American Journal of Essential Oils and Natural Products

Available online at www.essencejournal.com

AJEONP American Journal of Essential Oils and Natural Products

ISSN: 2321-9114
AJEONP 2020; 8(4): 11-18

Received: 07-08-2020
Accepted: 18-09-2020

Shimshon Ben-Yehoshua
Department of Post-harvest Science, Agricultural Research Organization (ARO), The Volcani Center, Rishon LeZion, Israel

Beatrice Nafussi
Department of Post-harvest Science, Agricultural Research Organization (ARO), The Volcani Center, Rishon LeZion, Israel

Michael Grabarnick
Chemical R&D Adama Agricultural Solutions, Be'er Sheva, Israel

Victor Rodov
Department of Post-harvest Science, Agricultural Research Organization (ARO), The Volcani Center, Rishon LeZion, Israel

Corresponding Author:
Victor Rodov
Department of Post-harvest Science, Agricultural Research Organization (ARO), The Volcani Center, Rishon LeZion, Israel

Chlorophyll-catalyzed photooxidation of limonene determines the interaction of citrus fruit with a wound pathogen

Shimshon Ben-Yehoshua, Beatrice Nafussi, Michael Grabarnick and Victor Rodov

Abstract
Citrus exocarp (flavedo) contains numerous cavities filled with essential oil comprising predominantly the monoterpene hydrocarbon limonene. Limonene discharge from ruptured cavities is known to stimulate the wound pathogen *Penicillium digitatum*, the major cause of citrus postharvest losses. Limonene content reaches maximum in full-sized green-colored (mature-green) citrus fruit. However, in spite of their high limonene content, the mature-green fruit are less prone to decay than the fully colored ones. Mechanisms counteracting the decay-promoting effect of limonene in mature-green citrus were investigated in this work. Two antifungal spots were detected by bioautography assay in the flavedo extract of mature-green lemon, but not of the yellow one. Limonene hydroperoxides (LHP) were identified in these spots. The LHP were absent in intact oil cavities. They were generated by the interaction of the cavities' content with the ingredients of green flavedo, but only under light. Activity-driven fractioning of the deterpenated green flavedo extract revealed chlorophyll and phaeophytin as photosensitizers catalyzing the LHP generation. We suggest that the light-dependent conversion of limonene into potent antifungal and defense-activating LHP may neutralize and even reverse the disease-promoting effect of limonene. Aqueous 1% LHP emulsions showed efficacy comparable with synthetic fungicides in controlling decay of artificially pathogen-inoculated agricultural produce.

Keywords: flavedo, essential oil, monoterpenes, lemon, wounding, photodynamics, *Penicillium digitatum*, green mold, organic hydroperoxides, fruit-pathogen relations, decay control, fungicides

1. Introduction

The external colored layer of citrus peel-flavedo (exocarp)-contains numerous cavities (glands) filled with essential oil comprising various ingredients, predominantly monoterpenes. Mechanical wounding of the fruit results in the release of the cavities' content. Several authors suggested that the content of the oil cavities is involved in fruit-pathogen interaction. However, the nature of this interaction is disputable, especially for different oil ingredients. Some compounds, such as the monoterpene aldehyde citral, exert potent inhibition towards a wound pathogen *Penicillium digitatum* Sacc. [1], a causative agent of the green mold disease, the major cause of postharvest losses in citrus [2]. Citral was suggested to be involved in antifungal defense of mature-green lemon fruit [3]. At the same time, other citrus oil constituents may stimulate the development of *P. digitatum*. Furthermore, it was shown that even the same compound may either suppress or enhance pathogen development depending on its concentration, and the balance between the inhibitory and the stimulatory activities depends on chemical nature of the compound. Thus, in low concentrations ranging between 0.005 and 0.01 µL plate^{-1}citral stimulated the germination of *P. digitatum* conidia but completely inhibited this process in the doses of 0.05 µL plate^{-1} or higher [31]. On the other hand, stimulatory concentrations of the monoterpene alcohol linalool ranged between 0.5 to 2 µL plate^{-1}, while its minimal inhibitory dose was as high as 5 µL plate^{-1}. Similar dose-dependent shift from stimulation to inhibition of *P. digitatum* was demonstrated for neryl acetate [3].

The monocyclic hydrocarbon limonene (1-methyl-4-(prop-1-en-2-yl) cyclohex-1-ene) is the most abundant citrus monoterpene typically accounting for more than 90% of the oil. Pure limonene has no antimicrobial activity [5]. Furthermore, limonene was shown to enhance the germination of *P. digitatum* spores [31, 6] and to facilitate the green mold development [7-8]. Down-regulation of limonene biosynthesis resulted in reduced susceptibility of transgenic oranges to *P. digitatum* [9] and enhanced induced defense mechanisms [10]. The content of D-limonene in the transgenic fruit inversely correlated with their resistance to pathogens [11].

These results support the understanding that the release of limonene-rich oil gland content encourages the fungal decay of wounded citrus fruit, even if some less abundant ingredients (e.g. citral) are fungitoxic. However, this apparently clear picture leaves some questions unanswered. In particular, limonene content in the peel of full-size green-colored (mature-green) oranges was insignificantly different or even slightly higher [9-10] than in the fully colored mature fruit. If limonene unconditionally determines the *P. digitatum* sensitivity, one might expect the mature-green citrus to be prone to green mold disease at least to the same extent as fully colored fruit. In fact, the opposite is true; mature-green citrus is much less susceptible to *P. digitatum* than fully colored fruit at the stage of commercial maturity [2-3, 12-13] showing two- to eight-fold lower decay incidence. The explanations relating this phenomenon to somewhat higher citral content [3] or enhanced production of phytoalexin umbelliferone [13] seem insufficient, considering the overwhelming prevalence of limonene in citrus oil. Mechanisms counteracting the decay-promoting effect of high limonene content in mature-green citrus are still unknown.

