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Histological Detection of Phytoalexin Scoparone from Heat-Treated and UV-Illuminated Lemon Fruits After Inoculation with *Penicillium digitatum*

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Phytoalexin scoparone (6,7-dimethoxycoumarin) was induced in flavedo tissue of lemon fruit inoculated with *Penicillium digitatum* during heat treatment for 3 days at 36°C. The compound was also induced in the flavedo tissue after UV illumination. Induction of scoparone was detected in the flavedo tissue by histological analysis. This fluorescent scoparone accumulated only on the 4-5 layers of cells adjacent to the inoculation site. Pre-inoculation with *P. digitatum* and subsequent heat-treatment induced resistance in the lemon fruit tissues after challenge-inoculation at the site of the first infection. The data obtained in the study suggest that lemon fruit acquired resistance against *P. digitatum* parallel with the scoparone production at the infection site.

Keywords **:** fluorescent compound, histological analysis, phytoalexin, resistance, scoparone (6,7-dimethoxycoumarin).

Phytoalexins, the low-molecular anti-microbial compounds of various chemical structures, are elicited in plant tissues by either biotic (pathogen challenge) or abiotic (wounding, chemicals, irradiation, etc.) stresses (Bailey and Mansfield, 1982). It has been well documented that phytoalexins play an important role in resistance against pathogens (Darvill and Albersheim, 1984; Hahlbrock and Scheel, 1987; Kuc, 1987). The presence of phytoalexins in citrus tissues has also been reported previously (Afek and Sztejnberg, 1988; Arimoto et al., 1986; Kahn et al., 1985; Kim et al., 1991; Rodov et al., 1992).

Phytoalexin, scoparone (6,7-dimethoxycoumarin) was isolated from orange peel infected with *Guignardia citricarpa* Kiely (De Lange et al., 1976). Scoparone was also induced in fruit, leaves and twigs of *Satsuma mandarin* infected with *Diaporthe citri*, but not in the uninfected fruits of citrus (Arimoto et al., 1986). Afek and Sztejnberg (1988) observed the accumulation of scoparone in the bark of various citrus species following inoculation with *Phytophthora citrophthora*. The concentration of scoparone was higher and increased more rapidly in resistant cultivars than in susceptible ones.

Heat treatment of sealed citrus fruits with relatively high temperatures accelerated healing of fruit wounds and markedly reduced green mold decay caused by *Penicillium digitatum* Sacc. (Ben-Yehoshua et al., 1987; 1989). Kim et al. (1991) demonstrated the relationship of this effect to the induction of phytoalexin scoparone in lemon fruit.

Scoparone was also induced in lemon and kumquat peel after UV illumination (Kim et al., 1991; Rodov et al., 1992). The scoparone in UV-illuminated kumquat fruit was associated with the reduction of its decay susceptibility (Rodov et al., 1992). Short wave ultraviolet light is known as a nonspecific phytoalexin elicitor. Elicitation of phytoalexins by UV illumination increased disease resistance of genetically susceptible genotypes of several plant species (Andebrhan and Wood, 1980; Bridge and Klarman, 1973).

Generally, the detection of induced and/or increased compounds in plant tissues by biotic or abiotic stresses was done through chromatography studies. On the other hand, several compounds, which emit fluorescence among the substances, could also be detected by microscopic analysis (Lummerzheim et al., 1993; Von Röpenack et al., 1998).

The purpose of this study was to histologically confirm the induction of fluorescence compounds in lemon flavedo after inoculation with *P. digitatum* or UV illumination. It also sought to determine the contribution of these compounds to the acquired resistance in the inoculated site of the flavedo tissue.

Materials and Methods

Plant materials. Mature, light-green lemons (*Citrus limon* L. Burm., cv. Eureka) were obtained from orchards or packing houses in Israel, prior to any postharvest treatment. Samples of lemon fruit of uniform size and appearance, originating from one orchard, were subjected to different treatments.

Pathogen, fungal suspension, and inoculation. *Penicillium digitatum* Sacc. was isolated from naturally infected lemon fruit. Single spore culture on potato dextrose agar (PDA) was identified according to Raper et al. (1968). Pure culture was kept on PDA

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slants at 4°C. The preparation of conidial suspension of the fungus and procedure of inoculation were done according to Kim et al. (1991).

