Preformed and Induced Antifungal Materials of Citrus Fruits in Relation to the Enhancement of Decay Resistance by Heat and Ultraviolet Treatments†

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Two mechanisms of the resistance of citrus fruits against pathogens—preformed and induced antifungal materials—were investigated. Flavedo tissue of lemon contains the following preformed antifungal materials: citral, limettin, 5-geranoxy-7-methoxycoumarin, and isopimpinellin, which act as the first line of defense against pathogens. Exogenous application of citral to *Penicillium*-inoculated lemon fruit prevented development of decay. Being subjected to fungal challenge and/or abiotic stress, citrus fruits are elicited to produce the phytoalexin scoparone, considered as another line of fruit defense. According to median effective dose (ED_{50}) of the inhibition of germ-tube elongation or percent germination, scoparone had higher fungitoxicity against *Penicillium digitatum* Sacc. than the preformed antifungal materials. Different citrus species (lemon, orange, grapefruit, lime, kumquat) varied in their capacities to produce scoparone responding to the combined *Penicillium* inoculation and heat treatment or to UV illumination. UV illumination of lemon fruit reduced its susceptibility to *P. digitatum*. Expression of this effect was directly related to the level of scoparone in illuminated fruit. UV light and citral application were visibly injurious to the flavedo tissues in high dose.

INTRODUCTION

Through evolution the species has developed a mechanism to preserve itself. The preservation of fruit should be of high importance in such a scheme. If fruit rots before completion of seed development, the existence of this species would be endangered. Consequently, our objective should be to discover what are the mechanisms that evolution engineered to fight decay. By utilizing these mechanisms, one could more effectively control decay using less fungicide, with a consequent reduction of toxic residues in fruit.

The presence of antifungal substances in plant tissues has been shown to play a role in resistance against disease (Darvill and Albersheim, 1984; Kuc, 1991). Previous investigations demonstrated a substantial level of antifungal activity in the flavedo of just-harvested citrus fruit, indicating the presence of preformed antifungal materials (Ben-Yehoshua et al., 1988; Kim et al., 1991). However, the nature of these substances was not studied. Arimoto et al. (1986a) identified antifungal compounds in the peel of uninfected Satsuma mandarin as citrinol, naringin, and hesperidin. Ben-Yehoshua et al. (1988) isolated from flavedo tissues of pomelo several coumarin-derived preformed antifungal materials: osthol (7-methoxy-8-prenylcoumarin), auraptene (2,3-epoxy-7-methoxy-8-prenylcoumarin), 7-[(6,7-epoxy-3,7-dimethyl-2-octyl)oxy]coumarin, and 7-geranoxycoumarin.

The level of antifungal activity in lemon flavedo gradually declined during storage (Kim et al., 1991), correlating with the decrease of disease resistance (Ben-Yehoshua et al., 1988). The heat treatment, which was developed to reduce decay of harvested citrus fruit (Ben-Yehoshua et al., 1987, 1989a), inhibited this decline in antifungal activity. Heat-treated sealed lemon maintained its initial antifungal activity from harvest time during 70 days of storage (Kim et al., 1991). Moreover, a marked increase in antifungal activity of flavedo extract was observed in heat-treated sealed lemon, when the fruit was previously inoculated with *Penicillium digitatum*. Recent work of Kim et al. (1991) demonstrated the relation of this effect to the induction of phytoalexin scoparone in lemon. Scoparone was induced in lemon and kumquat peel also after UV illumination (Ben-Yehoshua et al., 1991; Kim et al., 1991). Accumulation of scoparone in UV-illuminated kumquat fruit was associated with the reduction of its decay susceptibility (Rodov et al., 1992).

The purpose of this work is to study the chemical basis of antifungal activity in various citrus fruit and of its changes as caused by abiotic stresses.

