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# Umbelliferone, a phytoalexin associated with resistance of immature Marsh grapefruit to *Penicillium digitatum*

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**Abstract**

A low concentration (2 $\mu g^{-1}$ FW) of 7-hydroxycoumarin (umbelliferone) was found in albedo of non-inoculated mature yellow Marsh grapefruit (*Citrus paradisi*). The concentration of umbelliferone in albedo of immature green grapefruit (2 months before full maturity) was 17 $\mu g\ g^{-1}$ FW. Four days following inoculation with *Penicillium digitatum*, the accumulation of umbelliferone in the albedo of mature and immature grapefruit was 28 and 250 $\mu g\ g^{-1}$ FW, respectively. By that period, the infected area in the yellow fruit was as large as 230 $mm^2$, as compared with 8 $mm^2$ in the green fruit. The $EC_{50}$ of umbelliferone to *P. digitatum* growth in vitro was 95 $\mu g\ ml^{-1}$. Additionally, umbelliferone inhibited growth of several other pathogenic fungi *in vitro*. It is suggested that umbelliferone plays a role in the defense mechanisms of immature grapefruit against *P. digitatum*. 

*Keywords:* *Citrus paradisi*; Rutaceae; Marsh grapefruit; Umbelliferone; 7-Hydroxycoumarin; Phytoalexin; Resistance; *Penicillium digitatum*

## 1. Introduction

The fungus *Penicillium digitatum* Sacc. is well known as the major pathogen causing decay (green mould disease) of citrus fruit during storage (Eckert, 1975; Eckert & Eaks, 1989). Several authors reported that scoparone (6,7-dimethoxycoumarin) is the main phytoalexin involved in induced defense mechanisms of citrus against pathogens such as *Phytophthora citrophthora* (Smith and Smith) Leonian (Afek, Sztejnberg, & Carmely, 1986; Afek & Sztejnberg, 1988), *Guignardia citricarpa* Kiely (De Lange, Vincent, Du Plessis, Van Wyk, & Ackerman, 1976), *Penicillium digitatum* Sacc. (Kim, Ben-Yehoshua, Shapiro, Henis, & Cameli, 1991; Rodov, Ben-Yehoshua, Kim, Shapiro, & Ittah, 1992) and *Diaporthe citri* (Wolf) (Aritmo, Homma, & Ohsawa, 1986). Xanthyletin (6,7-dimethylpyranocoumarin) (Khan, Kunesch, Chuilon, & Ravise, 1985), seselin (7,8-dimethylpyranocoumarin) (Vernenghi, Ramiandrasoa, Chuilon, & Ravise, 1987) and scopoletin (6-methoxy, 7-hydroxycoumarin) (Rodov et al., 1992; Rodov, Burns, Ben-Yehoshua, Fluhr, & Ben-Shalom, 1996) were also isolated from citrus tissues challenged by pathogen or UV light and showed an antifungal activity in vitro. Along with induced antimicrobial materials (phytoalexins), the tissues of immature fruits may contain preformed compounds (phytoanticipins) contributing to their resistance against pathogens (Prusky, 1996). Ben-Yehoshua et al. (1988) and Ben-Yehoshua, Rodov, Kim, and Carmeli (1992) isolated from citrus fruit several preformed antifungal substances of a phenolic or terpenoid nature. The relative importance of phytoanticipins and phytoalexins in defense mechanisms of citrus fruit still awaits investigation.

The outer colored peel layer (flavedo) is considered the major barrier for pathogen invasion and has been the main object of investigating the defense mechanisms of citrus fruit. Recently, Rodov et al. (1996) have demonstrated the capability of the inner white layer of citrus peel (albedo) to produce phytoalexins (scoparone and scopoletin) and PR-proteins. This study examines the role of umbelliferone (7-hydroxycoumarin) as another phytoalexin of citrus fruit accumulated in albedo and involved in resistance of immature grapefruit to *P. digitatum*.

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## 2. Results and discussion

In this study we investigated the phenomenon that immature green grapefruit (2 months before full maturity) was resistant to *P. digitatum*, but became susceptible to the pathogen after maturation accompanied by yellowing. Light-green fruit (3 weeks before full maturity) was found to be less susceptible to *P. digitatum* than the yellow one. Four days after inoculation, the infected areas in yellow, light-green and green fruit peels were 230, 110 and 8 $mm^2$, respectively (Fig. 1).

Our results showed that the concentration of umbelliferone in albedo of non-inoculated yellow grapefruit was as low as 2 $\mu g\ g^{-1}$ FW. The concentration of umbelliferone in albedo of light-green grapefruit was 8 $\mu g\ g^{-1}$ FW and in the albedo of green grapefruit 17 $\mu g\ g^{-1}$ FW. Four days following inoculation with *P. digitatum* Sacc., the accumulation of umbelliferone in the albedo of yellow, light-green and green fruit was 28, 90 and 250 $\mu g\ g^{-1}$ FW, respectively. The concentration of umbelliferone after 4 days of storage started to decline. Mechanical injury had no effect on umbelliferone production. The $EC_{50}$ of umbelliferone to *P. digitatum* was found to be 95 $\mu g\ ml^{-1}$. Additionally to *P. digitatum*, umbelliferone inhibited growth of several other fungi in vitro (Table 1).

