of green lemons, indicating that the production of this antifungal material was an oxidation reaction that converted the limonene into limonene hydroperoxides.

Eliciting the Production of Phytoalexins. Puncturing the oil glands in the peel of mature green lemons with a needle induced the production of the citrus phytoalexin scoparone in the adjacent tissue in highly significant way ($P = 0.01$) as compared either with uninjured peel or with peel that was injured between the oil glands (**Figure 6**). The scoparone concentration was low in untreated peel or in peel that was injured only between the oil glands, so as to avoid release of the oil. Induction of scoparone production by injuring the oil glands was also observed in the yellow fruit, but the scoparone concentration was significantly ($P = 0.01$) lower than that in the green ones. Scoparone concentration in the peel of green lemons, reached a level of 54 μg/g of fresh weight, which is above the median effective dose (29 μg/mL) reported for the inhibition of germ tube elongation of *P. digitatum* spores (*20*). In yellow lemons, however, the scoparone concentration induced by the oil gland injury was only 25 μg/g of fresh weight.

Treating uninoculated lemons by injecting 5 μL of sun-exposed limonene into the albedo tissue elicited significant production of scoparone and scopoletin, up to levels adequate to protect the fruit from the pathogen. Similar results were achieved by injecting the sun-exposed crude dichloromethane extract of lemon flavedo or synthetically prepared limonene

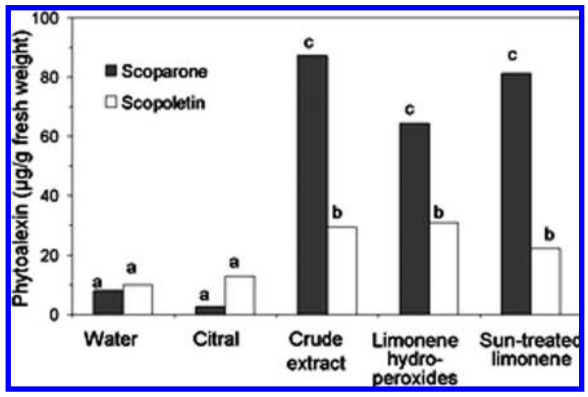

Figure 7. Effect of several treatments on the phytoalexins content of fruit flavedo of mature-green lemons. Different letters above columns indicate statistically significant differences according to Duncan's multiple-range test ($P = 0.05$).

hydroperoxides (**Figure 7**). Interestingly, injecting 5 μL of citral or water into the albedo tissue did not elicit the same protective response and did not induce the production of the phytoalexins.

In Vivo Effects of Limonene Hydroperoxides on Decay of Kumquat Fruit. Freshly harvested kumquat fruits were dipped in an aqueous solution of 5000 μL/L sun-exposed limonene in 25% ethanol plus 5000 μL/L of Tween 20, and this treatment reduced decay in a highly significant way ($P = 0.01$), from 12.0% in the control fruit to 1.6% in the treated fruit, after storage for 11 days at 10 °C followed by 6 days of shelf life at 20 °C (*15*). The fruit treated with 25% ethanol showed 7.9% decay.

DISCUSSION

Injury to Oil Glands Elicits the Production of Phytoalexins. In the present study the release of the contents of the oil glands in the peel of mature-green lemons induced a rapid production of peroxides which, in turn, were shown to elicit the accumulation of the phytoalexins scoparone and scopoletin. The production of peroxides started as early as the first minute after the injury (**Figures 3** and **4**), and they exerted a direct antifungal activity on the spores on the fruit surface. These limonene hydroperoxides had a strong biocidal activity against *Staphylococcus aureus* with a MIC of 32 μL (*16*). Similarly, peroxides were reported to inhibit spore germination of three kinds of fungi almost completely (*4*). Thus, the injury to the oil glands in the peel of mature green lemon fruit induced a local defense response in the adjacent peel tissues, expressed as accumulation of the phytoalexins scoparone and scopoletin (**Figures 6** and **7**). This phytoalexin accumulation was sufficient to inhibit *P. digitatum* growth (*20*). Scoparone accumulation is recognized as a biochemical marker of resistance induction in citrus (*22*), and it is probable that other molecules may be involved in the resistance of citrus fruit against pathogens.