MATERIALS AND METHODS

Plant Materials. Mature, light-green lemons (*Citrus limon* L. Burm., cv. Eureka) and mature fully colored fruits of kumquat (*Fortunella margarita* L. Swingle, cv. Nagami), orange (*Citrus sinensis* L. Osbeck, cvs. Shamouti and Valencia), lime (*Citrus aurantifolia* L. Swingle, cv. Tahiti), and grapefruit (*Citrus paradisi* Macf, cv. Marsh) were obtained from orchards or packing houses before any postharvest treatment had been applied. Samples of fruit of uniform size and appearance, originating in one orchard, were subjected to different treatments at random.

Inoculation. Fruit was inoculated by piercing the flavedo with a tool incorporating three 0.5-mm-diameter needles to a depth of 1.5 mm at four sites near the stem end of the fruit. The tool was immersed in a suspension of *P. digitatum* (10^6 spores mL^{-1}) prior to each piercing operation.

Fruit Treatments. Seal packaging and heat treatment were carried out according to the procedure of Ben-Yehoshua et al. (1987).

UV illumination of fruit with various doses of UV light (254 nm) was carried out in a chamber with four G15T8 lamps (each having a UV output of 3.6 W), supplied by Tana Industries, Netiv Halamed-He, Israel. Fruit was placed 25 cm from the UV source. UV doses were measured with a UVX radiometer (UV Products, Inc., San Gabriel, CA).

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Citral treatment was conducted by dipping fruit for 2 min in an aqueous emulsion of citral (0.05–1.0% v/v with addition of L-77 detergent, 0.05% v/v). The control fruit was dipped in water with 0.05% detergent.

Isolation of the Antifungal Substances. Flavedo tissues were extracted with petroleum ether as described previously by Ben-Yehoshua et al. (1987) and Kim et al. (1991).

The preliminary fractioning of crude extract was performed by flash chromatography on a column of silica gel (Kieselgel D, 400-mesh ASTM, Riedel-de-Haen). The elution was made stepwise with petroleum ether, petroleum ether–ethyl acetate (9:1, 8:2, 7:3, and 1:1, v/v) and, finally, ethyl acetate.

Fractions obtained were separated by TLC (Kieselgel 60 F_{254}) using toluene–ethyl acetate (4:1). The developed plates were exposed to UV light (366 and 254 nm). Individual bands were collected and extracted with dichloromethane. The extract was dried by a stream of nitrogen.

The final purification of active fractions was performed on a Varian 5000 HPLC instrument equipped with a variable-wavelength UV-50 detector. The materials were purified on a C-18 reversed-phase column using a constant elution gradient, from 60% methanol in water to methanol, and a constant flow rate of 0.5 mL min^{-1}. Peaks were detected at 335 nm. The absorbance spectrum of the compound isolated by HPLC was obtained with a Uvikon spectrophotometer using methanol as solvent.

Detection of Antifungal Activity on TLC Plates. Antifungal materials were detected in the crude extract directly on TLC plates using *Cladosporium cladosporioides* G. A. De Vries as the test organism. The isolated materials were tested for their ability to inhibit growth of *P. digitatum* in Petri dishes.

Antifungal activity was evaluated quantitatively by percent inhibition of *P. digitatum* spore germination and germ-tube elongation, and median effective doses (ED_{50}) of substances were calculated. The procedures were carried out according to those of Kim et al. (1991).

Identification of Active Fractions. Crude extract of lemon flavedo was analyzed on a Varian 3300 gas chromatograph equipped with a flame ionization detector and fitted with an OV-17, Chrom W-HP steel column (6 ft × 1/4 in. o.d.). Nitrogen was used as carrier gas at a flow rate of 50 mL min^{-1}. The initial temperature of 120 °C was held for 5 min, and then the temperature was increased to 250 °C at 2.5 °C min^{-1} and held at 250 °C for an additional 20 min. The temperature of the injector was 250 °C and of the detector 270 °C. Peaks were identified on the gas chromatogram by comparing their retention times with those of authentic standard materials. The structure of the tested compound was confirmed by its ^1H NMR spectrum at 360 MHz and ^{13}C NMR spectrum at 90 MHz in $CDCl_3$ on a Bruker WM-360 spectrometer. GC–MS analysis was performed on the Finnigan 5100 instrument with an Rt_x5 capillary column (dimethyldiphenylpolysiloxane).