In our previous work, a transient formation of limonene hydroperoxides (LHP) was demonstrated in the peel of mechanically wounded mature-green lemons [14]. The LHP were labile on the fruit surface and disappeared within antimicrobial activity, in particular towards *P. digitatum*. Ten minutes after oil gland injury, the LHP level on the surface of mature green lemon was sufficient to inhibit the fungal spores [14]. Furthermore, wounding the oil glands or injecting LHP into the lemon peel elicited the production of the citrus phytoalexins scoparone and scopoletin in amounts effective against *P. digitatum*. These phenomena were absent or less expressed in the peel of older yellow fruit. The production of hydroperoxides by photosensitized oxidation of limonene was reported earlier [15], without any relevance to their antifungal activity.

However, no explanation has been given until now on mechanisms of the LHP formation in citrus fruit, their relationship with fruit maturity and possible involvement in enhanced disease resistance of mature-green citrus fruit. In the present work, we investigate the LHP formation upon injury of citrus oil glands and test their potential for controlling postharvest spoilage of fresh agricultural produce caused by fungal pathogens.

2. Materials and Methods

2.1 Plant material

Lemon (*Citrus limon* (L.) Burm., cv. Eureka) served as an example of citrus fruits since due to the extended flowering period of this species it is harvested throughout the year [16] that allows simultaneously obtaining fruit of different maturity from the same tree. Mature-green and fully mature (bright-yellow) lemon fruit were obtained from an orchard in Palmachim, Israel. All experiments were done within one day after harvest and the fruit were surface sterilized by wiping with 70% ethanol, and air-dried.

2.2 Flavedo extraction

Samples of flavedo tissue (1 g) excised with a scalpel from green or yellow lemons were incubated in dichloromethane (DCM) at a ratio of 1:10 (weight/volume) for one day at room temperature. Then the tissue was homogenized in DCM by an Ultra Turrax TP 18/10 instrument (Janke and Kunkel, Staufen, Germany). The homogenate was filtered in vacuo through Whatman No. 1, dried with anhydrous magnesium sulfate and concentrated under vacuum on a rotary evaporator (Buchi, Flawil, Switzerland). For preparing light-exposed extract, the flavedo excision and further extraction was performed under white fluorescent light of intensity 40 µmol m^{-2} s^{-1} (LI-185B instrument, LI-COR, Inc., Lincoln NE, USA) and the extracts were incubated in transparent glass containers at room temperature under the same illumination conditions. For obtaining the extracts with minimal light exposure, the extracts were prepared and treated under a dim light and kept in amber glass containers.

2.3 Oil gland content evacuation

The direct evacuation of the oil gland content from 0.8-cm lemon peel discs was performed using a stainless 26-gauge hypodermic needle (0.45×12mm) attached to a custom-made instrument comprising a luer-fitted polypropylene tube containing ca. 0.3 mL dichloromethane, connected to a potentiometer-regulated miniature micro-diaphragm air pump. The needle was gently inserted into a gland cavity and its content was drawn into dichloromethane by a weak suction force. The efficacy of the oil evacuation method was tested in preliminary trials and was proven to collect at least 90% of the essential oil content of the peel discs. After the oil gland content evacuation, the remainder nearly oil less discs were extracted with dichloromethane as described above.

2.4 Thin-layer chromatography and bio-autography assay

The DCM flavedo extracts were concentrated to the final volume of 1 mL, and 50-µL aliquots of these extracts were applied in duplicate onto thin-layer chromatography (TLC) plates Silica gel 60 F_{254} (Merck, Darmstadt, Germany). The plates were developed in DCM/ethyl acetate (2:98, v/v) system. After the development and drying the plates, the parallel duplicate TLC runs were separated. One-half of the plate was sprayed with 0.2% vanillin solution in concentrated sulfuric acid for visualization of the spots, in particular of terpenoid nature [17]. The other half was used for detection of the antifungal activity by a bioautography assay using *Cladosporium cladosporioides* as a test organism [14]. *Cladosporium cladosporioides* spore suspension in a Czapek-Dox medium (10^6 spores/mL) was sprayed on the plate that was subsequently incubated at 24 °C and high humidity for 2 days, to allow the fungus development on the plate. White areas indicated fungal growth inhibition due to the presence of antifungal materials. *Cladosporium* is typically used as a model object in this assay due to its capacity to grow uniformly and to sporulate on the surface of a TLC plate, in contrast to many other plant pathogens, e.g. *P. digitatum*.

2.5 GC/MS analysis

Extracts were analyzed on a Hewlett Packard G1800A GCD system fitted with a mass spectrometer. Using an auto-sampler, aliquots (1 µL) of test samples were injected in a spitless mode at 220 °C onto a 15m × 0.25mm, 5% phenyl/95% methyl silicone HP-5 capillary column (J&W Scientific, Folsom CA). Column temperature was held at 80 °C (2 min) and was programmed to increase, at 15 °C/min, to 160 °C, at which temperature the column was maintained for a further 10 min. Mass spectra were recorded at 70 eV. Mass range was from *m/z* 35 to 425. Library search was carried out using the Wiley GC/MS Library. Identification of limonene hydroperoxides (LHP) was performed by comparing the mass-spectra obtained with the data published by Schieberle *et al.* [15] using spectral contrast angle's cosines as similarity criteria [18]. The spectral contrast angle cosine (*cos* θ)

represents the degree of similarity between the experimental mass-spectra and the mass-spectra of limonene hydroperoxides published by Schieberle *et al*. [15]. The *cos* θ values vary between zero (no similarity) and one (ideal match).