Treating noninoculated lemons by injection of limonene hydroperoxides, sun-treated limonene, or crude extract of lemon peel into the albedo tissue, just below the flavedo, also elicited production of scoparone and scopoletin up to levels adequate to protect the fruit from the pathogen (**Figure 7**). However, if these treatments did not include the limonene hydroperoxides, there was no inhibition of the pathogens (*15*). Thus, the antifungal activity as well as the induction of phytoalexin accumulation is related to these newly formed limonene hydroperoxides (*15*). Although these limonene hydroperoxides had a short life, they were still able to elicit phytoalexin production and to inhibit decay, as demonstrated in previously reported in vivo experiments (*15, 16*). These new hydroperoxides showed a higher antifungal activity than those of scoparone or scopoletin, which are the endogenous phytoalexins of citrus fruits, or that of citral, which is known as the most active constitutive antifungal material in lemon fruit (*15, 18*). However, different results were obtained for peaches: postharvest application of limonene hydroperoxides did not succeed in reducing decay caused mainly by *Monilinia fructicola* (unpublished data).

Elicitation of phytoalexins is of great importance because it enhances resistance of the fruit against pathogens. Furthermore, such elicitation is probably a more reasonable way of utilizing these phytoalexins in disease control than by direct application of these materials to fruit or to plants, because the latter may result in phytotoxicity as well as rapid degradation of these compounds.

Interestingly, limonene itself, which is the major component of the citrus oil gland contents, does not exhibit activity against filamentous fungi (*15*). The antifungal activity detected in some publications is related mainly, according to the opinion of these authors, to possible oxidized limonene contaminants or products and not to the actual antifungal activity of limonene itself (*14, 15*). Because limonene has insecticidal activity, it has been suggested that its possible role is to protect fruit from various insects (*14*). The limonene hydroperoxides were generated upon oil gland injury, and it was hypothesized that such injury is probably the trigger, developed through evolution, to initiate action against the pathogen (*15, 16*). Thus, evolution enabled the selection of this better survival attribute of injury-triggered conversion of limonene into limonene hydroperoxides, which were found to be the most active antifungal compound in citrus fruit (*15, 16*). It appears that, through evolution, citrus fruit has developed a special capacity to maintain near its surface, inside the oil gland, nontoxic limonene that serves as the precursor to the active antipathogen compound, which is thus available at the proper location at the time of attack by the pathogen. Furthermore, this precursor is compartmentalized in the oil gland because of the phytotoxicity of both the precursor and the much greater phytotoxicity of the converted oxidized derivative. Further experiments are still needed to test this hypothesis.

Reactive Oxygen Species and Resistance against Pathogens. The limonene hydroperoxides are ROS. The demonstration in the present study of in vivo production of such ROS after injury to oil glands, its induction of phytoalexins, and the inhibition of decay are the first reported example for citrus fruits, and the process was tested by its application to kumquat fruit. Kumquat fruit cannot be exposed to any fungicides, because the peel of the fruit is also eaten, which was the reason for its selection for this experiment.

Rapid production of the peroxide H_2O_2 was also reported in soybean cells (*1*), parsley cells (*24*), and larch cells (*25*) following the application of elicitors from fungal cell walls. This rapid accumulation of peroxides indicates the occurrence of an oxidative burst, and numerous studies showed that the oxidative burst is implicated as regulator of the phytoalexin production (*1, 2, 26–30*). Exogenous application of H_2O_2 to the pericarp of avocado fruit induced, similarly to fungal inoculation, an increase in PAL activity and a parallel increase in epicatechin levels, indicating that the presence of H_2O_2 can lead to the activation of the phenylpropanoid pathway (*3*). Activation of phenylpropanoid metabolism by elicitor-induced H_2O_2 accumulation has also been reported in *Pinus radiata* cells (*7*). However, other studies showed that although elicitors and

pathogens induced both processes, phytoalexin synthesis was not always dependent on the production of ROS (*9, 31–33*).

