# Environmentally Friendly and Safe Technologies for Quality of Fruits and Vegetables

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The papers contained in this book report some of the peer reviewed Proceedings of the International Conference "Environmentally friendly and safe technologies for quality of fruit and vegetables", but also other papers related with the subject were included. The manuscripts were reviewed by the Editor and Editorial Board, and only those papers judged suitable for publication were accepted. The Editor wish to thank to all the reviewers and authors for their contribution.

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# SECTION 4. ENVIRONMENTALLY FRIENDLY AND SAFE METHODS TO CONTROL POSTHARVEST LOSSES

# 26. ANTIFUNGAL ACTIVITY OF CITRUS ESSENTIAL OIL COMPONENTS *IN VITRO* AND *IN VIVO* AGAINST *PENICILLIUM DIGITATUM* PERS. (SACC.)

*V Rodov*, B Nafussi, P Burns, S Ben-Yehoshua*
Dep of Postharvest Science of Fresh Produce, Agricultural Research Organization - the Volcani Center, P.O.Box 6, Bet Dagan 50250, Israel
* E-mail: vrodov@agri.gov.il

## Abstract

*In vitro* studies were conducted on 37 compounds present in citrus essential oil, to test their activity against *Penicillium digitatum* by three methods: agar diffusion, amended growth medium and vapor assay. The aliphatic alcohols 1-nonanol, 1-decanol and especially 1-octanol exhibited the highest activities, as assayed by all the methods used. The terpenoid compounds perillalcohol, perillaldehyde, citral, terpineol, carveol and citronellol, as well as the reference aromatic compound cinnamaldehyde also exhibited high activity against *P. digitatum*. Neither hydrocarbons nor esters inhibited this fungus. The mode of action of 1-octanol, perillaldehyde, citral, perillalcohol and terpineol against *P. digitatum* was fungicidal, whereas 1-decanol, 1-nonanol, carveol and citronellol were only fungistatic. Application of biocidal formulations comprising 1-octanol and citral either separately or together inhibited decay of *P. digitatum*-inoculated lemons for three weeks after inoculation.

**Keywords:** antifungal activity, *Citrus*, essential oil components, green mold disease, *Penicillium digitatum*

## Introduction

The flavedo of citrus fruit contains large quantities of essential oils compartmentalized inside the oil glands. 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, 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 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 & Liguori 1958; Subba *et al.* 1967; Dabbah *et al.* 1970) and its investigation is continued at present (Romano *et al.* 2005; Sharma & Tripathi 2006, 2008; Viuda-Martos *et al.* 2008; Chutia *et al.* 2009). The subject has been recently reviewed by Fisher & 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. Citrus oils were reported to inhibit *in vitro* the development of the main postharvest citrus pathogen, *Penicillium digitatum* Pers. (Sacc.), the causative agent of the green mold disease (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

& Narasimham 1986; Caccioni & Guizzardi 1994; Caccioni *et al.* 1995), the information on this subject is still limited. Moreover, 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 & Rodov 2006).

The aim of this study was to identify the compounds that would be potentially suitable for use as a postharvest fungicide on citrus fruits. Three different *in vitro* assay techniques were used to evaluate 37 individual components of citrus essential oils for activity against *P. digitatum*. The selected promising compounds were further tested *in vivo* as active ingredients of microbiocidal formulations on *P. digitatum*-inoculated lemons.

## Materials & Methods

Most of the essential oil components 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.

The antifungal activity of the essential oil compounds against *P. digitatum* was assayed *in vitro* by three methods: agar diffusion (Maruzzella & Henry 1958), vapor assay (Maruzzella & Sicurella 1960) and amended growth medium ("poisoned food") assay (Grover & Moore 1962). In the first two methods, a 5-mg (if not specified differently) sample of each substance was pipetted onto a sterile 1.3-cm antibiotic assay paper disc which was placed either on the center of the inoculated agar (the agar diffusion assay) or onto the lid of the inverted Petri dish (the vapor assay). The activity was evaluated by visual evaluation of fungal growth and measuring the growth-free zones.