2.6 Isolation of the photosensitizing agents from green lemon flavedo

Flavedo tissues of five green lemons were extracted with hexane (1:10 weight/volume ratio, Ultra Turrax TP 18/10 homogenizer) to remove the endogenous limonene. The deterpenated tissue residue was extracted with DCM as described above. The DCM extract was dried with anhydrous magnesium sulfate and concentrated *in vacuo* on a rotary evaporator. The DCM extract was loaded onto a silica gel column preliminarily equilibrated with DCM. The fractions were sequentially eluted from the column using 100-mL portions of the solvents of increasing polarity: DCM, ethyl acetate, ethanol and methanol, and concentrated to the volume of 20 mL. Purified limonene (10 µL) was added to 1-mL aliquots of the fractions, and their photosensitizing capacity was tested after overnight light exposure (40 µmol m^{-2} s^{-1}) by TLC-vanillin/sulfuric acid assay as described above. The fine fractionation of the DCM extract was performed by column chromatography using a series of eluents comprising DCM and ethyl acetate in the following ratios: 100:0, 90:10, 85:15, 80:20, 75:25, 50:50, 0:100. The fractions were collected from the column and tested for photosensitizing activity as described above. Further purification of the active fraction was performed by high-performance liquid chromatography (HPLC) using the Varian HPLC 5000 instrument (Varian, Inc., Palo Alto CA, USA) with reverse phase RP-18 LiChrosorb 250-10 (7µm) column (Merck, Darmstadt, Germany) and refractive index detector. DCM was used as the eluent with flow rate of 1 mL min^{-1}. The eluate fractions were collected according to the detector signals and evaluated for photosensitizing activity as described above. The UV-visible absorption spectra of the active fractions were recorded using the Uvikon 820 spectrophotometer (Kontron AG, Munich, Germany).

2.7 LHP synthesis by chlorophyll-catalyzed limonene photooxidation

Limonene (5 mL) purchased from Sigma Israel (Rehovot, Israel) was loaded onto a silica gel column (Kieselgel D, 400 mesh ASTM, Merck) preliminarily equilibrated with hexane. Pure limonene was washed from the column with 50 mL hexane while the impurities (including the autoxidation products) were kept in the column and could be further eluted with DCM. Hexane was removed on a rotary evaporator, and the purified limonene (500 µL) was dissolved in 1.5 mL of absolute ethanol. Chlorophyll (50 µL) extracted from the leaves of bitter orange *Citrus aurantium* [19] was added to the limonene solution to reach the final chlorophyll concentration of 2.2 µg mL^{-1}. The control samples included limonene and chlorophyll solutions alone. The solutions were incubated for 3 h under light (40 µmol m^{-2} s^{-1}) or in the dark. Afterwards, 2-µL aliquots of the solutions were loaded onto TLC plates and analyzed as described above, with spot visualization by vanillin assay and activity detection by bioautography.

2.8 Preparing LHP by sun exposure of limonene or flavedo crude extract

Limonene was purified by column chromatography as presented above. The purified limonene (5 mL) was placed in a glass Petri dish and exposed for 3 h to direct sunlight of intensity 1500 µmol m^{-2} s^{-1}. The exposure resulted in a conversion of approximately 5% of the limonene into oxidation products, predominantly LHP.

The crude DCM flavedo extract was prepared as described above, and exposed to sunlight for about 14 hours. The extract was concentrated by evaporating the solvent and cleaned by passing through a silica gel 60 column using DCM as a carrier. A green-colored fraction showing a LHP-typical positive reaction in vanillin-sulfuric acid assay was collected from the column and concentrated by solvent evaporation.

2.9 Chemical synthesis of LHP

Limonene hydroperoxides were synthesized by the heterogeneous catalytic method as described by Ben-Yehoshua *et al*. [14] in ethanolic solution, using sodium molybdate ($Na_2MoO_4 \bullet 2H_2O$) as a catalyst and hydrogen peroxide as an oxidation agent. The reaction was performed for 5-6 h with continuous stirring at 50 ºC under atmospheric pressure. Nearly complete conversion of limonene into the hydroperoxides was reached.

2.10 Testing the antifungal efficacy of LHP preparations *in vivo*: citrus fruit

The LHP-containing emulsions for testing the antifungal activity were prepared as follows. The LHP preparations (synthetic LHP or sun-treated flavedo crude extract) were mixed 1:1 with Tween-20 detergent and further diluted with 25% aqueous ethanol using a vigorous stirring. The diluted emulsions contained 0.25, 0.5 and 1% (w/v) of LHP preparations. The capacity of LHP-containing emulsions to control the green mold disease of citrus fruit *in vivo* was tested as described previously [5]. The lemon fruit were inoculated with *P. digitatum* by piercing the flavedo to a depth of 1.5 mm with a three-needle tool immersed in a fungal spore suspension (10^6 spores mL^{-1}) prior to each piercing [1]. After overnight incubation at 20 °C, the inoculated lemons were treated by 2-min dipping in aqueous LHP-containing emulsions. In the control, the inoculated fruit were dipped in water or in aqueous 25% ethanol-Tween-20 solution. For comparison, some samples were treated with emulsions containing 0.1% of synthetic fungicide imazalil. After drying, the dipped fruit were arranged in four replications of 20 lemons each, and stored at 20 °C in cartons covered with polyethylene bags. The disease development was evaluated daily by calculating the decay incidence (percentage of symptomatic fruit out of the total number of inoculated lemons in a replication) and the decay index. The decay index was calculated as a weighted average of the indices assigned to each individual fruit in a replication according to the scale: 0-healthy fruit, 1-soft lesion, 2-white mycelium, 3-green sporulation.

