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Essential Oil Components as Potential Means to Control *Penicillium digitatum* Pers. (Sacc.) and Other Postharvest Pathogens of Citrus Fruit

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ABSTRACT

The studies were conducted on 37 compounds present in citrus essential oils, to test their activity against *Penicillium digitatum* Pers. (Sacc.) by three *in vitro* assays: agar diffusion, amended growth medium ("poisoned food") and vapor assay. The aliphatic alcohols 1-nonanol, 1-decanol and especially 1-octanol exhibited the highest activities against *P. digitatum*, as assayed by all the methods used. The terpenoid compounds perillalcohol, perillaldehyde, citral, terpineol, carveol and citronellol, as well as the aromatic compound cinnamaldehyde, which is not a component of citrus oil but was used as a reference material, exhibited high activity against *P. digitatum.* The hydrocarbons d-limonene and myrcene, and the esters octyl acetate, decyl acetate, geranyl acetate, neryl acetate and linalyl acetate did not inhibit this fungus. 1-Octanol, perillaldehyde, citral, perillalcohol and terpineol were fungicidal, whereas 1-decanol, 1-nonanol, carveol and citronellol were only fungistatic. 1-Octanol, 1-decanol, perillaldehyde and citral also showed antifungal activity against *Alternaria citri* Ell. et Pierce and *Penicillium italicum* Wehmer. In addition 1-octanol and perillaldehyde were active against *Geotrichum candidum* Link. The selected promising compounds 1-octanol and citral were further tested *in vivo* on *P. digitatum*-inoculated lemons as active ingredients of microbiocidal formulations. Application of formulations comprising 1-octanol and citral either separately or together inhibited decay of *P. digitatum*-inoculated lemons for three weeks after inoculation without visible phytotoxic damage or perceivable off-odor, although total control was not achieved. The results obtained demonstrated the potential of essential oil components as postharvest citrus fungicides.

Keywords: aliphatic alcohols and aldehydes, *Alternaria citri*, antifungal activity, esters, *Geotrichum candidum*, hydrocarbons, *in vitro*, *in vivo*, *Penicillium digitatum*, *P. italicum*, terpenoids
Abbreviations: GLM, general linear model; **MIC**, minimal inhibitory concentration; **PDA**, potato dextrose agar

INTRODUCTION

The flavedo of citrus fruit contains large quantities of essential oils compartmentalized inside the oil glands. The essential oil is composed of complex mixtures of chemical compounds that may vary within a species as a result of environmental and genetic factors, or with maturity. The composition of citrus peel oils has been studied for many years and the major components are generally established (Shaw 1977; Huet 1991). Chemically the components of essential oils fall into several distinct groups. The terpenoids are the most abundant and are present mostly as monoterpene hydrocarbons, both cyclic (e.g., limonene) and non-cyclic (e.g., myrcene), and as their oxygenated derivatives such as aldehydes (e.g., citral, perillaldehyde), alcohols (e.g., terpineol, perillalcohol), ketones (e.g, carvone), esters (e.g., geranyl acetate). Quantitatively, the monoterpene hydrocarbons are the predominant group in citrus essential oils: the cyclic monoterpene limonene comprises ~80-95% of the oils of citrus fruits, whereas many of the other compounds are present only as minor or trace constituents. The most important contribution to the oil's distinct flavor and fragrance comes from oxygenated terpenoid compounds; removal of the hydrocarbon fraction improves its quality and stability (Arce and Soto 2008; Danielski *et al.* 2008). The non-terpenoid compounds include many organic materials, such as aliphatic aldehydes (e.g., 1-octanal or 1-nonanal), alcohols (e.g., 1-octanol, 1- nonanol), esters (e.g., octyl acetate, nonyl acetate), as well as phenolics (e.g., coumarins and psoralens).

The antimicrobial activity of citrus peel oils has been known for a long time (Maruzzella and Liguori 1958; Subba *et al.* 1967; Dabbah *et al.* 1970) and its investigation is continued at present (Romano *et al.* 2005; Sharma and Tripathi 2006; Sharma and Tripathi 2008; Viuda-Martos *et al.* 2008; Chutia *et al.* 2009). The subject has been recently reviewed by Fisher and Phillips (2008). Application of natural compounds such as essential oils to control postharvest pathogens attracts attention because of the increasing concern on the health hazards of synthetic fungicide residues. *Penicillium digitatum* Pers. (Sacc.), the causative agent of the green mold disease, is the main postharvest citrus pathogen, specific to this genus that may account for most of the citrus decay in storage (Barkai-Golan 2001). Citrus oils were reported to inhibit *in vitro* the development of this fungus (Caccioni *et al.* 1998). However, the interaction of citrus oil with *P. digitatum* is complex. The major compound of citrus oils, limonene, was found to stimulate the pathogen's development (Arimoto 1996; Droby *et al.* 2008). At the same time, products of limonene oxidation and other oxygenated monoterpenes exhibit strong antimicrobial activity (Ben-Yehoshua *et al.* 2008). The monoterpene aldehyde citral was found to be one of the preformed antifungal materials in lemon peel (Ben-Yehoshua *et al.* 1992); its level was suggested to affect the fruit sensitivity to the green mold disease (Rodov *et al.* 1995). Although the *in vitro* activity of several essential oil components against *P. digitatum* was investigated in the past (Moleyar and Narasimham 1986; Caccioni and Guizzardi 1994; Caccioni *et al.* 1995), the information on this subject is still limited. More-

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over, high *in vitro* activity does not guarantee the efficient disease control *in vivo* due to the possible phytotoxicity of the essential oil compounds (Ben-Yehoshua *et al.* 1992; Plaza *et al.* 2004). Applying essential oil compounds within a formulation reducing their phytotoxicity resulted in high microbiocidal efficacy (Ben-Yehoshua 2001; Ben-Yehoshua and Rodov 2006).