In yellow and light green fruit, *P. digitatum* expanded during the whole 8-day period following the inoculation (no checks were performed after 8 days since most of the yellow fruit were infected). On the other hand, in green fruit the pathogen expansion was stopped after 3 days Fig. 1. The concentration of umbelliferone in the albedo of green fruit by that time was 143 $\mu g\ g^{-1}$ FW (Fig. 2). This concentration was sufficient to inhibit the growth of the pathogen in the peel, since the $EC_{50}$ value of umbelliferone for *P. digitatum* was 95 $\mu g\ ml^{-1}$. In yellow and light-green fruit the amounts of umbelliferone after the inoculation were insufficient for pathogen inhibition. Still, the expansion rate of *P. digitatum* in light-green fruit was slower as compared to yellow fruit, in agreement with higher umbelliferone accumulation (Fig. 1).

Table 1
Effective concentrations of umbelliferone for obtaining 50% growth inhibition ($EC_{50}$) of 5 pathogenic fungi of citrus and other crops

| Fungal species | $EC_{50}$ of umbelliferone ($\mu g\ ml^{-1}$) |
|---|---|
| *Penicillium digitatum* | 95 |
| *Penicillium italicum* | 110 |
| *Phytophthora citrophthora* | 125 |
| *Botrytis cinerea* | 70 |
| *Alternaria alternata* | 85 |

Our results indicate that umbelliferone is accumulated in the albedo of pathogen-challenged grapefruit, additionally to scoparone and scopoletin described earlier (Rodov et al., 1996). We assume that this complex of phytoalexins plays a role in defense mechanisms of immature grapefruit against wound pathogens such as *P. digitatum* when the fungus penetrates the fruit through mechanical injuries of the flavedo. Little attention was paid to umbelliferone in previous studies with citrus fruits, possibly since the extraction of the peel was usually conducted using less polar solvents, such as dichloromethane. At the same time, in other plant species umbelliferone was shown earlier to participate in defense mechanisms against pathogens (Hamerski, Schmitt, & Matern, 1990; El-Modafar, Clerivet, Fleuriet, & Macheix, 1993).

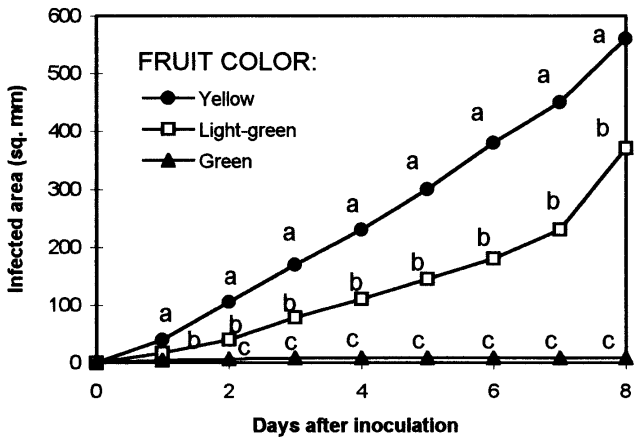


Fig. 1. The infected area ($mm^2$) in yellow, light green and green grapefruit peels 1–8 days following inoculation with *P. digitatum* and incubation at 20°C and 95% RH. Numbers are means of 5 replicates (50 fruits in each replicate). Different letters within the same evaluation day indicate significant differences among the values according to Fisher's protected least significant difference ($P=0.05$).

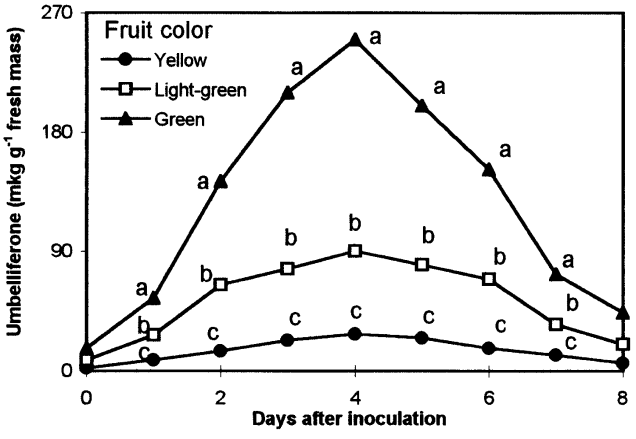


Fig. 2. Accumulation of umbelliferone ($\mu g\ g^{-1}$ FW) in the albedo of yellow, light-green and green grapefruit 0–8 days following inoculation with *P. digitatum* and incubation at 20°C and 95% RH. Numbers are means of 5 replicates (50 fruits in each replicate). Different letters within the same evaluation day indicate significant differences among the values according to Fisher's protected least significant difference ($P=0.05$).