Quantitative Determination of Scoparone Extracted from Lemon. Quantitative analysis was performed on a SPF-125 spectrophotofluorometer (Aminco) as described by Kim et al. (1991).

RESULTS AND DISCUSSION

Preformed Antifungal Materials of Lemon Fruit. Extract of lemon flavedo contained several antifungal materials. TLC bioassay using *C. cladosporioides* as the test organism enabled us to detect several spots with antifungal activity. Materials eluted from these zones also inhibited the growth of *P. digitatum* in Petri dishes.

These materials were isolated and purified by column, thin-layer, and high-performance liquid chromatography. Their identity was determined by ^1H NMR and ^{13}C NMR spectra and by GC relative retention time. The following materials were found in lemon flavedo: two 5,7-substituted coumarin derivatives, a) 5,7-dimethoxycoumarin (limettin) and (b) 5-geranoxy-7-methoxycoumarin, and (c) monoterpene aldehyde citral (3,7-dimethyl-2,6-octadienal), present as a mixture of two geometric isomers, geranial and neral. A trace amount of (d) isopimpinellin (5,8-dimethoxypsoralen), known as the main fungitoxic compound of lime leaves (Martin, 1966), was tentatively identified in lemon flavedo extract by the relative GC retention time. Much higher quantities of isopimpinellin (up to 20 μg g^{-1} of fresh weight) were detected in the flavedo extract of lime.

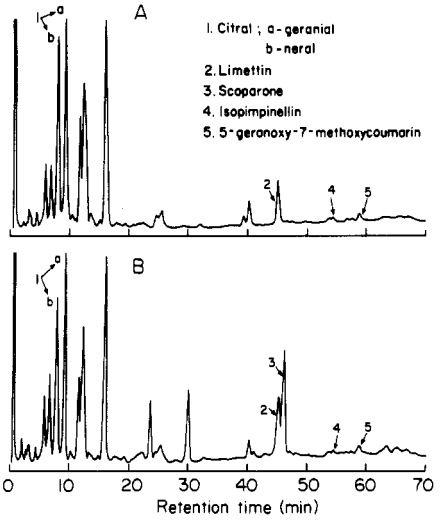

Figure 1. Gas chromatogram of the petroleum ether extract from lemon flavedo: A, nontreated; B, inoculated and heat-treated. Extract was from 1 g of flavedo dissolved in 0.2 mL of dichloromethane. Injection volume was 1 μL. An OV-17, Crom W-HP steel column was used. Carrier gas was nitrogen (50 mL min^{-1}). The initial temperature of 120 °C was held for 5 min, then increased to 250 °C at 2.5 °C min^{-1}, and held at 250 °C for 20 min. The temperature of the detector was 270 °C and of the injector 250 °C.

Figure 1A illustrates the chromatogram of an extract prepared from lemon flavedo just after harvest. The location of antifungal materials is shown as well as some indication about their relative abundance in the extract. The content of the named substances in lemon flavedo was evaluated, according to fluorometric and gas chromatographic data, as 867 ± 80 μg g^{-1} for citral, 136 ± 9 μg g^{-1} for limettin, and 52 ± 11 μg g^{-1} for 5-geranoxy-7-methoxycoumarin (all related to the fresh weight of flavedo tissue). Levels of these materials ranged between 581 and 1205 μg g^{-1} for citral, between 110 and 165 μg g^{-1} for limettin, and between 30 and 113 μg g^{-1} for 5-geranoxy-7-methoxycoumarin. The *Cladosporium* bioassay exposed several other antifungal materials. However, vague zones of fungus inhibition displayed by these materials indicated that either their concentration or specific activity was relatively low.

The activity of limettin and citral was evaluated in the bioassay with *P. digitatum* spores as the inhibition of germ-tube elongation. Median effective doses (ED_{50}) of these compounds were calculated to equal 860 and 242 μg mL^{-1}, respectively.