Fruit treatments after inoculation. Seal packaging of lemon fruits in D-950 film (Cryovac division, Passirana di Rho, Milan, Italy) was done immediately after inoculation. The fruits were then kept for 24 h at 17°C and 85% RH to allow the pathogen to grow after inoculation. Heat treatment (36°C, 72 h) of the inoculated and sealed fruits was done as described by Ben-Yehoshua et al. (1987). After the heat treatment, the fruits were transferred to 17°C and 85% RH. The inoculated and non-sealed fruits were maintained under water-saturated conditions achieved by holding the fruits in a tray covered with a plastic film.

UV illumination. UV illumination of the fruit with a dose of 3×10^3 J m^{-2} was carried out in a chamber with four G15T8 UV lamps (254 nm, each of UV output 3.6 W, Tana Industries, Netiv Halamed-He, Israel). The fruit was placed 25 cm from the UV source. UV dose was measured with a UVX Radiometer (UV products, Inc., San Gabriel, CA, USA). After the UV treatment, the fruit was transferred to 17°C and 85% RH.

Histological analysis. Tissue removed from the peel of the fruit was placed in a plastic mold ($25 \times 20 \times 5$ mm, Miles Scientific, USA). The mold was filled with a Tissue-Tek O.C.T. embedding medium (Miles Scientific, USA) with liquid nitrogen placed on top until frozen solidly. The prepared tissues were sectioned with a Reichert-Jung cryostat model 2800 Frigocut (Cambridge Instruments GmbH, Germany) at −20°C and the sections (20-30 μm thick) were mounted on a poly-L-lysine-coated slide. For observation of fluorescence, the sections were examined with a fluorescent microscope (Universal Photomicroscope Ultraphot, Zeiss, Germany). Photomicrographs were prepared by using Kodak T-Max 400 ASA film.

Extraction and isolation of phytoalexin. Procedures were carried out using the modified method of Kim et al. (1991) and Rodov et al. (1992). Flavedo tissues of the inoculated sites of lemon fruits (5 g fresh weight) and of the UV illuminated fruits were excised with scalpel and extracted with dichloromethane at a ratio of 1 : 4 (w/v) for 48 h. The tissues were homogenized in dichloromethane with an Osterizer Cyclotrol blender (Oster, Milwaukee), and the homogenate was filtered *in vacuo* through Whatman No. 1 filter paper. The filtrate was dried using anhydrous $MgSO_4$ and concentrated *in vacuo* on a Buchi Rotavapor RE 120 evaporator (Buchi, Flawil, Switzerland). The extract was recovered with dichloromethane and dried under nitrogen gas. The dry, crude extracts were kept at −15°C in vials with nitrogen gas until used.

The separation of crude extracts was carried out by thin layer chromatography (TLC) on silica gel (60_{254}, 0.2 mm thickness, Merck, Darmstadt, Germany). Plates developed with toluene:ethyl acetate (1 : 1, v/v) were dried and exposed to UV light (366 nm) for detection of the fluorescent compounds. Individual bands were scraped and extracted with dichloromethane, and then concentrated *in vacuo*.

Detection of antifungal activity of induced fluorescent compound. Antifungal activity of the TLC bands was detected directly on the plates with *Cladosporium cladosporioides* G.A. De Vries as the test organism, according to Dahiya et al. (1984). A sample of 20 μl of induced band extract dissolved in dichloromethane (5 mg ml^{-1}) was separated by TLC as described above. After air-drying overnight at room temperature, the plate was sprayed with *C. cladosporioides* spore suspension in Czapek-Dox medium (10^6 spores ml^{-1}). The presence of antifungal activity was indicated by the absence of mycelium around the spot of the compound on the plate.

Phytoalexin analysis. The active fraction induced by inoculation and UV illumination was purified by HPLC on Varian 5000 equipped with variable wavelength UV-50 detector and a C-18 reverse phase column (250×4 mm, Merck). The elution-solvent mixture was methanol : water (4 : 1, v/v), and the flow-rate was held at 0.5 ml min^{-1} for 12 min. Peaks were detected with a fixed wavelength of 335 nm. The structure of phytoalexin was confirmed by ^{1}H-NMR spectrum at 360 MHz and ^{13}C-NMR spectrum at 90 MHz in $CDCl_3$ on a Bruker WM-360 spectrometer.