The aim of the present study was to identify the compounds potentially suitable for use as a postharvest fungicide on citrus fruits. The work comprised two stages. At the first stage, three different *in vitro* assay techniques were used to evaluate 37 individual components of citrus essential oils for activity against *P. digitatum* and against additional citrus pathogenic fungi (*P. italicum*, *Geotrichum candidum* and *Alternaria citri*). At the second stage, the selected promising compounds were further tested *in vivo* as active ingredients of microbiocidal formulations on *P. digitatum*-inoculated lemons.

MATERIALS AND METHODS

Chemicals

All the essential oil components used in this study, except geraniol and nootkatone, were supplied by Sigma, Rehovot, Israel. Geraniol was obtained from Frutarom, Haifa, Israel and nootkatone from Aromor, Kibbutz Givat Oz, Israel. Potato dextrose agar (PDA) was obtained from Difco, USA.

Agar diffusion assay

The agar diffusion assay was performed in accordance with Maruzzella and Henry (1958). The PDA medium was prepared and sterilized. The medium was then cooled in a water bath to 50°C, prior to inoculation by the addition of a suspension of *P. digitatum* spores in sterile water. The final concentration of spores in the medium was 10^4 ml^{-1}. The medium was gently mixed to disperse the spores evenly and 15 ml were dispensed into each 90-mm-diameter Petri dish. A 5-mg sample of each substance to be tested was pipetted onto a sterile 1.3-cm antibiotic assay paper disc (Whatman, Piscataway, NJ, USA) which was placed on the center of the inoculated agar. The Petri dishes were incubated at 24°C for 2 days. Antifungal compounds created a fungal growth-free zone around the paper disc, and the antifungal activity was evaluated by calculating the area of growth inhibition according to the equation:

Growth inhibition area (cm^2) = $\pi (R_d+W)^2 - A_d$,

where R_d – the radius of the paper disc (0.65 cm), W- the width of the clear zone from the edge of the paper disc to the area of fungal growth, A_d – the area of the paper disc (1.3 cm^2). Actually the whole area of inhibition including the area of the paper disc was calculated, and then the disc area was subtracted to get the real area of inhibition. In case of total growth inhibition, maximal achievable inhibition area was 62.3 cm^2. The relationship between the concentration of the compound and its antifungal activity was tested for citral and for 1-octanol. In these experiments the amounts of compound placed on the paper discs were 2, 4, 6, 8, 10, and 20 mg.

Vapor assay

In order to test the effects of the compounds in the vapor phase, a slightly different version of the agar diffusion assay was used as described by Maruzzella and Sicurella (1960). The Petri dish was inverted and the paper disc was placed centrally on its lid, so as to prevent the direct transport into agar of the test compound by diffusion from the paper disc. The inoculated Petri dishes were incubated at 24°C for 2 days. Antifungal activity was monitored by visual evaluation of fungal growth and if a clear zone of inhibition was present, the area of this zone was calculated according to the equation:

Growth inhibition area (cm^2) = πR^2,

where R- radius of the fungal growth-free area.

Amended growth medium ("poisoned food") assay

The amended growth medium ("poisoned food") assay was performed in accordance with Grover and Moore (1962). Samples of the compounds to be tested, dissolved in 0.5 ml of acetone, were added to sterile Petri dishes (90 mm), each containing 15 mL of molten PDA (50°C), to a final concentration of 1 mg mL^{-1}. The dish was gently agitated to ensure even distribution of the test compound and the agar was then left to set. The dishes were inoculated with mycelial discs (8 mm) cut from an agar plate culture of *P. digitatum* which had not yet begun to sporulate. A mycelial disc was placed at the center of each test dish. In some trials, a drop (25 µL) of *P. digitatum* spore suspension (10^5 mL^{-1}) was placed in the center of each test dish instead of the mycelial disc. The dishes were held at 24°C and the diameter of the hyphal growth was measured after 7 days of incubation at this temperature. The antifungal activity was calculated as a percentage of that in the control (PDA dishes which contained only acetone at 1 mg mL^{-1}) according to the equation:

Growth inhibition (%) = 100% $(A_a - A_c)/A_a$, where

A_a - the area of fungal growth in the presence of acetone, A_c - the area of fungal growth in the presence of the test compound.

For any compound that totally inhibited the fungal growth after 7 days an additional test was performed in order to investigate whether the compound was fungicidal or fungistatic: the mycelial disc of inoculum from the assay dish was transferred to another dish that contained only PDA. Growth was then monitored over the next 5 days, and the compound was classified as fungistatic or fungicidal, respectively, according to whether growth had or had not occurred.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of a compound was evaluated by using an adaptation of the amended growth medium method. Each compound was incorporated into the agar growth medium in the following concentrations: 1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, and 0.025 mg mL^{-1}. Seven days after the usual inoculation the plates were examined to establish the lowest concentration of compound at which no growth had occurred.

Tests on additional fungal species

The agar diffusion method and the amended medium method were also used to compare seven of the test compounds against three other fungal pathogens of citrus fruit: *Penicillium italicum* Wehmer, *Geotrichum candidum* Link and *Alternaria citri* Ell. et Pierce. The same procedures were used as for *P. digitatum*, but with the inoculum of spores or mycelial plugs from cultures of the other relevant species.