2.11 Testing the antifungal efficacy of LHP preparations *in vivo*

The 1.5-2 cm-thick discs were prepared from sweetcorn cobs. The discs were inoculated by spraying with a spore suspension of *Cladosporium herbarum* (an isolate from an infected sweetcorn cob), 10^5 spores mL^{-1}. After overnight incubation at 20 °C, the inoculated discs were treated by spraying with an emulsion prepared as described above, containing 0.1% synthetic LHP. In the control, the inoculated discs were sprayed with water or with aqueous 25% ethanol-Tween-20 solution. For comparison, some samples were treated with emulsions containing 0.1% citral prepared in the

same way as described above for LHP. In addition, the trial included non-inoculated control discs. The treated corn discs were arranged in three replications of six discs each, and stored at 20 °C in plastic trays covered with polyethylene bags. The fungal growth was evaluated after 5 days of storage by evaluating the surface darkening in the center of the disc measured as lightness (*Parameter* L) decline using a Chroma Meter CR-300 instrument (Konica-Minolta, Osaka, Japan). In addition, a visual fungal growth index was calculated as a weighted average of the indices assigned to each individual disc in a replication according to the scale from zero (no fungal growth, white disc center) to 4 (profound fungal growth, black disc center).

2.12 Statistical analysis
Experiments were repeated three times, each with at least four separate replicates. The results were subjected to Duncan's multiple range test. The arcsin transformation was performed for statistical analysis of decay incidence data.

3. Results
3.1 Detection of active compounds and their origin
The bioautography assay revealed several antifungal compounds in flavedo extract of mature-green lemons that were absent or negligible in the flavedo extracts of yellow fruit (Fig. 1A). Two major antifungal spots were stained in purple color by vanillin/sulfuric acid reagent (Fig.1B). For the sake of brevity, the two active fractions were encoded as V + (upper) and V + (lower).

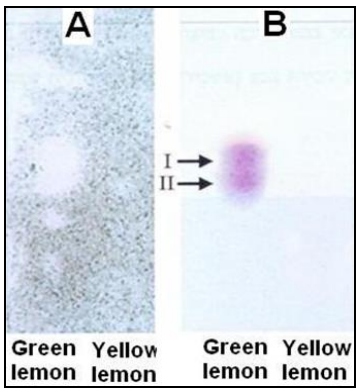

Fig 1: Detection of active antifungal compounds in citrus peel extracts by *Cladosporium* bioassay (A) and vanillin staining (B). The active fractions: I - V+ (upper); II – V+ (lower)

The combination of TLC with the vanillin assay was used for investigating the origin of these active compounds (Fig. 2). As mentioned above, they were absent in the peel of yellow fruit. Furthermore, they were not detected in the extract of green fruit prepared under faint light and kept strictly without light exposure, in containers wrapped with aluminum foil. Since vanillin stain is typical for terpenoid essential oil compounds, the active principles were expected to be located inside the oil cavities. However, direct collection of cavities' contents revealed no V + reaction, even after light exposure (Fig. 2). On the other hand, extraction of the green lemon's flavedo after evacuation of the oil cavities content showed upon illumination very limited V + response. At the same time, the intense reaction occurred when such oil less green flavedo extract was combined with the content of the oil cavities and exposed to light. Furthermore, substituting the oil cavities content with pure limonene showed the same light-dependent reaction. Thus, it was shown that the active V + principles were not present in the lemon peel as preformed compounds, but were generated through the light-dependent interaction of limonene from the oil cavities with the compound(s) present in the green flavedo outside the cavities. Most probably, the latter compound(s) acted as a photosensitizer in the light-limonene interaction.

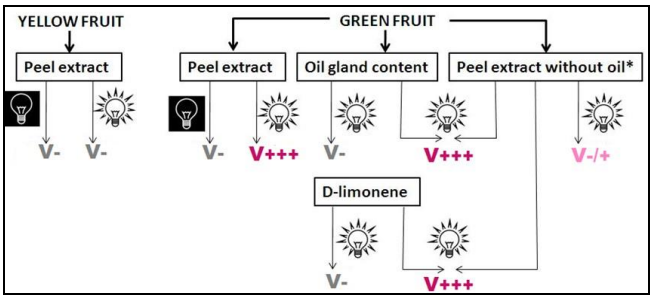

Fig 2: Effect of light, fruit maturity and peel components on the generation of active antifungal compounds in citrus peel extracts

3.2 Analysis of the active compounds
The GC/MS analysis of the compounds eluted from the active spots revealed limonene hydroperoxides and other products of monoterpene oxidation. As an example, Fig. 3 presents the gas chromatogram of the compounds eluted from the lower V+ spot. The structures of the compounds identified in the active spots are shown in Fig. 4. In addition, the mass-spectra of the limonene hydroperoxides are presented in Table 1. They were practically identical to the spectra of some hydroperoxides described by Schieberle *et al.* [15] as products of limonene photooxidation catalyzed by the synthetic photosensitizer Rose Bengal. The lower V+ spot contained also the product of chlorophyll degradation phytol.

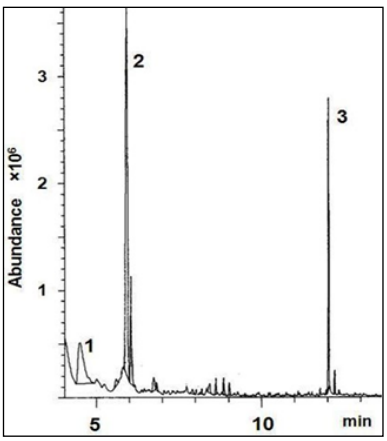

Fig 3: Gas chromatogram of the compounds isolated from the lower V+ spot: 1-4-isopropyl-2-cyclohexenone (cryptone); 2-(1*R*, 4*R*)-*p*-mentha-2, 8-diene-1-hydroperoxide; 3-phytol

Table 1: Mass-spectra of limonene hydroperoxides identified in the antifungal fractions