Results

***Penicillium* decay.** As observed in previous studies (Ben-Yehoshua et al., 1987; Kim et al., 1991), heat treatment at 36°C for 72 h prevented decay of lemon fruit inoculated with *Penicillium* for over 2 months at 17°C. Non-treated fruit decayed in 3-7 days after inoculation.

Induction of fluorescent compounds in inoculated and heat-treated fruit. Fluorescent compounds accumulated in the cells of flavedo of inoculated and subsequently heat-treated lemon fruit. As shown in Fig. 1, the fluorescent compounds accumulated in the cells adjacent to inoculated site of the fruit. Much lower intensity of fluorescence was also observed in the normal cells of non-treated fruit.

Induction of fluorescent compounds in UV-illuminated fruit. Fluorescent compounds accumulated in the cells of flavedo of lemon fruit illuminated with a UV dose of 3×10^3 J m^{-2}. As shown in Fig. 2, the illuminated cells emitted brighter fluorescence than the normal cells of non-treated fruit.

Antifungal activity and identification of the induced fluorescent compound. Antifungal activity of dilution series of the induced fluorescent compound was carried out with the fungus *C. cladosporioides* by TLC-bioassay. The inhibitory zones of fungal growth at R_f 0.5 on the TLC plate were larger at higher concentrations of the induced compound (Fig. 3). The induced compound with high antifungal activity was found to correspond to scoparone (6,7-dimethoxycoumarin) by NMR spectra data (Afek and Sztejnberg, 1988; Kim et al., 1991).

Effect of re-inoculation in the site of pre-inoculation on decay development. Inhibition of pathogen development in the sites of phytoalexin accumulation was examined by re-inoculation of *P. digitatum*. Accumulation of scoparone was induced by the combined inoculation and heat treat-

ment as described above. The re-inoculation was applied 6 days after heat treatment into non-necrotic flavedo tissue between the sites of previous inoculation, as shown in Fig. 4.

Decay development following re-inoculation at the site of the first inoculation was markedly inhibited as compared with that of the non-treated fruit kept for 10 days at 17°C (Fig. 5). While 100% of decay occurred in the non-treated fruit 6 days after inoculation, only 11% of decay was developed in the pre-inoculated and heat-treated fruit.

Discussion

This study demonstrated that the fluorescent compound, scoparone, was induced in the flavedo tissue of lemon fruit inoculated with *P. digitatum* following heat treatment at 36°C for 72 h. Heat treatment completely inhibited the decay development of *P. digitatum* on lemon fruit.

The scoparone accumulated at the inoculation sites of lemon fruit, but was not detected in the tissue at the site opposite to the inoculation site or even 1 cm away from the inoculation site (Kim et al., 1991). Previous study revealed that the level of scoparone accumulating at the inoculation site was sufficient to inhibit the growth of *P. digitatum* in the fruit. In this present study, however, histological observations showed that fluorescent compounds accumulated only in about 4 or 5 layers of cells adjacent to the inoculation site of the lemon fruit (Fig. 1). This finding was well consistent with the previous report of Ingham (1973) that phytoalexin biosynthesis is confined to the infected cells and their immediate vicinity in most plant species.

The increased resistance in re-inoculated fruit that had recovered from the first inoculation because of the heat treatment, indicated that the lemon had acquired local resistance against *P. digitatum* at the site of the first inoculation (Fig. 5). Since no heating was applied to the re-inoculated fruit, the inhibition of decay development on this site may be due in part to pathogen-induced scoparone.