Rapid lipid peroxidation has been reported in bean cells (*28, 34*) and tomato cells (*35*), following elicitor application. Association of peroxidation of lipids with induction of phytoalexin accumulation has been reported in a number of papers (*27, 30, 36*). Recently, interesting data were reported regarding the production of hydrogen peroxide by citrus fruits as a response to injury or inoculation with two *Penicillium* species, *P. digitatum* and *P. expansum* (*23*). *P. digitatum* suppressed the production of the peroxides, whereas *P. expansum* markedly enhanced it. The authors (*23*) suggested that this suppression of the production of H_2O_2 was strongly associated with the ability of *P. digitatum* to suppress the host defense. This study does not relate to our present one because, as shown by catalase application (**Figure 5**), the findings reported in our present work were related to the organic peroxide, limonene hydroperoxide, rather than to H_2O_2. The release of the essential oil from ruptured oil glands followed by immediate generation of hydroperoxides (ROS) may be a signal (oxidative burst) for the activation of a defense response (phytoalexin accumulation) in lemons. The present results are consistent with the suggestion that "the initial signal that leads to a defense response is not an organic compound that is synthesized as a result of inoculation but a compartmentalized compound which is released as a result of injury" (*37*).

In nature, *P. digitatum* penetrates the citrus fruit through injuries in its peel (*38*). Because fruit peel contains an average of 2−3 oil glands/mm^2 (*39*), injuries almost inevitably involve damage to the glands and the release of their essential oil content. Injuries also expose the fruit to inoculation with *P. digitatum* spores that may be present on the fruit surface. Thus, this injuring of oil glands may represent the real conditions of invasion of fungi into the citrus fruit when the penetrating pathogen causes the injury to the oil glands.

Oil Gland Injury, Maturity, and Resistance against Pathogens. This study may explain, for the first time, the differences between green and yellow citrus fruit in their host−pathogen interactions. In the young fruit, injury to the oil glands induces the rapid production of peroxides and the accumulation of scoparone up to a concentration that is sufficient to inhibit *P. digitatum*. The peroxides concentration in mature-green lemon reached 34.8 μM 10 min after the injury to the oil glands (**Figure 5**). Such an in situ dosage might be able to markedly inhibit pathogen spores (*20*). In addition to these newly formed hydroperoxides, the injury or pathogen penetration brings into action several other components of the essential oil that exude out of their compartment and that could inhibit the spores of any pathogen, as shown in previous studies (*10, 15*). Furthermore, the induction of marked accumulation of phytoalexins by the newly formed limonene hydroperoxides, as reported in the present paper, could also account for the relatively high resistance of green lemons to *P. digitatum*.

In contrast, in the older yellow fruits both the level of peroxides was low and scoparone accumulation was not sufficient to inhibit the pathogen, and the injury led to fruit decay. These results are consistent with the early findings that fully colored citrus fruit rotted easily when a wound included injury to the oil glands (*38, 40*). Furthermore, recent studies showed that the oil glands of yellow lemons contained compounds that facilitated the decay caused by *P. digitatum*, compounds such as prangolarin and limonene (*41*). Yellow lemons have already completed their biological task of protecting the developing seeds, and their more rapid decay may even help the dissemination of these seeds.

The differences between green and yellow lemons may be related to the photooxidative mechanism of the formation of limonene hydroperoxides and involvement of the chlorophyll that is present only in green fruit.

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LITERATURE CITED

(1) Apostol, I.; Heinstein, P. F.; Low, P. S. Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. *Plant Physiol.* **1989**, *90*, 109–116.

(2) Mehdy, M. C. Active oxygen species in plant defense against pathogens. *Plant Physiol.* **1994**, *105*, 467–472.

(3) Beno-Moualem, D.; Prusky, D. Early events during quiescent infection development by *Colletotrichum gloeosporioides* in unripe avocado fruits. *Biochem. Cell. Biol.* **2000**, *90*, 553–559.

(4) Peng, M.; Kuc, J. Peroxidase-generated hydrogen peroxide activity *in vitro* and on tobacco leaf disks. *Phytopathology* **1992**, *82*, 696–699.

(5) Wu, G.; Shortt, B. J.; Lawrence, E. B.; Levine, E. B.; Fitzsimmons, K. C.; Shah, D. M. Disease resistance conferred by expression of a gene encoding H_2O_2 generating glucose oxidase in transgenic potato plants. *Plant Cell* **1995**, *7*, 1357–1368.

(6) Bradley, D. J.; Kjellbom, P.; Lamb, C. J. Elicitor- and wound-induced oxidative cross-linking of proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* **1992**, *70*, 21–30.

(7) Hotter, G. H. Elicitor induced oxidative burst and phenylpropanoid metabolism in *Pinus radiata* cell suspension cultures. *Aust. J. Plant Physiol.* **1997**, *24*, 797–804.