Exogenous Application of Citral. Exogenous application of citral to mature green lemons inhibited development of decay in *Penicillium*-inoculated fruit (Figure 2). Dipping fruit in 1% (v/v) citral aqueous emulsion reduced the percentage of compatible inoculation from 100 to 9%. Smaller doses had lower activity but were still effective in the reduction of decay. Dipping lemon in 1% citral emulsion resulted in phytotoxic damage to the peel, visible on 68% of the fruit as dark spots. The incidence of damage did not exceed 7% at a citral dose of 0.5% and was negligible at lower concentrations.

Scoparone, the Phytoalexin of Citrus Fruit. Figure 1B presents the gas chromatogram of a flavedo extract

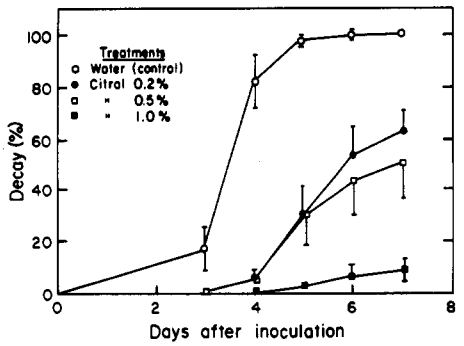

Figure 2. Effect of postharvest exogenous application of citral on *Penicillium*-inoculated green lemon fruit. Fruit was inoculated with *P. digitatum* spore suspension (10^6 spores mL^{-1}) and, 20 h after inoculation, dipped in citral emulsions. Fruit was stored at 17 °C and 85% relative humidity.

from the lemon fruit subjected to the *Penicillium* inoculation and heat treatment. Three additional peaks absent in nonstressed fruits were observed in this extract. The largest of them, seen near limettin, was found to correspond to scoparone (6,7-dimethoxycoumarin) by NMR and GC–MS data. The elicitation of scoparone in stressed lemon fruit was earlier described by Kim et al. (1991). Two other induced compounds shown in Figure 1B are now in the process of identification and testing of their antifungal activity.

Scoparone is the most studied phytoalexin of citrus fruit and is described by several authors (Afek et al., 1986; Arimoto et al., 1986b; Kim et al., 1991). Other inducible antifungal stress metabolites also were observed in citrus fruit. Dubery et al. (1988) reported the presence of 4-(3-methyl-2-butenoxy)isonitroacetofenone in the damaged peel of γ-irradiated orange and lemon. Recently, Stange et al. (1991) found in extracts of healed tissue of citrus fruit two materials with antifungal activity. They identified one as a prenylated coumaral, 3-[4-hydroxy-3-(3-methyl-2-butenyl)phenyl]-2(*E*)-propanal.

According to Kim et al. (1991), the median effective dose of scoparone against germ-tube elongation of *P. digitatum* is 29 μg mL^{-1}. Comparing the values of median effective doses, one could see the prevalence in the antifungal activity of the induced compound over the preformed ones. That is, the "weapons" developed during enemy attack are much more effective than those available for defense in "peaceful time". However, since the flavedo has significant amounts of these preformed antifungal materials, their importance as first line defense cannot be ignored. In fact, the endogenous decline in the level of these antifungal materials is the most reasonable explanation for the increase in the sensitivity of fruit to pathogen attack during its senescence on or off the tree (Ben-Yehoshua et al., 1989b).

Scoparone Level in Various Citrus Species. Since scoparone was demonstrated as the phytoalexin of citrus plant with a strong body of evidence supporting its important role in resistance against pathogens (Afek et al., 1986; Arimoto et al., 1986b; Kim et al., 1991), a survey was made of the level of its accumulation in various citrus species (Table I).

Different citrus fruits varied greatly in their capacity to produce scoparone. Certain amounts of this substance were detected in nonstressed oranges and kumquats. The occurrence of scoparone in just-harvested oranges or its appearance after several days of storage was earlier reported by Tatum and Berry (1977). However, quantities of this substance observed in such cases were insufficient to bring about the significant antifungal activity of scoparone, considering its median effective dose. Grapefruit was distinguished by the relatively low level of scoparone production.