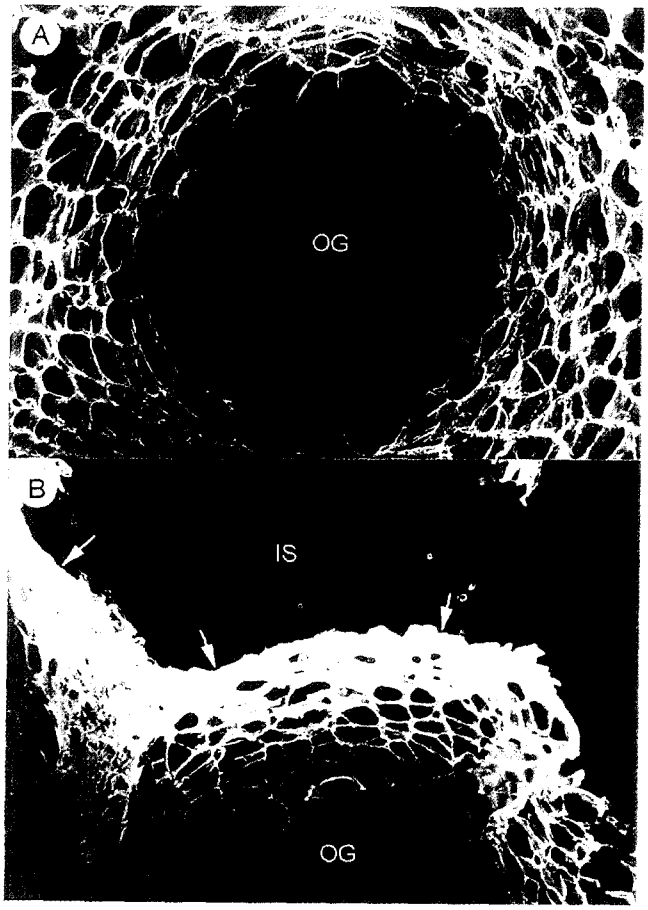

Fig. 1. Accumulation of fluorescent compound in the cells of lemon fruit inoculated with *Penicillium digitatum*. (**A**) non-inoculated fruit; (**B**) inoculated and heat-treated fruit. Arrows indicate the accumulation of fluorescent compounds near inoculated site (IS). OG, oil gland. (× 79).

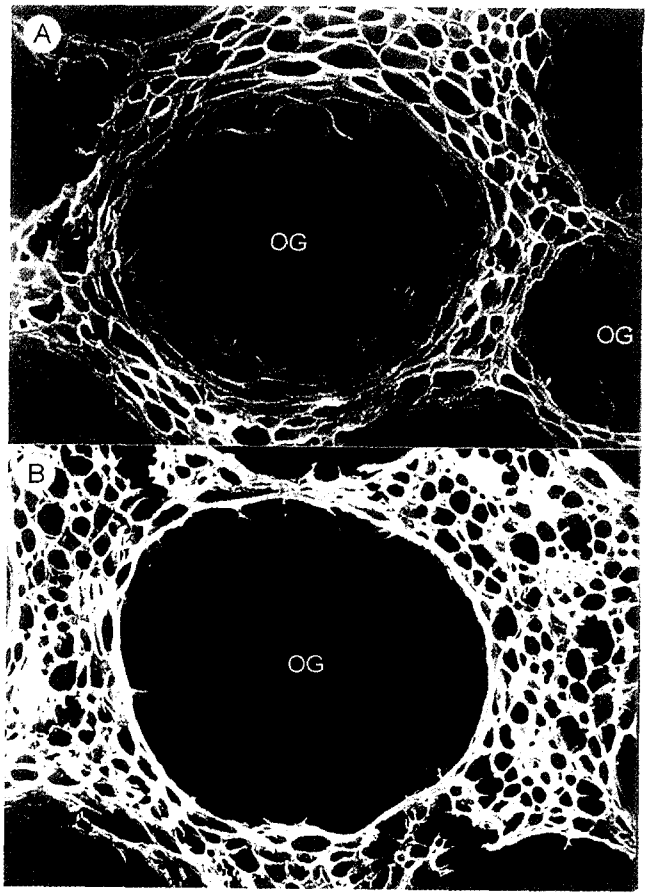

Fig. 2. Accumulation of fluorescent compounds in the cells of UV-illuminated lemon fruit. Section taken from the tissue located 100 μm under the surface. UV dose, 3×10^3 J m^{-2}. (**A**) non-illuminated fruit; (**B**) illuminated. OG; oil gland. (× 79).