In vivo application

Mature-green lemon fruit *Citrus limon* (L.) Burm. f. cv. 'Eureka' were harvested from Maslul (North-West Negev, Israel), brought to the laboratory on the day of harvest, washed with tap water and surface sterilized by wiping with 70% ethanol. The fruit were wound-inoculated by piercing the peel to a depth of 1.5 mm with a 3-needle instrument immersed in the spore suspension of *Penicillium digitatum* (10^4 spores mL^{-1}) as described by Ben-Yehoshua *et al*. (1992). After overnight storage at 20°C, the fruit were treated with 1-octanol and/or citral by dipping for 2 min in emulsion formulations reported to prevent the essential oil phytotoxicity, including 25% ethanol and 5000 ppm Tween-20 (Ben-Yehoshua 2001; Ben-Yehoshua and Rodov 2006). The control fruit were dipped in water with addition of 5000 ppm Tween-20 or in 25% ethanol with addition of 5000 ppm Tween-20. After drying the fruit were arranged in four replications of 20 fruit each, and stored at 20°C in cartons covered with plastic bags. The decay incidence was evaluated weekly.

Statistics

All tests were conducted at least in triplicate. Conventional statistical methods were used to calculate means, standard deviations and 95% *t*-confidence intervals by means of the Microsoft Office Excel spreadsheet. Significant differences between the results of *in vitro* assays were determined by Tukey's 5% HSD ("honestly significant difference") test following one-way ANOVA using the PASW Statistics 18 software (SPSS 2009). The results of the *in vivo* trial were analyzed by the GLM (General Linear Model) Repeated Measures procedure (SPSS 2009) and the critical HSD value calculated according to Urdan (2005).

RESULTS

Agar diffusion assay

Of the 37 compounds tested 19 showed a zone of total inhibition of *P. digitatum* growth after 48 hours of incubation (**Fig. 1**). The largest zones of inhibition were produced by the primary aliphatic alcohols 1-heptanol, 1-octanol, and 1-nonanol. 1-Octanol was particularly active, and caused almost total inhibition of growth. In contrast, the secondary alcohol 2-octanol was only slightly antifungal. The tertiary terpenoid alcohol linalool showed no activity at all. The primary aliphatic alcohols 1-hexanol and 1-decanol showed zero or low inhibitory activity. The aliphatic aldehydes octanal and nonanal also inhibited fungal growth, but to a markedly smaller extent than the corresponding alcohols (growth inhibition areas of 52.7 cm^2 and 8.3 cm^2 for 1-octanol and octanal; respectively; 14.6 cm^2 and 4.4 cm^2 for 1-nonanol and nonanal, respectively). Aldehydes with main chain of less than seven (hexanal) or more than 10 (undecanal and dodecanal) carbon units showed no activity against *P. digitatum*.

The most effective terpenoid compounds against *P. digitatum* growth were perillaldehyde, carvone and citral, but their inhibition activities were much lower than that of 1-octanol. The compounds perillalcohol, terpineol, terpinene-4-ol and carveol exhibited only weak inhibitory activities. The hydrocarbon compounds limonene and myrcene did not cause any inhibition at a concentration of 10^4 spores mL^{-1}. The acetate derivative compounds also showed no inhibition. The synthetic fungicide Imazalil showed strong activity with almost total inhibition of the fungus. This fungicide was used as a reference compound against which the antifungal activities of the natural compounds were compared. Another reference compound was cinnamaldehyde, a component of the essential oil of cinnamon, known for its antifungal potency (Moleyar and Narasimham 1986; Kurita *et al*. 1981). Cinnamaldehyde showed high antifungal activity, but 1-octanol was much more active, and in fact 1-octanol was as effective as imazalil in inhibiting *P. digitatum* growth in this assay.

A saturation curve described the correlation between the antifungal activity and the concentrations of citral and 1-octanol (**Fig. 2**). The antifungal activity increased with concentration of the compound until a certain saturation level was reached. The compound 1-octanol was much more active than citral in this test, and it totally inhibited the growth of *P. digitatum* at 8 mg/disc, while even 20 mg/disc of citral inhibited growth in an area of only 7.7 cm^2.