Table I. Effect of the Combined Inoculation and Heat Treatment or UV Illumination on Scoparone Level (μg g^{-1} of Fresh Weight of Flavedo) in Citrus Fruits[a]

species	control	inoculation and heat treatment[b]	UV illumination[c]
lemon, cv. Eureka	0	178 ● 7.3	247 ± 19.3
grapefruit, cv. Marsh	0	114 ± 7.3	10.3 ± 2.3
orange, cv. Shamouti	4.6 ± 0.2	1676 ± 97.9	130 ± 15.6
orange, cv. Valencia	3.8 ● 0.4	848 ● 56.7	162 ± 38.9
lime, cv. Tahiti	trace	545 ± 65.1	23 ± 5.2
kumquat, cv. Nagami	2.1 ± 0.8	101 ± 5.3	418 ± 50

[a] Result was obtained 10 days after inoculation or UV illumination. Means ● SE. [b] Fruits were heat-treated at 36 °C for 3 days 24 h after inoculation with *P. digitatum*. [c] UV dose: 4.5×10^3 J m^{-2}.

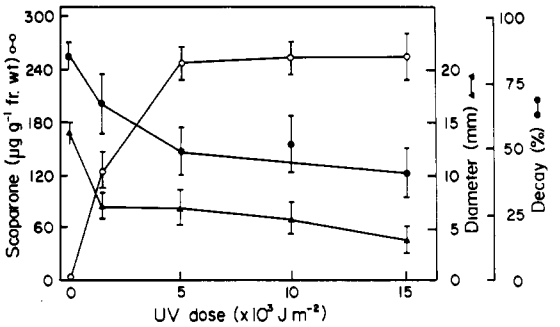

Figure 3. Effect of UV dosage on scoparone level, decay percentage, and diameter of infected area in lemon fruit. Fruit was UV-illuminated and stored in the dark at 17 °C and 85% relative humidity. Scoparone was measured fluorometrically 9 days after illumination. Inoculation was performed 2 days after UV treatment. Decay percentage was determined 6 days after inoculation.

The reaction of different fruits to the two kinds of stresses was also specific. Thus, lemon produced similar amounts of scoparone responding either to combined inoculation and heat treatment or to UV illumination. With lime, orange, and grapefruit, however, the first treatment was much more effective in eliciting scoparone on the stressed site. Only with kumquat was the effectiveness of these two treatments inversely related, stressing the genealogical difference between kumquat (genus *Fortunella*) and genuine *Citrus* species.

UV-Induced Scoparone and Decay Reduction. The accumulation of scoparone in UV-illuminated lemon was accompanied by a decrease of its decay susceptibility.

Figure 3 illustrates the level of phytoalexin and inoculation efficiency (expressed as decay percentage) in lemons treated with different doses of UV light. The inverse relation between scoparone concentration and fruit decay is clearly evident. Both parameters were affected by UV dose only up to the level of 5×10^3 J m^{-2}, further dose increase being ineffective. Additionally, UV illumination inhibited the rate of pathogen growth in fruit tissues, as reflected by the diameter of infected area. The lowest UV dose tested was sufficient to bring about 50% inhibition of fungus expansion. It is reasonable that the rate of fungal growth could be more sensitive to UV illumination than decay percentage.

Time of inoculation greatly affected the disease susceptibility of UV-treated fruit (Table II). UV was not effective in reducing decay percentage of lemon fruit inoculated either 1 day or immediately before illumination. However, pathogen applied 2 or 8 days after illumination was inhibited markedly and significantly as compared with the fruit inoculated without (control) or before UV

Table II. Effect of Inoculation Time on the Decay Percentage[a] of UV-Illuminated Lemon Fruit by the Dose of 5×10^3 J m^{-2}

	time of inoculation (before or after UV illumination)					
	1 day before	immediately before	2 days after	8 days after	17 days after	37 days after
UV treatment	88 A ab	93 A a	48 B c	53 B c	62 A c	69 A bc
control[b]	88 A a	88 A a	85 A a	77 A a	82 A a	90 A a

[a] Fruit was stored in the dark at 17 °C and inoculated as described. Decay percentage was determined 6 days after inoculation. Values within columns (capital letters) and lines (small letters) separated by Duncan's multiple range test, $p = 1\%$. [b] Nonilluminated fruit inoculated after the same storage period as corresponding UV-treated fruit.