#	V+ spot	Mol. ion	Major mass-spectrum ions	*Cos* θ	Identity
1	higher	152	53(19); 55(46); 65(8); 67(37); 69(16); 77(20); 79(46); 81(35); 91(30); 93(100); 105(10); 107(30); 109(10); 119(6); 135(7); 150(2)	0.98	(2*S*,4*R*)-*p*-mentha-[1(7),8]-diene-2-hydroperoxide
2	higher	152	51(12); 53(27); 55(73); 67(80); 69(42); 77(36); 79(95); 81(55); 89(55); 93(100); 95(32); 107(22); 109(33); 119(27); 123(19); 134(47); 135(37); 150(5)	0.95	(2*R*,4R)-*p*-mentha-[1(7),8]-diene-2-hydroperoxide
3	lower	152	53(13); 55(34); 65(11); 67(15); 69(13); 77(36); 79(59); 91(49); 93(100); 94(29); 107(80); 119(8); 121(14); 135(86)	0.98	(1*R*,4*R*)-*p*-mentha-2,8-diene-1-hydroperoxide

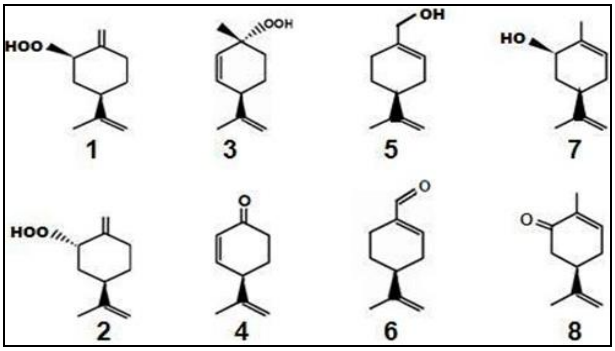

Fig 4: Limoneneoxidation products detected in the active spots

3.3 Isolation of photosensitizing compounds

Photosensitizing compounds were isolated from deterpenated dichloromethane extract of green flavedo by activity-driven fractioning using column chromatography and HPLC (Fig. 5). The capacity to facilitate the generation of V+ compounds in light-exposed limonene as detected by vanillin reagent stain served a marker for selecting the active fractions. The photosensitizing activity was present in the fraction eluted from the column with ethyl acetate or with dichloromethane/ethyl acetate (9:1) mix. Further HPLC fractioning isolated two active compounds, of green and yellowish color, identified by their absorption spectra as chlorophyll b and phaeophytin b. To confirm this identification, the generation of antifungal V+ compounds was reproduced by the interaction of limonene, chlorophyll and light (Fig. 6).

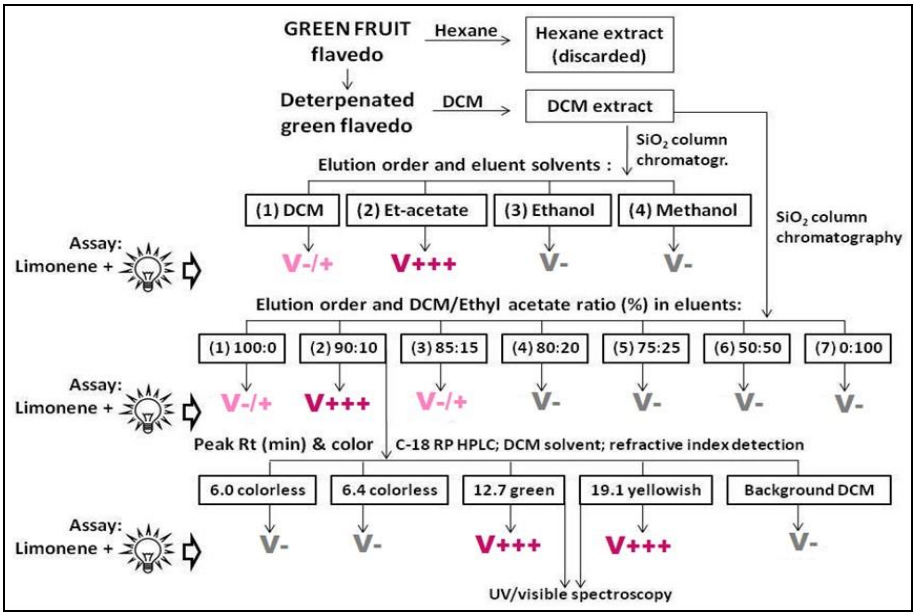

Fig 5: Activity-guided isolation of photosensitizing compounds

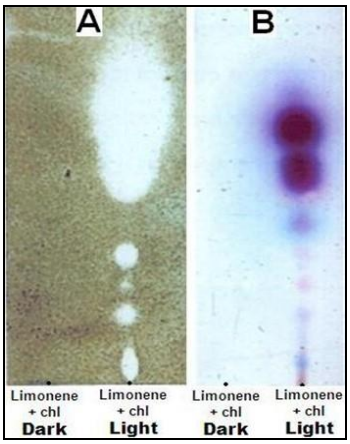

Fig 6: Synthetic reproduction of the antifungal vanillin-reactive compounds by chlorophyll-sensitized photooxidation of limonene. A-*Cladosporium* bioassay, B-vanillin assay

3.4 Controlling fungal pathogens by applying LPH preparations *in vivo*

The antifungal activity of the limonene photooxidation products was sufficient to control the decay development in lemon fruit inoculated with *P. digitatum*. Figure 7 shows that treating the inoculated fruit with aqueous emulsion containing 25% ethanol, 0.5% Tween-20 detergent and 5000 ppm (0.5%) of sun-treated dichloromethane crude flavedo extract of green lemons was twice as efficient in decay control as the 25% ethanol alone. Increasing the crude extract concentration to 1% resulted in complete decay prevention in inoculated lemons stored for 16 days at 20 °C. This efficacy was comparable with that of 0.2% of the synthetic fungicide imazalil.