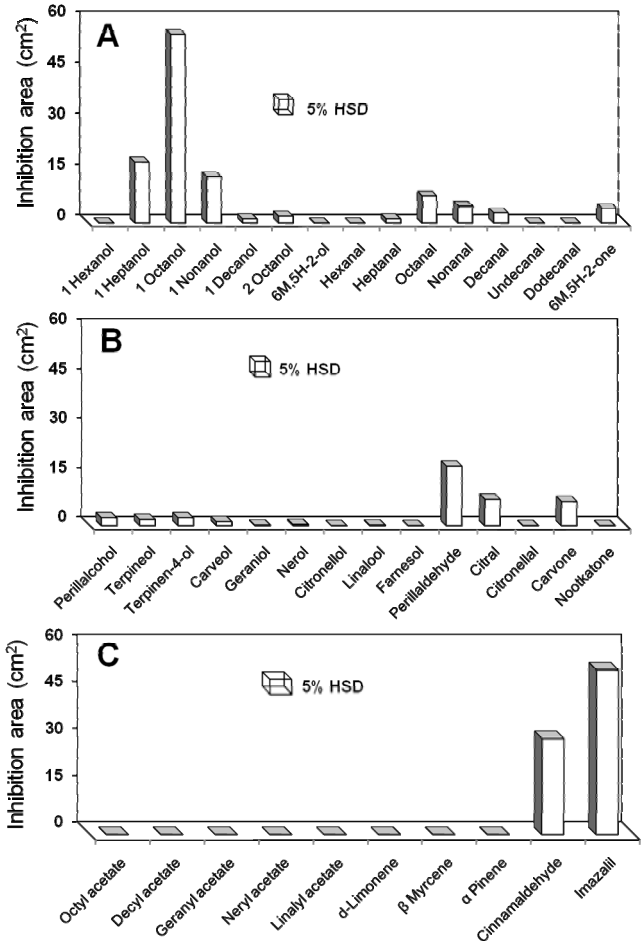

Fig. 1 The antifungal effect of essential oil compounds on *Penicillium digitatum* in the agar diffusion assay. (**A**) Aliphatic alcohols, aldehydes and ketone. (**B**) Terpenoid alcohols, aldehydes and ketones. (**C**) Aliphatic and terpenoid esters, terpenoid hydrocarbons and reference compounds cinnamaldehyde and imazalil. The abbreviations 6M,5H-2-ol and 6M,5H-2-one stand for 6-methyl,5-hepten-2-ol and 6-methyl,5-hepten-2-one, respectively. The results represent averages of three replications. The bar represents the 5% Tukey's HSD value.

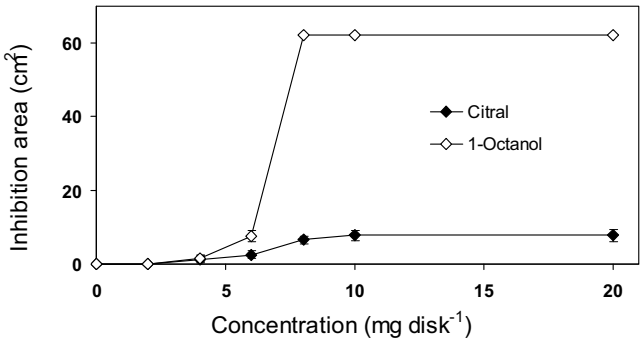

Fig. 2 The effect of 1-octanol and citral concentrations on the growth inhibition of *P. digitatum* in the agar diffusion assay. Bars indicate the 95% *t*-confidence intervals.

Vapor assay

In the agar diffusion assay the activities of the vapors of the compounds were markedly similar to those of their liquids, although there were some changes in the activity strength. The most active compounds were again the aliphatic alcohols 1-octanol and 1-nonanol, especially 1-octanol, with perilladehyde, carvone and citral being the most active of the terpenoid compounds (**Fig. 3**). The vapors of the aldehydes octanal and nonanal showed much lower inhibition activities than the corresponding alcohol vapors (octanal and 1-octanol inhibited the fungal growth in areas of 4.9 and 54 cm^2, respectively; and nonanal and 1-nonanol inhibited growth in 0.78 cm^2 and 28 cm^2, respectively). However, 1-nonanol was twice as active in a vapor phase as in a liquid one. Other compounds which showed stronger antifungal activity as vapors than as liquids were cinnamaldehyde (1.6-fold), citral (1.7-fold), carvone (1.9-fold), carveol (2.7-fold) and especially perillalcohol (3-fold). The compound nerol was active only as vapor. Other compounds, such as 1-heptanol, were more active as liquids; in particular imazalil was five times active as a liquid than as a vapor.

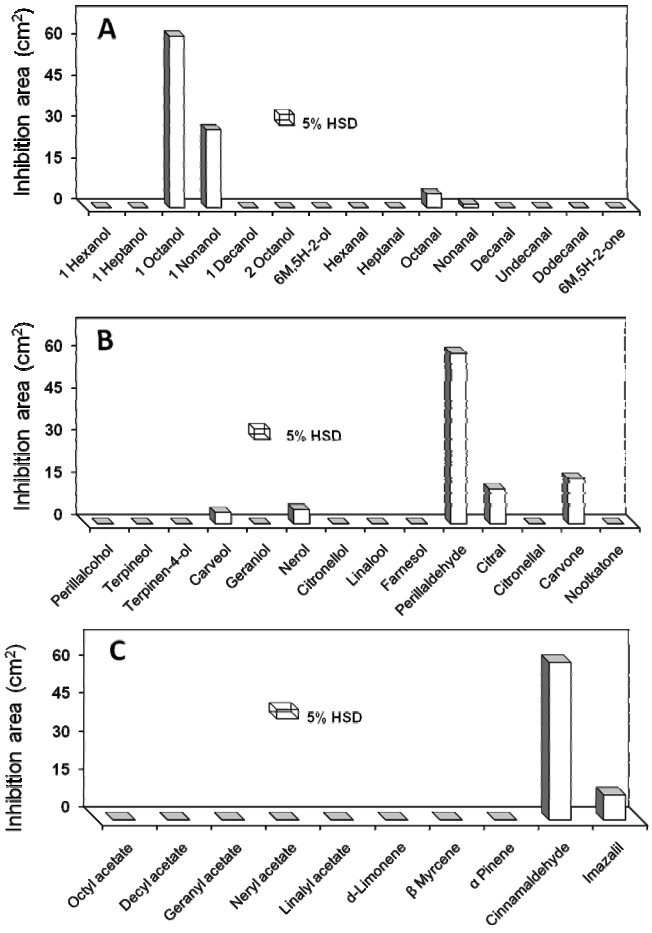

Fig. 3 The antifungal effect of essential oil compounds on *P. digitatum* in the vapor assay. (**A**) Aliphatic alcohols, aldehydes and ketone. (**B**) Terpenoid alcohols, aldehydes and ketones. (**C**) Aliphatic and terpenoid esters, terpenoid hydrocarbons and reference compounds cinnamaldehyde and imazalil. The abbreviations 6M,5H-2-ol and 6M,5H-2-one stand for 6-methyl,5-hepten-2-ol and 6-methyl,5-hepten-2-one, respectively. The results represent averages of three replications. The bar represents the 5% Tukey's HSD value.