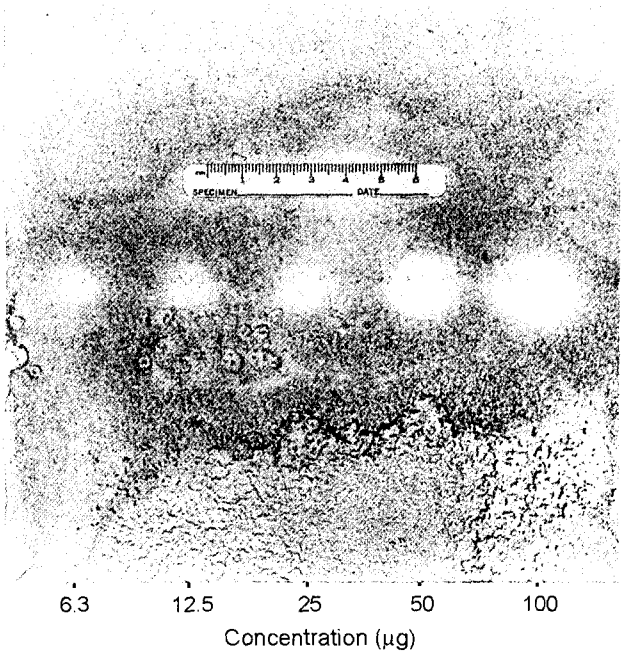

Fig. 3. Antifungal activities of the fluorescent compounds induced by a combined inoculation and heat treatment in lemon fruit. Samples (20 μl) of the dilution series of the compound were placed on a TLC plate, which was then developed by a solvent system of toluene : ethyl acetate (1 : 1, v/v). The developed plate was sprayed with *C. cladosporioides* spore suspension, followed by incubation at 24°C for 3 days.

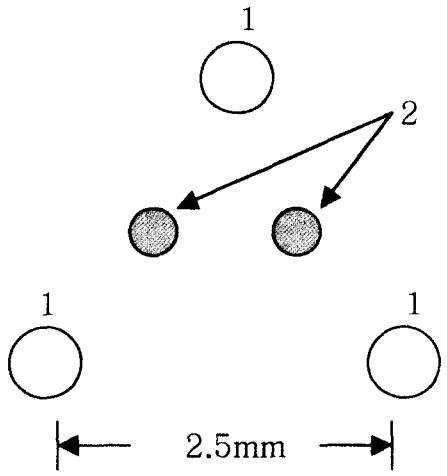

Fig. 4. Scheme of pre-inoculation (1), and re-inoculation (2).

Short wave (254 nm) UV illumination was also shown as an effective elicitor of phytoalexin production (Fig. 2). This is in agreement with many earlier reports starting with Hadwiger and Schwochau (1971). Unlike heat, UV illumination strongly induced production of scoparone even without *Penicillium digitatum* inoculation in lemon (Fig. 2) and other citrus fruits (Ben-Yehoshua et al., 1992).

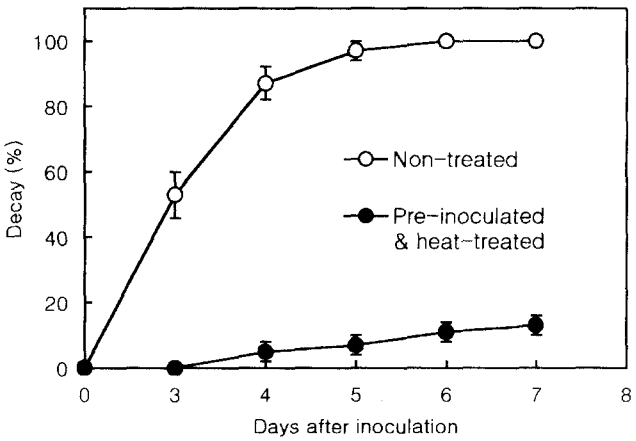

Fig. 5. Effect of re-inoculation with *Penicillium digitatum* on the decay development of lemon fruit. The pre-inoculation was done with a suspension (1×10^6 spores ml^{-1}) of *P. digitatum* at three locations near the stem-end of the fruit. The re-inoculation was done 6 days after heat-treatment on the pre-inoculated site as described in Figure 4. Non-treated fruit was inoculated 10 days after storage at 17°C and 85% RH. Vertical bars indicate standard errors.

The concentration of scoparone in UV-illuminated lemon was dependent on the dose of UV illumination. However, illumination in excess of 5×10^3 J m^{-2} did not stimulate further increase in scoparone level (Kim et al., 1991). The accumulation of scoparone in UV-illuminated lemon was accompanied by a decrease in its decay susceptibility (Ben-Yehoshua et al., 1992). This clearly showed that the inverse relationship between scoparone concentration and fruit decay. Rodov et al. (1992) demonstrated that the decay of kumquat fruit was lowered by UV-treating the fruit prior to inoculation, i.e., without direct contact of pathogen with UV light. Moreover, illumination of previously inoculated fruit failed to prevent its decay.

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