Furthermore, efficient decay control was demonstrated by the solution containing synthetic LHP prepared by heterogeneous catalytic oxidation of limonene (Fig. 8). The same solution

was helpful for controlling fungal development and preventing blackening of sweet corn slices inoculated with *Cladosporium herbarum* (Fig. 9).

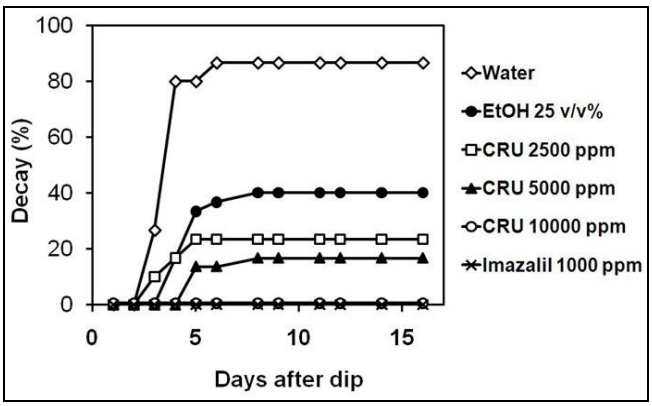

Fig 7: The effect of the emulsion containing sun-treated dichloromethane crude extract (CRU) from green lemon flavedo, on decay percentage in *P. digitatum*-inoculated lemon fruit

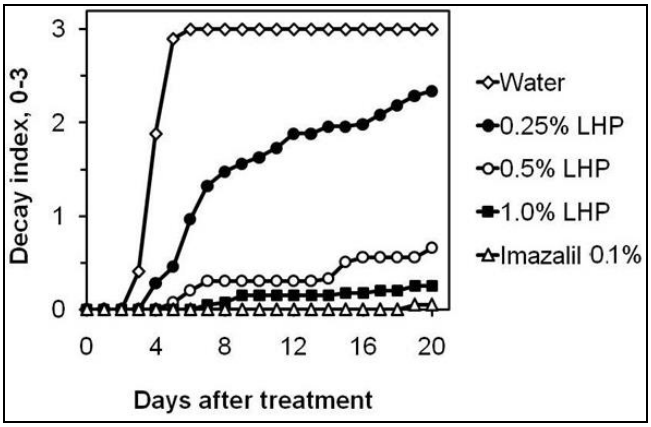

Fig 8: The effect of synthetic limonene hydroperoxides (LHP) on the decay percentage of *P. digitatum*-inoculated lemon fruit

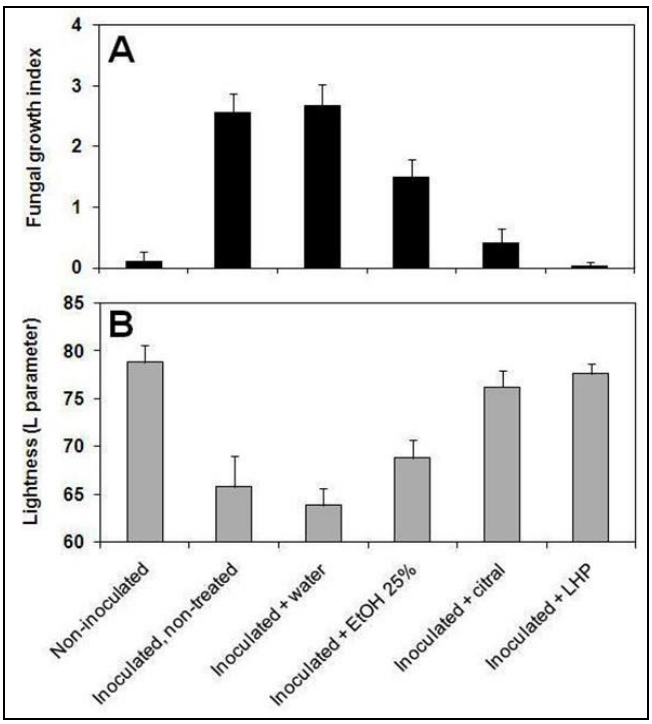

Fig 9: Effect of synthetic limonene hydroperoxides on fungal development on *Cladosporium*-inoculated sweet corn discs

4. Discussion

This study has revealed a novel pathway generating antifungal materialsin the citrus peel from preexisting non-toxic compound. This newly described pathway may explain the controversy between high limonene content in mature-green citrus and its reduced susceptibility to *P. digitatum*, as compared to fully colored commercially mature fruit. We suggest that presence of chlorophyll in the peel of the mature-green citrus may be one of the factors contributing to this phenomenon. Limonene release from the ruptured oil cavities plays a critical role in encouraging the development of green mold infection in fully mature citrus [7-8, 10]. However, photooxidation may prevent and even reverse the disease-facilitating effect of limonene by its conversion into potent antifungal hydroperoxide derivatives.

As shown in the present study, limonene may undergo spontaneous photooxidation also in the absence of chlorophyll, but then this process requires durable exposure to intense light, preferably comprising ultraviolet rays in its spectrum, e.g. sunlight. The photosensitizing effect of chlorophyll greatly enhances light sensitivity of limonene, allowing generation of limonene hydroperoxide seven upon a brief exposure to diffuse indoor illumination (almost 40 times as weak as the outdoors sunlight at noon). Shomer and Erner [20] demonstrated that essential oil from ruptured oil cavities of mechanically wounded green-colored citrus fruit gets in contact with chloroplasts, affecting their structure and resulting in the oleocellosis disorder.