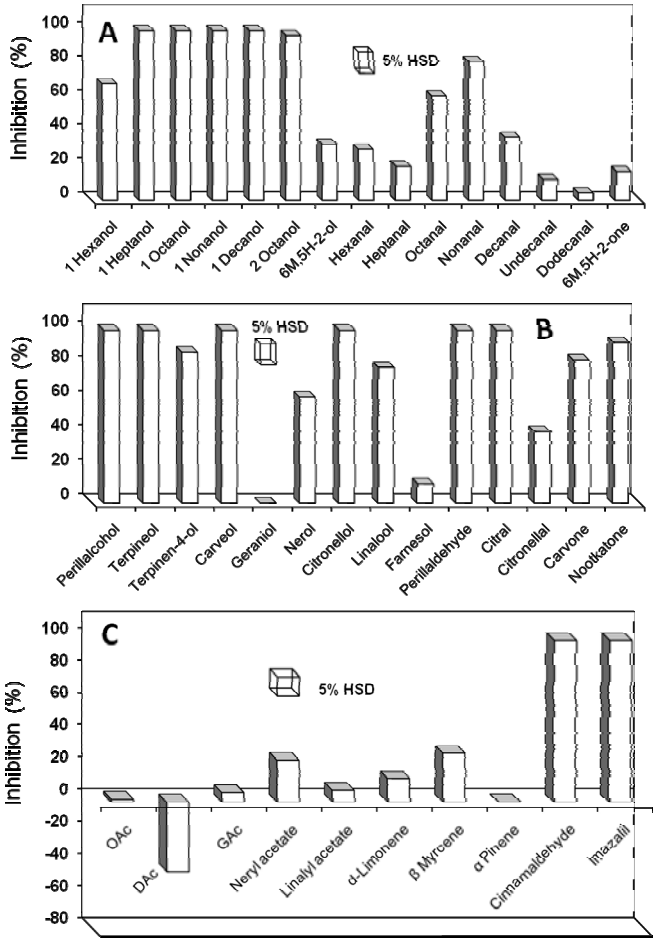

Fig. 4 The antifungal effect of essential oil compounds on *P. digitatum* in the amended growth medium assay. (**A**) Aliphatic alcohols, aldehydes and ketone. (**B**) Terpenoid alcohols, aldehydes and ketones. (**C**) Aliphatic and terpenoid esters, terpenoid hydrocarbons and reference compounds cinnamaldehyde and imazalil. The abbreviations 6M,5H-2-ol and 6M,5H-2-one stand for 6-methyl,5-hepten-2-ol and 6-methyl,5-hepten-2-one, respectively. The results represent averages of three replications. The bar represents the 5% Tukey's HSD value.

The compounds 1-octanol, perillaldehyde and cinnamaldehyde totally inhibited the growth of the fungus, and as vapors they were much more active than imazalil. The vapors of the hydrocarbons d-limonene and myrcene, as well those of all the esters had no effect at all on the growth of *P. digitatum* in this assay. In most cases, when the vapor of a compound produced a zone of total inhibition then it also inhibited the growth outside this zone (data not shown). An exception to this behavior was imazalil which allowed dense growth outside a sharply delineated zone of total inhibition. With several compounds, notably 1-heptanol, 1-decanol and 2-octanol, although there was no zone of total inhibition, the fungal growth on the plate was generally much less than that on the control (PDA only).

Amended growth medium assay

Four of the primary aliphatic alcohols – 1-heptanol, 1-octanol, 1-nonanol and 1-decanol – totally inhibited the growth of *P. digitatum* in this assay. The corresponding aliphatic aldehydes only partially inhibited the fungal growth (**Fig. 4**). Six terpenoid compounds also completely inhibited *P. digitatum* growth, they comprised four alcohols- perillalcohol, terpineol, carveol, citronellol- and two aldehydes, perillaldehyde and citral. The fungus was also totally inhibited by cinnamaldehyde and imazalil. Most of the other compounds tested allowed varying degrees of growth, but less than that in the control (acetone only). Decyl acetate was the only compound tested that actually stimulated *P. digitatum* growth. The use of acetone as a solvent for the tested compounds resulted in only a small inhibitory effect as compared to the PDA medium without acetone.

The amended growth medium assay was applied to seven compounds – 1-octanol, 1-decanol, perillaldehyde, citral, octanal, cinnamaldehyde and imazalil – to compare their activity against *P. digitatum* spore germination with that against mycelial growth. Most of these compounds totally inhibited spore germination, quite consistent with their effects on mycelial growth. The exception was octanal, which was more active against spore germination than against fungal growth (data not shown). The activities of 1-octanol and citral against germination of *P. digitatum* spores increased with their concentrations, but 1-octanol was much more active than citral (**Fig. 5**). 1-Octanol not only showed a higher inhibition activity at all the concentrations tested, but also it totally inhibited the fungus growth at 0.6 mg mL^{-1}, whereas total inhibition required citral at 1 mg mL^{-1}.