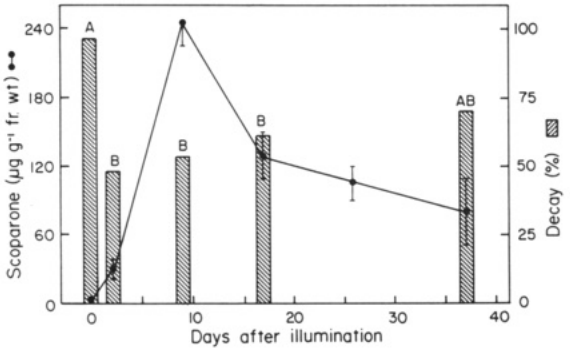

Figure 4. Effect of UV-induced scoparone accumulation on decay percentage of *Penicillium*-inoculated lemon fruit. Fruit was illuminated with a UV dose of 5×10^3 J m^{-2}, stored and inoculated as described in Figure 2. Scoparone was measured fluorometrically. The position of histograms indicates the day of inoculation. Decay percentage was determined 6 days after inoculation. Values were separated by Duncan's multiple range test ($p = 1\%$).

treatment. With later inoculations—17 and 37 days after the illumination—the fruit resistance decreased again and decay reduction was not significant as compared to that of control (nonilluminated fruit). This indicates that the decay-inhibiting effects of UV decrease with time.

Figure 4 demonstrates the relation between decay susceptibility of UV-treated lemons and the level of scoparone in their flavedo at the inoculation day. Fruit inoculated 2 days after illumination displayed the lowest decay percentage, although scoparone content at that period was far below its maximal level. Nevertheless, the content was comparable to the median effective dose of scoparone and, furthermore, increased rapidly during the period of pathogen development. Although an effective inhibitory level of scoparone was established in the tissues, the lack of a greater effect in reducing decay may be related to the gradual development of UV damage which is described below. This damage, visible and possibly also nonvisible, attenuated the lemon so that the fruit succumbed to the pathogen.

Disease reduction by UV treatment was evidently related to the induced internal defense mechanisms rather than to the germicidal effect of ultraviolet illumination. Decay was lowered by treating the fruit prior to inoculation, i.e., without direct contact of pathogen with UV light. Moreover, illumination of previously inoculated fruits failed to prevent their decay. The final result of fruit–pathogen interaction depended on the relative rates of fungal growth and resistance development. The inoculation of fruit prior to UV treatment gave adequate advantage to the pathogen, while fruit illuminated 2 days before inoculation was capable of resisting infection.

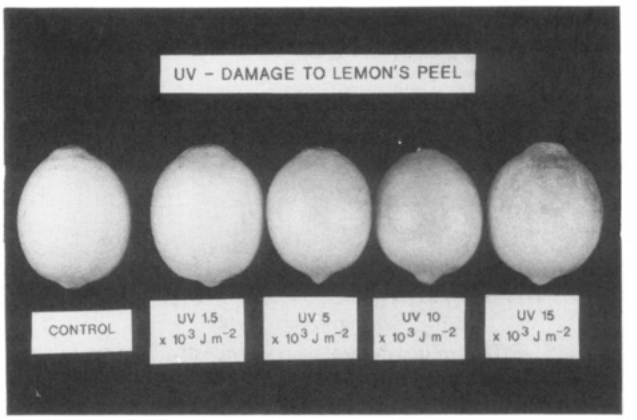

Figure 5. UV-induced damage to lemon peel. Light-green fruit was illuminated in the vertical position, stem end facing the UV source, at the distance of 25 cm. Control: nonilluminated fruit.