(8) Wu, G. S.; Shortt, B. J.; Lawrence, E. B.; Leon, J.; Fitzsimmons, K. C.; Levine, E. B.; Raskin, I.; Shah, D. M. Activation of host defense mechanisms by elevated production of H_2O_2 in transgenic plants. *Plant Physiol.* **1997**, *115*, 427–435.

(9) Levine, A.; Tenhaken, R.; Dixon, R.; Lamb, C. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **1994**, *79*, 583–593.

(10) Rodov, V.; Ben-Yehoshua, S.; Fang, D. Q.; Kim, J. J.; Ashkenazi, R. Preformed antifungal compounds of lemon fruit: citral and its relation to disease resistance. *J. Agric. Food Chem.* **1995**, *43*, 1057–1061.

(11) Arimoto, Y. Chemical facilitator for infection of epicarp of *Citrus limon* by *Penicillium digitatum*. *Ann. Phytopathol. Soc. Jpn.* **1996**, *62*, 593–596.

(12) Ben-Yehoshua S.; Rodov, V.; Peretz, J. Constitutive and induced resistance of citrus fruits against pathogens. In *Disease Resistance in Fruit*; Johnson, G. I., Highly, E., Joyce, D. C., Eds.; *Proceedings of an International Workshop*, Chiang Mai, Thailand; ACIAR: Canberra, Australia, 1998; p 78–93.

(13) Nafussi, B. Study of defense mechanisms of citrus fruit against *Penicillium digitatum* Sacc. and their involvement in reducing decay by hot water dip. Doctoral thesis, Hebrew University, Jerusalem, Israel, 2001.

(14) Ben-Yehoshua, S.; Mercier, J. UV irradiation, biological agents and natural compounds for controlling postharvest decay in fresh fruits and vegetables. In *Environmentally Friendly Technologies for Produce Quality*; Ben-Yehoshua, S., Ed.; CRC, Taylor & Francis Group: Boca Raton, FL, 2005; pp 265–299.

(15) Ben-Yehoshua, S. Microbiocidal formulation comprising an essential oil or their derivatives. Israeli Patent 145767 Jerusalem, Israel; registered also in 15 other countries and pending in few other countries; 2001; http://www.freepatentsonline.com/EP1434486.html.

(16) Ben-Yehoshua, S.; Rodov, V. Developing a novel environmentally friendly microbiocidal formulation from peel of citrus fruit. *Acta Hortic.* **2006**, *712*, 275–284.

(17) Schieberle, P.; Maier, W.; Firl, J.; Grosch, W. HRGC separation of hydroperoxides formed during the photosensitized oxidation of (*R*)-(+)-limonene. *J. High Resolut. Chromatogr.* **1987**, *10*, 588–594.

(18) Ben-Yehoshua, S.; Rodov, V.; Kim, J. J.; Carmeli, S. Preformed and induced antifungal materials of citrus fruits in relation to enhancement of decay resistance by heat and ultraviolet treatment. *J. Agric. Food Chem.* **1992**, *40*, 1217–1221.

(19) Scora, R. W.; Coggins, C. W.; Knapp, J. C. F. Influence of maturation upon the essential rind oil components in *Citrus sinensis Proceedings of the 1st International Citrus Symposium*, Riverside, CA, 1969; Vol. 3, pp 1171–1176. In *Proceedings of the International Citrus Symposium*; Chapman, H. D., Ed.; University of California Press: Riverside, CA, Vol. 1.

(20) Kim, J. J.; Ben-Yehoshua, S.; Shapiro, B.; Henis, Y.; Carmeli, S. Accumulation of scoparone in heat-treated lemon fruit inoculated with *Penicillium digitatum* Sacc. *Plant Physiol.* **1991**, *97*, 880–885.

(21) Root, R. K.; Metcalf, J.; Oshino, N.; Chance, B. H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantification and some regulating factors. *J. Clin. Invest.* **1975**, *55*, 945–955.

(22) Ali, M. K.; Lepoivre, P.; Semal, J. Fosetyl-Al treatment of *Phytophthora citrophthora* releases a higher scoparone elicitor activity from a fosetyl-Al sensitive strain than from an insensitive mutant. *Fruits* **1991**, *46*, 51–55.

(23) Macarisin, D.; Cohen, L.; Eick, A.; Rafael, G.; Belausov, E.; Wisnieski, M.; Droby, S. *Penicillium digitatum* suppresses production of hydrogen peroxide in host tissue during infection of citrus fruit. *Phytopathology* **2007**, *97*, 1491–1498.