Minimum inhibitory concentrations and modes of action of citrus peel oil compounds

Compounds that totally inhibited the mycelial growth of *P. digitatum* at a concentration of 1 mg mL^{-1} were further tested to determine their minimum inhibitory concentration (MIC) and to learn if their activity was fungicidal or fungistatic. The most active of the components of citrus peel essential oil was 1-decanol, followed by 1-octanol and 1-nonanol. Citral, perillalcohol and perillaldehyde, were the

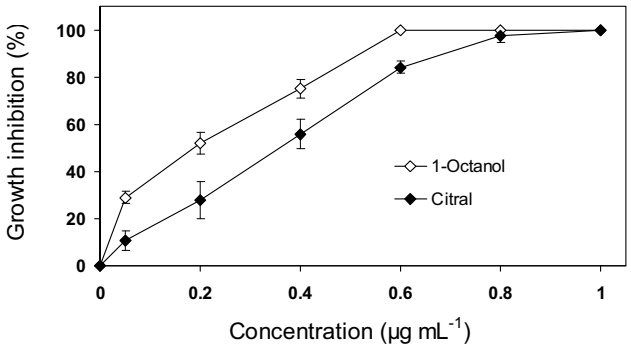

Fig. 5 The effect of different concentrations of 1-octanol and citral on the inhibition of *P. digitatum* spores in the amended growth medium assay. Bars indicate the 95% *t*-confidence intervals.

Table 1 MIC values and modes of action of essential oil compounds towards *P. digitatum*.

Test compound	MIC (mg mL^{-1} medium)	Mode of action
1-Decanol	0.05	Fungistatic
1-Octanol	0.1	Fungicidal
1-Nonanol	0.2	Fungistatic
Citral	0.4	Fungicidal
Perillaldehyde	0.4	Fungicidal
Perillalcohol	0.4	Fungicidal
Cinnamaldehyde	0.4	Fungicidal
Citronellol	0.6	Fungistatic
Terpineol	0.8	Fungicidal
Carveol	1.0	Fungistatic
Imazalil	<0.025	Fungicidal

most active of the terpenoid compounds (**Table 1**). None of the natural compounds tested exhibited an MIC close to that of imazalil, which gave total inhibition even at the lowest concentration tested. As for the mode of action, it was found that 1-octanol, citral, perillaldehyde, perillalcohol and terpineol as well as imazalil and cinnamaldehyde were fungicidal, whereas 1-decanol, 1-nonanol, citronellol and carveol were fungistatic (**Table 1**).

Tests on additional citrus pathogenic fungi

Four of the most active citrus oil compounds – 1-octanol, 1-decanol, perillaldehyde and citral – were tested on three other major citrus pathogenic fungi, by means of both the agar diffusion assay (**Table 2**) and the amended growth medium assay (**Table 3**). Their effects were compared with those of octanal, which exhibited moderate activity against *P. digitatum*, and with the reference antifungal compounds cinnamaldehyde and imazalil. In most cases the inhibitory activities of the compounds against the other pathogens correlated with their activities against *P. digitatum*. Imazalil showed very strong antifungal activity, especially against the *Penicillium* species and also against *Alternaria citri*, but only low inhibitory activity against *Geotrichum candidum*, particularly in the agar diffusion assay.

The compounds citral and 1-decanol were also much more active against *P. italicum* and *A. citri* than against *G. candidum* in the agar diffusion assay, whereas they were equally active against all fungi in the amended growth medium assay. It is interesting to note that octanal, which exhibited moderate inhibitory activity against the *Penicillium* species, totally inhibited the growth of *G. candidum* in the amended growth medium assay. 1-Octanol and perilladehyde totally inhibited all the fungi tested in both assays.

In vivo application

Ninety-seven percent of the inoculated fruit in the control (water + Tween-20) rotted six days after their inoculation (**Fig. 6**). Treatment with 25% ethanol without the essential

Table 2 Inhibitory effects of essential oil compounds against different species of citrus pathogens in the agar diffusion assay.

Test compound	Growth inhibition area of the pathogen (cm^2)		
	G. candidum	*P. italicum*	*A. citri*
1-Octanol	Total inhibition[a]	Total inhibition	Total inhibition
1-Decanol	1.0 ± 0.2	Total inhibition	Total inhibition
Octanal	3.6 ± 0.3	3.6 ± 0.2	5.3 ± 0.4
Perillaldehyde	Total inhibition	Total inhibition	Total inhibition
Citral	10.6 ± 1.3	**Total inhibition**	Total inhibition
Cinnamaldehyde	24.2 ± 1.8	Total inhibition	Total inhibition
Imazalil	8.3 ± 0.6	Total inhibition	42.8 ± 3.4
Control (PDA)	0	0	0

[a] Total inhibition: inhibition area of 62.3 cm^2

Table 3 Inhibitory effects of essential oil compounds against different species of citrus pathogens in the amended growth medium assay.