In the amended medium ("poisoned food") assay the samples dissolved in 0.5 mL of acetone, were added to molten PDA to a final concentration of 1 mg $mL^{-1}$. The medium was inoculated with mycelial discs (8 mm) cut from the fungal agar plate cultures. The antifungal activity was calculated as a percentage of fungal growth inhibition. For any compound that totally inhibited the fungal growth after 7 days fungicidal or fungistatic mode of its action was determined by monitoring the fungal recovery after transferring the mycelial disc to fresh PDA medium. The minimum inhibitory concentration (MIC) of a compound was evaluated by using concentrations from 0.025 to 1.0 mg $mL^{-1}$ PDA.

For *in vivo* tests lemon fruit were washed with tap water, surface sterilized by wiping with 70% ethanol and inoculated with *P. digitatum* ($10^4$ spores $mL^{-1}$). After overnight storage at 20 °C, the fruit were treated with l-octanol and/or citral by dipping for a minute in emulsion formulations including 25% ethanol and 2500 or 5000 ppm Tween-20 (Ben-Yehoshua 2001; Ben-Yehoshua & Rodov 2006). After drying the fruit were arranged in four replications of 15 fruit each, and stored at 20 °C in cartons covered with plastic bags. The decay incidence was evaluated daily.

## Results

### Agar Diffusion Assay

Of the 37 compounds tested 19 showed a zone of total inhibition of *P. digitatum* growth after 48 h incubation (Table 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. The effect of l-octanol was similar to that of the reference compound, the synthetic fungicide Imazalil, and exceeded the activity of another reference compound, cinnamaldehyde, known for its antifungal potency (Kurita *et al.* 1981; Moleyar & Narasimham 1986). 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. Aldehydes with main chain of less than seven (hexanal) or more than ten (undecanal and dodecanal) carbon units showed no activity. The most effective terpenoid compounds against *P. digitatum* 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. Neither hydrocarbons limonene and myrcene nor acetate esters caused any inhibition.

A saturation curve described the correlation between the antifungal activity and the concentrations of citral and 1-octanol (data not shown). 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 per disc, while even 20 mg per disc of citral inhibited growth in an area of only 7.7 $cm^2$.

**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 (Table 1). 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. The vapors of the aldehydes octanal and nonanal showed much lower inhibition activities than the corresponding alcohol vapors However, 1-nonanol was twice as active as a vapor as as a liquid. 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 (three-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 gas. 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 the esters had no effect 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 (Table 1). 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.

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. 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 $mL^{-1}$.

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 (MIC 0.05 mg $mL^{-1}$ medium), followed by 1-octanol (0.1 mg $mL^{-1}$) and 1-nonanol (0.2 mg $mL^{-1}$). Citral, perillalcohol and perillaldehyde, were the most active of the terpenoid compounds (0.4 mg $mL^{-1}$). None of the natural compounds tested exhibited an MIC close to that of imazalil, which gave total inhibition even at the lowest concentration tested (0.025 mg $mL^{-1}$). 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.

### *In Vivo* Application

Ninety-seven percent of the inoculated fruit in the control treatment of a water dip rotted six days after their inoculation. Treatment with 25% ethanol without the essential oil compounds resulted in decay incidence of 38% six days after the inoculation and 60% on a day 20. Treatments with 2500 ppm of 1-octanol, citral or their combination reduced the decay of inoculated lemons 20 days after inoculation to 17-20%. None of the fruit in these experiments had visible damage (data not shown).