Transient generation of limonene hydroperoxides on the surface of wounded mature-green lemons was reported in our previous publication [14]. The present work explains for the first time the mechanism of this generation and its relationship with fruit maturity based on the photosensitizing effect of chlorophyll in mature-green fruit. Chlorophyll b and phaeophytin b were found in this study as photosensitizing agents in the green lemon flavedo extract. However, we believe that both chlorophyll a and chlorophyll b, as well as their derivatives, possess this activity, and isolation of less polar chlorophyll b as the major photosensitizing agent was just due to the extraction procedure applied.

Up to date, two major types of defense mechanisms were described in plant host-pathogen relations and in particular in citrus fruit: preformed and induced ones [1]. The phenomenon described in this paper cannot be associated with any of these types. LHP are absent in the peel of non-challenged citrus fruit and obviously are not preformed compounds. On the other hand, the induced defense mechanisms e.g. production of phytoalexins, PR-proteins and lignin, are associated with cascades of signal transduction and gene expression [21], typically being manifested one to two days after the challenge. Therefore, realization of disease-controlling potential of these delayed responses in mature fruit depends on additional factors (e.g. heat treatment) that slow down the pathogen growth and change the force balance in favor of fruit [22]. In contrast to the induced defense mechanisms, chlorophyll-catalyzed LHP generation is an instant non-enzymatic reaction involving no gene expression. It allows immediate "disinfection" of the wound by reducing the viability of fungal spores around the ruptured oil cavity [14]. Furthermore, LHP are capable of eliciting the induced defense mechanisms e.g. phytoalexin production [14]. Thus, this newly described photodynamic process brings together the preformed and induced defenses, converting a constitutive oil ingredient (limonene) into active LHP derivatives that, on one hand, exert direct antifungal effect on the pathogen and, on

the other hand, elicit the induced protective mechanisms. This consolidated defense may be the basis of the enhanced resistance of mature-green citrus fruit against *P. digitatum*.

Organic hydroperoxides, including LHP, belong to the category of reactive oxygen species (ROS). Like other ROS, the organic hydroperoxides possess antimicrobial activity [23] and at the same time function as signaling molecules, in particular in plant-microorganisms interaction [24]. Antibacterial activity was reported in particular for the hydroperoxides derived from monoterpene hydrocarbons, including limonene [25]. Furthermore, it has been recognized relatively recently that antimicrobial properties ascribed to limonene as an active ingredient of some industrial formulations are in fact due to the spontaneous generation of limonene hydroperoxides [26]. Photosensitizing activity of chlorophyll and its derivatives has been also known and implemented, in particular, in photodynamic therapy [27].However, to the best of our knowledge, it is the first time that photooxidation is considered a part of natural plant-pathogen interaction. Our work does not dispute the concept of limonene being a natural decay facilitator aimed to stimulate seed release from fully mature citrus fruit [9-11]. It addresses just the special case of mature green fruit that maintain relatively low disease susceptibility in spite of their high limonene content.

Practical outcomes of this work deserve special attention. Nature-based solutions are extensively sought nowadays as sustainable alternatives for synthetic biocides [28-29]. In view of this, generating potent antimicrobial compounds *in situ* by interaction of three non-toxic factors-limonene, chlorophyll and light-may be of special interest. The experimental results presented in this work demonstrate high efficacy of LHP compounds in controlling plant pathogens. On the other hand, their potential practical implementation should consider the contact allergenicity of limonene hydroperoxides [30]. Methods and areas of application minimizing the potential undesirable effects should be elaborated, e.g. generation of LHP directly on the surface of treated objects with fast subsequent degradation. We believe that optimal application methods will allow practical utilization of the antimicrobial potential of LHP revealed in this work.