Test compound	Growth inhibition (%)		
	G. candidum	*P. italicum*	*A. citri*
1-Octanol	100	100	100
1-Decanol	100	100	100
Octanal	100	88.9 ± 7.2	56.9 ± 4.8
Perillaldehyde	100	100	100
Citral	100	100	100
Cinnamaldehyde	100	100	100
Imazalil	95.3 ± 2.9	100	100

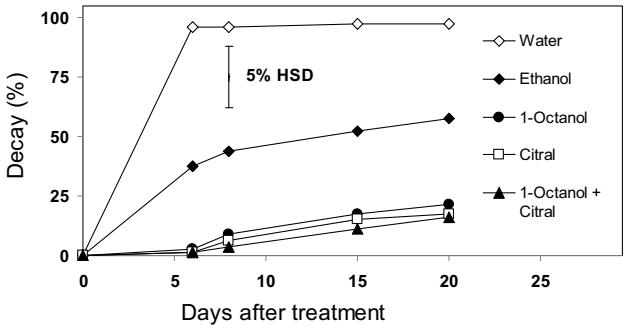

Fig. 6 The effects of aqueous formulations comprising citral, 1-octanol or their combination on decay of mature green lemons inoculated with *P. digitatum*. The formulations comprised essential oil compounds (total concentration of 2500 ppm), 25% ethanol, 5000 ppm Tween-20 and water (the rest). The control fruit were dipped in water with addition of 5000 ppm Tween-20 or in 25% aqueous ethanol with addition of 5000 ppm Tween-20. The bar represents the 5% Tukey's HSD.

oil compounds resulted in decay incidence of 38% six days after the inoculation; the incidence reached 60% by a day 20. The effect of ethanol was statistically significant but obviously insufficient from the practical viewpoint. Treatments with formulations comprising 2500 ppm of 1-octanol, citral or their combination had a curative effect reducing the decay of lemons 20 days after inoculation to 17-20%. No statistically significant difference was observed between the active ingredients tested, but each of them significantly differed from the controls (water and ethanol). None of the fruit in these experiments had visible damage or noticeable off-odor. Although 25% ethanol by itself was insufficient for effective pathogen control, its presence in the formulation was necessary for the success of the treatment. Applying similar concentrations of 1-octanol or citral as water emulsions with addition of a detergent but without ethanol resulted by the end of storage in decay incidence of 60 to 70% and visible peel damage, in particular with citral (data not shown).

DISCUSSION

Among the 37 tested components of the essential oil of citrus flavedo, the aliphatic alcohols 1-decanol, 1-nonanol and especially 1-octanol, exhibited the highest inhibitory acti-

vity against *P. digitatum,* as measured by the three in vitro assays used. The terpenoid compounds perillalcohol, perillaldehyde, citral, terpineol, carveol and citronellol, and also the aromatic aldehyde cinnamaldehyde, which is not a component of citrus oil, exhibited high activity against *P. digitatum.* The hydrocarbons d-limonene and myrcene and the esters that were tested did not inhibit the fungus. 1-Octanol, perilladehyde, citral, perillalcohol and terpineol were fungicidal, whereas 1-decanol, 1-nonanol, carveol and citronellol were only fungisatic. 1-Octanol, 1-decanol, perillaldehyde and citral also showed antifungal activity against *Alternaria citri* and *Penicillium italicum*, and 1-octanol and perillaldehyde also against *Geotrichum candidum*. The descending order of antimicrobial activity of the major oil components according to Faid *et al*. (1996) was: phenols > alcohols > aldehydes > ketones > ethers > hydrocarbons. The present results conform to this general pattern, although phenols and ethers were not tested. Esters were found to have low antifungal activity, very much like the ethers. The aromatic compound cinnamaldehyde exhibited high inhibitory activity and is probably ranked near the phenols.

The primary aliphatic alcohols were much more active than their corresponding aldehydes, and the primary alcohol 1-octanol showed markedly higher activity than 2-octanol (in the agar diffusion assay). These present results confirm the findings of Kurita *et al*. (1981), who examined a range of essential oil compounds for antifungal activity against seven species of fungi, none of which were citrus pathogens, and reported that primary alcohols were considerably higher in antifungal activity than secondary and tertiary alcohols. They also reported that compounds with an α,β saturated carbonyl group had a much lower antifungal activity than their corresponding alcohols (octanal vs. 1-octanol; nonanal vs. 1-nonanol; decanal vs. 1-decanol; and also citronellal vs. citronellol), in complete agreement with the results presented in this paper. The reason for the higher antifungal activity of a hydroxyl than of a carbonyl group among these compounds is still not clear. In contrast, Davis and Smoot (1972) reported that the C_5, C_6, C_7 and C_8 aliphatic aldehydes inhibited spore germination of *P. digitatum*, whereas the corresponding alcohols showed no effect. The terpenoid aldehyde citral showed much higher activity than its corresponding alcohol geraniol. Perhaps the carbonyl group is more effective than the hydroxyl group in α,β unsaturated compounds.

As for the terpenoid aldehydes, perillaldehyde showed the highest antifungal activity, followed by citral that was fairly potent. In contrast, the antifungal activity of citronellal was low. This is in line with the results of Kurita *et al*. (1981) that suggested that aldehydes which have one or more double bonds conjugated to their carbonyl group have a much higher antifungal activity than those which have not. Moleyar and Narasimham (1986) also reported that the CHO group in conjugation with a carbon to carbon double bond was found to be responsible for the antifungal activity of citral. Similar results were reported for the unsaturated aldehydes 2-hexenal and 2-nonenal, which had a much more potent activity than hexanal and nonanal (Hamilton-Kemp *et al*. 1992). The presence of an α,β unsaturated bond adjacent to the carbonyl moiety enhanced the antifungal activity of these aldehydes (Anderson *et al*. 1994). The effective inhibitory activity of citral against *P. digitatum* and other fungi was reported in several papers (Moleyar and Narasimham 1986; Onawunmi 1989; Ben-Yehoshua *et al*. 1992; Caccioni *et al.* 1995; Rodov *et al*. 1995). In contrast, French *et al*. (1978) reported that citral and nonanal stimulated the germination of *P. digitatum* spores in a water agar medium. However, addition of sucrose to the growth medium caused these aldehydes to be inhibitory. A stimulatory effect of citral on *P. digitatum* growth was reported also by Rodov *et al*. (1995), but only at very low concentrations. Thus, the growth medium used and the concentration of the compound have profound effects on the results.