**Table 1.** The activity of essential oil components against *P. digitatum*.

| Component | AD $cm^2$ | VA $cm^2$ | AGM % | Component | AD $cm^2$ | VA $cm^2$ | AGM % |
|---|---|---|---|---|---|---|---|
| Aliphatic alcohols | | | | Terpenoid alcohols | | | |
| 1-Hexanol | 0 | 0 | 69 | Perillalcohol | 2 | 0 | 100 |
| 1-Heptanol | 18 | 0 | 100 | Terpineol | 2 | 0 | 100 |
| 1-Octanol | 56 | 63 | 100 | Terpinen-4-ol | 2 | 0 | 87 |
| 1-Nonanol | 14 | 28 | 100 | Carveol | 1 | 4 | 100 |
| 1-Decanol | 1 | 0 | 100 | Geraniol | 0 | 0 | 0 |
| 2-Octanol | 2 | 0 | 97 | Nerol | 0 | 5 | 61 |
| 6-Meth-5-hepten-2-ol | 0 | 0 | 33 | Citronellol | 0 | 0 | 100 |
| Aliphatic aldehydes and ketone | | | | Linalool | 0 | 0 | 79 |
| Hexanal | 0 | 0 | 30 | Farnesol | 0 | 0 | 11 |
| Heptanal | 1 | 0 | 20 | Terpenoid aldehydes and ketones | | | |
| Octanal | 8 | 5 | 61 | Perillaldehyde | 18 | 61 | 100 |
| Nonanal | 5 | 1 | 81 | Citral | 7 | 12 | 100 |
| Decanal | 3 | 0 | 37 | Citronellal | 0 | 0 | 41 |
| Undecanal | 0 | 0 | 13 | Carvone | 7 | 16 | 83 |
| Dodecanal | 0 | 0 | 4 | Nootkatone | 0 | 0 | 93 |
| 6-Meth-5-hepten-2-one | 4 | 0 | 17 | Terpenoid hydrocarbons | | | |
| Aliphatic and terpenoid esters | | | | d-Limonene | 0 | 0 | 14 |
| Octyl acetate | 0 | 0 | 2 | β-Myrcene | 0 | 0 | 30 |
| Decyl acetate | 0 | 0 | -43 | α-Pinene | 0 | 0 | 0 |
| Geranyl acetate | 0 | 0 | 6 | Controls (reference compounds) | | | |
| Neryl acetate | 0 | 0 | 26 | Cinnamaldehyde | 30 | 62 | 100 |
| Linalyl acetate | 0 | 0 | 7 | Imazalil | 52 | 10 | 100 |
| Octyl acetate | 0 | 0 | 2 | PDA (control) | 0 | 0 | 0 |

Assays used: AD - agar diffusion assay (inhibition area, $cm^2$), VA - vapor assay (inhibition area, $cm^2$), AGM – amended growth medium, or "poisoned food" assay (% growth inhibition)

## 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 activity 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. 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).

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 & 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 $\alpha,\beta$ 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 & 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.

It is important to note the major differences between the assayed methods used in the present study. In the agar diffusion method the test compound is applied centrally, therefore there is a gradient in concentration of test compound from the center to the edge of the Petri dish. This means that the results depend on the ability of the compound to diffuse in agar, and since agar mainly consists of water, the results greatly depend on the water solubility of the compound. Secondly the inocula used in this method are fungal conidia, so that the effects of the compounds on spore germination are being tested. In the amended growth medium method the test compound is evenly distributed throughout the agar and the inocula are mycelial plugs. These differences in concentration distribution and in the kinds of inocula undoubtedly affect 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 very 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 a compound that would be suitable for application as a postharvest fungicide on citrus fruits. The results of *in vitro* assays show that 1-octanol has 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. Citral also exhibited good fungicidal activity. *In vivo* applications of 1-ocnanol or citral or their combinations as active constituents of biocidal formulations were able to effectively inhibit the decay development of lemons inoculated with *P. digitatum*, although total control was not achieved. No phytotoxic effects were observed when the essential oil compounds were applied within formulations comprising ethanol and sufficient concentrations of a food-grade detergent. These encouraging results demonstrate the potential of essential oil compounds as postharvest fungicides.

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