Recently Stevens et al. (1991) and Droby et al. (1991) described certain reduction of green mold decay in UV-treated citrus fruits accompanied with PAL enzyme activation. This enzyme plays an important role in the defensive reactions of plants, including phytoalexin induction and cell-wall reinforcement (Matern, 1991).

UV Damage to Flavedo Tissue. UV illumination, beyond a certain threshold dose, exerted visible damage to the peel of citrus fruits. Only the flavedo tissues showed damage that appeared as brown or bronze-tan coloration (Figure 5). Additionally, UV induced a more shiny appearance on the surface of fruit and the peel seemed to become firmer. These changes may relate to a possible induction of a lignification-like processes.

At 17 °C, the damage started appearing 10–14 days after the illumination was applied. The extent of damage depended on the state of maturity, or on the color of the flavedo. Thus, yellow lemons showed much less damage than green or greenish yellow fruit. Damage was dependent also on the dosage of UV illumination. Symptoms of this damage in green lemons started appearing at a dose of 1.5×10^3 J m^{-2}. Yellow lemons required higher doses, such as 10^4 J m^{-2}, to show damage.

Similar types of damage were seen also with other citrus species. The yellow fruit of Marsh seedless grapefruit showed similar tan-bronze coloration in injurious doses. Shamouti and Valencia oranges and Nagami kumquat also showed moderate tanning, although less visible on the orange background. With kumquat an additional type of damage was seen after 2 weeks of storage at 17 °C as serious shriveling of the fruit. This shriveled appearance was not seen at 11 °C.

The present study demonstrates two components of the resistance mechanisms present in the flavedo tissues of various citrus fruits—preformed and induced antifungal materials. The first line of this resistance defending fruit from a pathogen attack is in the preformed antifungal materials illustrated in lemon fruit by citral, limettin, and 5-geranoxy-7-methoxycoumarin. The second line is in the phytoalexin scoparone induced upon invasion of the pathogen (Arimoto et al., 1986b; Kim et al., 1991). The relative contribution of these compounds to the resistance, along with other factors of plant defense, such as synthesis of lignin-like materials, inducible PR proteins like chitinase and β-1,3-glucanase, and probably several other mechanisms, awaits further research in citrus fruit.

The reduction of decay of *Penicillium*-inoculated lemons by dipping in citral solution may be of practical use if the phytotoxic damage of this application would be eliminated. It is interesting that the general rise in the decay of lemons

and other citrus fruits during the season was inversely related with the decline in the level of preformed antifungal substances in various citrus fruits (Ben-Yehoshua et al., 1988; Kim et al., 1991).

Abiotic stress like UV illumination may act as a nonspecific elicitor simulating in tissue the effect of pathogen challenge. Heat treatment by itself does not induce scoparone production, but it markedly enhances the elicitation induced by the pathogen and inhibits the degradation of the preformed antifungal materials (Kim et al., 1991). Other above-mentioned defensive mechanisms, synthesis of lignin-like materials (Brown and Barmore, 1983) and PR proteins (Hahlbrock and Scheel, 1987), as well as direct thermal inhibition of the pathogen (Ben-Yehoshua et al., 1987), may also participate in the decay-reducing effect of abiotic stresses.

The inhibition of decay development by both heat treatment and UV illumination is an important development encouraging the possibility of reducing exogenous fungicide residues by inducing the fruit to build its own defense against pathogens. Heat treatment (combined with seal packaging) was already demonstrated to enable export of pomelo free of toxic residues to any market in the world (Ben-Yehoshua et al., 1987). However, the application of the heat treatment might be commercially difficult because of the length of treatment (72 h) and its incompatability with the waxing treatment applied in citrus packing houses (Ben-Yehoshua et al., 1989a). Illumination with UV appears to be a more easily applied technology. UV damage, however, is a serious factor which may disturb possible commercial utilization of UV illumination as a new decay control. Further research aims at optimization of fruit treatment and storage conditions that would enhance positive UV effects and diminish undesirable ones.

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