The antimicrobial activity of essential oils is related to their damaging effects on the membrane structure and function (Andrews *et al*. 1980; Knobloch *et al*. 1988; Helander *et al*. 1998; Cox *et al*. 2000; Skandamis *et al*. 2001). It has been suggested that the antifungal activities of cinnamaldehyde, perillaldehyde and citral are due, at least in part, to their ability to form charge-transfer complexes with electron donors in a fungus cell (Kurita *et al*. 1981). The toxicity of unsaturated aldehydes in position α,β, such as citral, is generally based on interaction with sulfhydryl and amino groups of the target cells or fungi (Witz 1989; Anderson *et al*. 1994). It also appears that a certain degree of hydrophobicity is necessary for a potent antifungal effect to be achieved (Kurita *et al*. 1981), but is not sufficient in itself to inhibit the fungal growth effectively (Kurita and Koike 1983).

It is important to note the major differences between the assay methods used in the present study. In the agar diffusion method the test compound was applied centrally, therefore there was a gradient in concentration of test compound from the center to the edge of the Petri dish. This means that the results depended on the ability of the compound to diffuse in agar medium, and consequently on the water solubility of the compound. Secondly the inocula used in this method were fungal conidia, so that the effects of the compounds on spore germination were being tested. In the amended growth medium assay the test compound was evenly distributed throughout the agar and the inocula were mycelial plugs. These differences in concentration distribution and in the kinds of inocula undoubtedly affected the assay results. 1-Decanol, for example, showed a low activity in the agar diffusion assay, whereas it totally inhibited the growth of the fungi in the poisoned food assay. Moreover, 1-decanol was the most active compound tested, with the lowest MIC in the poisoned food assay, therefore its lack of activity in the agar diffusion assay was probably because of poor diffusion in the agar or poor effectiveness against the germination of *P. digitatum* spores. In fact, many compounds were more active in the amended growth medium assay than in the agar diffusion assay, which hints on distribution problems in the agar diffusion assay, as mentioned above, and/or activity against hyphal growth but not against spore germination. Reduced inhibitory activity against *P. digitatum* spore germination than against hyphal growth was exhibited by 1-octanol and citral.

When the agar diffusion assay was applied to the vapors of the tested compounds, they exhibited a similar pattern of activity levels to that of the liquid phase, although the actual levels were different. The antifungal effectiveness of a vaporized compound depends on its volatility, which determines the amount of vapor in the headspace, and on the ability of the vapor to diffuse in the agar as well as into the mycelium itself. Utama *et al*. (2002) reported that despite the relatively small amount of cinnamaldehyde found in the agar medium, compared with those of water-soluble volatiles, this compound was a strong growth inhibitor of *P. digitatum*, probably because of its high activity or/and because of its hydrophobic nature that may enable it to directly accumulate in the fungus. On the other hand, the inhibitory effect of imazalil in the vapor phase assay was limited by its low volatility. Some of the compounds (perillaldehyde, cinnamaldehyde, 1-nonanol and carvone) showed increased activity when used as vapors. This could be due to the high volatility of these compounds and/or a better penetration of the vapor than of the liquid into the agar. Alternatively, it could be that the exposure of the compound to air allows oxidation reactions to produce compounds with enhanced antimicrobial activities (Naigre *et al*. 1996).

The present study was aimed to identify compounds that would be suitable for application as postharvest fungicides on citrus fruits. The results of *in vitro* assays showed that 1-octanol had the potential to act as a fungicide: it was most active against *P. digitatum* growth in all the assay methods used, and was shown to have an MIC of 0.1 mg mL^{-1} and a fungicidal mode of action. 1-Octanol also totally inhibited *in vitro* growth of other citrus pathogens such as *G.*

candidum, *P. italicum* and *Alternaria citri*. Citral also exhibited good fungicidal activity. *In vivo* applications of 1-ocnanol or citral significantly inhibited the decay development in the inoculated fruit during a 3-week storage period demonstrating a curative effect. No visible phytotoxic damage or perceivable off-odor were found. The enhanced efficacy and reduced phytotoxicity of the essential oil compounds was achieved due to their application within optimized formulations combining adequate concentrations of a food-grade detergent and an organic solvent (Ben-Yehoshua 2001; Ben-Yehoshua and Rodov 2006). To the best of our knowledge, the potential of 1-octanol to control the citrus green mold disease hasn't been reported earlier, although both octanol and citral were shown to inhibit the sour rot of lemons caused by *Geotrichum candidum* (Suprapta *et al.* 1997). At the same time, only partial control of the pathogen was achieved in that work, as well as in our trials. Further efficacy improvement is needed in order to practically realize the potential of essential oil components as postharvest citrus fungicides. Such improvement may be reached in particular by augmentation of the formulations with additional active ingredients, for example, with hydroperoxide monoterpene derivatives (Ben-Yehoshua 2001). The latter compounds are generated either naturally or artificially by photooxidation of monoterpene hydrocarbons (e.g. limonene) and possess very high antimicrobial activity (Ben-Yehoshua *et al*. 2008). The promising results obtained with these improved formulations will be a subject of a separate publication.

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