References

1. Ben-Yehoshua S, Rodov V, Kim JJ, Carmeli S. Preformed and induced antifungal materials of citrus fruits in relation to enhancement of decay resistance by heat and ultraviolet treatment. Journal of Agricultural and Food Chemistry. 1992; 40(7):1217-1221.
2. Eckert JW, Eaks IL. Postharvest disorders and diseases of citrus fruits. In: Reuther W, Calavan EC, Carman GE (Eds.), The citrus industry, UC Press, Berkeley CA. 1989; 5:179-260.
3. Rodov V, Ben-Yehoshua S, Fang DQ, Kim JJ, Ashkenazi R. Preformed antifungal compounds of lemon fruit: citral and its relation to disease resistance. Journal of Agricultural and Food Chemistry. 1995; 43(4):1057-1061.
4. Teshome Y, Leweye A. A review on the status of post-harvest loss in vegetable crop and their management options. Int. J Agric. Food Sci. 2020;2(1):31-40. DOI: 10.33545/2664844X.2020.v2.i1a.32
5. Rodov V, Nafussi B, Ben-Yehoshua S. Essential oil components as potential means to control *Penicillium digitatum* Pers. (Sacc.) and other postharvest pathogens of citrus fruit. Fresh Produce. 2011; 5(1):43-50.
6. Eckert JW, Ratnayake M. Role of volatile compounds from wounded oranges in induction of germination of *Penicillium digitatum* conidia. Phytopathology. 1994; 84(7):746-750.
7. Arimoto Y. Chemical facilitator for infection of epicarp of *Citrus limon* by *Penicillium digitatum*. Japanese Journal of Phytopathology. 1996; 62(6):593-596.
8. Arimoto Y. Limonene: chemical facilitator for the enhanced development of infection in the epicarp of *Citrus limon* by *Penicillium digitatum*. In: Manicom B, Robinson J, Du Plessis SF, Joubert P, Van Zyl JL, Du Preez S. (Eds.), Proceedings of the International Society of Citriculture. 1996; 2:1197-1199.
9. Rodríguez A, San Andrés V, Cervera M, Redondo A, Alquézar B, Shimada T *et al*. Terpene down-regulation in orange reveals the role of fruit aromas in mediating interactions with insect herbivores and pathogens. Plant Physiology. 2011; 156(2):793-802.
10. Rodríguez A, Shimada T, Cervera M, Alquézar B, Gadea J, Gómez-Cadenas A *et al*. Terpene down-regulation triggers defense responses in transgenic orange leading to resistance against fungal pathogens. Plant Physiology. 2014; 164(1):321-339.
11. Rodríguez A, Shimada T, Cervera M, Alquézar B, Gadea J, Gómez-Cadenas A *et al*. Resistance to pathogens in terpene down-regulated orange fruits inversely correlates with the accumulation of D-limonene in peel oil glands. Plant Signaling & Behavior. 2015; 10(6):e102-8704.
12. Kaplan HJ, Dave BA. The current status of imazalil: a postharvest fungicide for citrus. Proceedings of the Florida State Horticultural Society. 1979; 92:37-43.
13. Afek U, Orenstein J, Carmeli S, Rodov V, Bar Joseph M. Umbelliferone, a phytoalexin associated with resistance of immature Marsh grapefruit to *Penicillium digitatum*. Phytochemistry. 1999; 50(7):1129-1132.
14. Ben-Yehoshua S, Rodov V, Nafussi B, Feng X, Yen J, Koltai T *et al*. Involvement of limonene hydroperoxides formed after oil gland injury in the induction of defense response against *Penicillium digitatum* in lemon fruit. Journal of Agricultural and Food Chemistry. 2008; 56(6):1889-1895.
15. Schieberle P, Maier W, Firl J, Grosch W. HRGC separation of hydroperoxides formed during the photosensitized oxidation of (R)-(+)-Limonene. Journal of High Resolution Chromatography. 1987; 10(11):588-593.
16. Carr MKV. The water relations and irrigation requirements of citrus (*Citrus* spp.): a review. Experimental Agriculture. 2012; 48(3):347-377.
17. Debenedetti SL. TLC and PC. In: Sampietro DA, Catalan CAN, Vattuone MA (Eds.), Isolation, identification and characterization of allelochemical/natural products. CRC Press, Boca Raton FL, 2009, 103-134.
18. Eidhammer I, Flikka K, Martens L, Mikalsen SO. Computational Methods for Mass Spectrometry Proteomics. John Wiley & Sons, Hoboken NJ, 2008, 296.
19. Amir-Shapira D, Goldschmidt EE, Altman A. Chlorophyll catabolism in senescing plant tissues: *in vivo* breakdown intermediates suggest different degradative pathways for citrus fruit and parsley leaves. Proceedings of the National Academy of Sciences of the United States of America. 1987; 84(7):1901-1905.
20. Shomer I, Erner Y. The nature of oleocellosis in citrus fruits. Botanical Gazette. 1989; 150(3):281-288.
21. Ballester AR, Izquierdo A, Lafuente MT, González-Candelas L. Biochemical and molecular characterization

of induced resistance against *Penicillium digitatum* in citrus fruit. Postharvest Biology and Technology. 2010; 56(1):31-38.
22. Nafussi B, Ben-Yehoshua S, Rodov V, Peretz J, Ozer BK, D'hallewin G. Mode of action of hot water dip in reducing decay of lemon fruit. Journal of Agricultural and Food Chemistry. 2001; 49(1):107-113.
23. Setlow B, Setlow CA, Setlow P. Killing bacterial spores by organic hydroperoxides. Journal of Industrial Microbiology and Biotechnology. 1997; 18(6):384-388.
24. Nanda AK, Andrio E, Marino D, Pauly N, Dunand C. Reactive oxygen species during plant-microorganism early interactions. Journal of Integrative Plant Biology. 2010; 52(2):195-204.
25. Chalchat JC, Chiron F, Garry RP, Lacoste J, Sautou V. Photochemical hydroperoxidation of terpenes. Antimicrobial activity of α-pinene, β-pinene and limonene hydroperoxides. Journal of Essential Oil Research. 2000; 12(1):125-134.
26. Chubukov V, Mingardon F, Schackwitz W, Baidoo EE, Alonso-Gutierrez J, Hu Q *et al*. Acute limonene toxicity in *Escherichia coli* is caused by limonene hydroperoxide and alleviated by a point mutation in alkyl hydroperoxidase AhpC. Applied Environmental Microbiology. 2015; 81(14):4690-4696.
27. Brandis AS, Salomon Y, Scherz A. Chlorophyll sensitizers in photodynamic therapy. In: Grimm B, Porra RJ, Rudiger W, Scheer H (Eds.), Chlorophylls and bacteriochlorophylls. Springer, Dordrecht, 2006, 461-483.
28. Castillo F, Hernández D, Gallegos G, Rodríguez R, Aguilar CN. Antifungal properties of bioactive compounds from plants. In: Dhanasekaran D (Ed.), Fungicides for plant and animal diseases. Intech Open, Rijeka, Croatia, 2012, 81-106. DOI: 10.5772/26598
29. Al-Samarrai G, Singh H, Syarhabil M. Evaluating eco-friendly botanicals (natural plant extracts) as alternatives to synthetic fungicides. Annals of Agricultural and Environmental Medicine. 2012; 19(4):673-676.
30. Karlberg AT, Shao LP, Nilsson U, Gäfvert E, Nilsson JLG. Hydroperoxides in oxidized d-limonene identified as potent contact allergens. Archives of Dermatological Research. 1994; 286(2):97-103.
31. Droby S, Eick A, Macarisin D, Cohen L, Rafael G, Stange R, *et al*. Role of citrus volatiles in host recognition, germination and growth of *Penicillium digitatum* and *Penicillium italicum*. Postharvest Biology and Technology. 2008; 49(3):386-396.