(24) Jabs, T.; Tschope, M.; Colling, C.; Halbrock, K.; Scheel, D. Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 4800–4805.

(25) Bach, M.; Seitz, H. U. Elicitor-induced defence response of a suspension-cultured woody plant (*Larix deciduas*) and possible mechanisms of signal transduction. *Can. J. Bot.* **1997**, *75*, 1243–1250.

(26) Doke, N. Involvement of superoxide anion generation in hypersensitive response of potato tuber tissues to infection with an incompatible race of Phytophthora infestans and to hyphal cell wall components. *Physiol. Plant Pathol.* **1983**, *23*, 345–357.

(27) Epperlein, M. M.; Noronha-Dutra, A. A.; Strange, R. N. Involvement of the hydroxyl radical in the abiotic elicitation of phytoalexins in legumes. *Physiol. Mol. Plant Pathol.* **1986**, *28*, 67–77.

(28) Rogers, K.; Albert, F.; Anderson, J. Lipid peroxidation is a consequence of elicitor activity. *Plant Physiol.* **1988**, *86*, 547–553.

(29) Guo, Z.; Nakagawara, S.; Sumitani, K.; Ohta, Y. Effect of intracellular glutathione level on the production of 6-methoxymellin in cultured carrot cells. *Plant Physiol.* **1993**, *102*, 45–51.

(30) Degousee, N.; Triantaphylides, C.; Montillet, J. Involvement of oxidative processes in the signaling mechanisms leading to the activation of glyceollin synthesis in soybean (*Glycine max*). *Plant Physiol.* **1994**, *104*, 945–952.

(31) Devlin, W. S.; Gustine, D. L. Involvement of the oxidative burst in phytoalexin accumulation and the hypersensitive reaction. *Plant Physiol.* **1992**, *100*, 1189–1195.

(32) Davis, D.; Merida, J.; Legendre, L.; Low, P. S.; Heinstein, P. Independent elicitation of the oxidative burst and phytoalexin formation in cultured plant cells. *Phytochemistry* **1993**, *32*, 607–611.

(33) Rusterucci, C.; Stallaert, V.; Milat, M.-L.; Pugin, A.; Ricci, P.; Blein, J.-P. Relationship between active oxygen species, lipid peroxidation, necrosis and phytoalexin production induced by elicitins in *Nicotiana*. *Plant Physiol.* **1996**, *111*, 885–891.

(34) Anderson, A. J.; Rogers, K.; Tepper, C. S.; Blee, K.; Cardon, J. Timing of molecular events following elicitor treatment of plant cells. *Physiol. Mol. Plant Pathol.* **1991**, *38*, 1–13.

(35) Vera-Estrella, R.; Blumwald, E.; Higgins, V. J. Effect of specific elicitors of *Cladosporium fulvum* on tomato suspension cells. *Plant Physiol.* **1992**, *99*, 1208–1215.

(36) Kondo, Y.; Hanawa, T.; Miyazawa, T.; Mizutani, J. Detection of rapid and transient generation of activated oxygen and phospholipid hydroperoxide in soybean after treatment with fungal elicitor by chemiluminescence assay. In *Mechanisms of Plant Defense Response*; Fritig, B., Legrand, M., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1993; pp 148–151.

(37) Kuc', J. Induced systemic resistance. In *Induced Resistance to Disease in Plants*; Hammerschmidt, R., Kuc', J., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1995; pp 169–175.

(38) Kavanagh, J. A.; Wood, R. K. S. The role of wounds in the infection of oranges by *Penicillium digitatum* Sacc. *Ann. Appl. Biol.* **1967**, *60*, 375–383.

(39) Turrell, F. M.; Klotz, L. J. Density of stomata and oil glands and incidence of water spot in the rind of Washington navel orange. *Bot. Gaz.* **1940**, *101*, 862–871.

(40) Shomer, I.; Erner, Y. The nature of oleocellosis in citrus fruits. *Bot. Gaz.* **1989**, *150*, 281–288.

(41) Arimoto, Y.; Sugawara, F.; Yoshida, S.; Yamaguchi, I. Prangolarin is a chemical facilitator for the enhanced development of the infection process in the epicarp of *Citrus limon* by *Penicillium digitatum*. *J. Agric. Food Chem.* **1995**, *43*, 2283–2285.

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