Brown (1985) suggested the biosynthetic pathway of umbelliferone and its metabolites as follows: 4-hydroxycinnamic acid→umbelliferone→aesculetin→scopoletin→puberulin. Scopoletin, in turn, was supposed to be a precursor of scoparone (Rodov et al., 1992). According to this scheme, the synthesis of umbelliferone precedes the formation of scoparone and scopoletion and therefore may be important for an early defense response of the fruit against pathogen invasion.

## 3. Experimental

### 3.1. Fungal and plant materials

The fungi, *Penicillium digitatum* Sacc., *Penicillium italicum* Wehmer, *Phytophthora citrophthora* (Smith and Smith) Leonian, *Botrytis cinerea* Pers. and *Alternaria alternata* (Fr.) Keissler (Lib). Dby were used in the experiments. The first three fungi are major pathogens causing rot diseases to grapefruit during storage and were isolated from naturally infected fruits from the Gilat Experiment Station, Israel, in January 1995. The fungi *B. cinerea* and *A. alternata* were isolated from naturally infected celery at the same time and from the same area.

Fruit were inoculated by wounding the peel upto a depth of 3 mm and injecting the conidia suspension of *P. digitatum* (Isolate G-14) 150 μl, $10^5$ spores/ml into the albedo layer. The fruit were incubated at 20°C and 95% RH.

### 3.2. Extraction, purification, isolation, identification and quantification of umbelliferone in grapefruit peel

Extraction was done 4 days after inoculation with *P. digitatum* for umbelliferone. Inoculated albedo with necrotic lesions (760 g FW) were extracted with distilled water, at 10 ml/g FW, for 2 h at 70°C and concentrated by evaporating the crude extract at 60°C in a Rotovac evaporator to a volume of 50 ml. This was followed by partition with EtOAc and concentration by evaporating the solvent.

NMR spectra were recorded on a Bruker ARX-500 spectrometer at 500.1 MHz for $^1$H. Preparative HPLC separations were preformed with Applied Biosystem instrument equipped with two model 150 pumps and 893 programmable detector. Mass spectra were recorded on a Pisons VG AutoSpec Q. The crude material (597 mg) was loaded onto a vacuum column (Merck silica H, 5 g, packed into a 2 cm i.d. × 30 cm high, sintered glass funnel; vacuum by water aspirator) and eluted in petroleum ether with an increasing EtOAc step gradient to yield 12 fractions (50 ml each). The fractions were assayed for antifungal activity against *P. digitatum*. Fractions 5, 6 and 7 eluted from the column with 30–40% EtOAc in petroleum ether, were the most active. Fraction 6 (22 mg) was purified on a preparative HPLC column (Althech Econosil $C_{18}$, 10 m, 250 × 22 mm) using 7:3 MeOH/water as the eluant (5 ml/min) with UV detection at 254 nm. The chromatogram yielded five major components. The second component ($t_R$ 20 min, 1.1 mg) was found to be the active component of the mixture and was identified as umbelliferone.

Umbelliferone: CIMS (methane) $m/z$ 162 ($MH^+$, 100%), 135 (21), 109 (33), 107 (35), 95 (30); identified by comparison with NIST data base and with the NMR of authentic sample as umbelliferone; $^1$H NMR data ($CDCl_3 + CD_3OD$) $\delta$ (multiplicity, $J$ in Hz, assignment): 7.57 (d, $J$=9.5 Hz, H-3), 7.23 (d, $J$=9.4 Hz, H-4), 7.27 (d, $J$=8.4 Hz, H-5), 6.67 (dd, $J$=8.4, 2.3 Hz, H-6), 6.65 (d, $J$=2.3 Hz, H-8), 6.07 (d, $J$=9.5 Hz, H-2).

Umbelliferone was also identified by $R_f$ values on TLC plates (0.5 mm, Art. 7730, Kieselgel 60 GF254, Merck), as compared with the authentic sample. Ascending TLC was developed in a mixture of toluene/EtOAc (1:1), then dried and inspected under UV light (365 nm) and $R_f$ was found to be 0.50.

Quantification of umbelliferone in the albedo during 8 days of storage at 20°C was carried out by means of analytical HPLC using an L-6200 pump, an L-4200 UV-VIS detector and a D-200 Chromat-Integrator (Merk-Hitachi, Tokyo, Japan). A Machery-Nagel (Duren, Germany) Nucleosil silica column (250 × 4 mm) containing $C_{18}$ reverse-phase packing of 5-m particle size, with a 15 × 4-mm guard column filled with the same packing material was used. All standards and samples were dissolved in MeOH. For standard we used umbelliferone which was purchased from Sigma. The standard was eluted isocratically with MeOH–$H_2O$ (45:55, mixed by HPLC pump) at a flow rate of 1.0 ml/min. Peaks were monitored and quantified at 254 nm.

### 3.3. Ultraviolet (UV) spectrometry

Measurements of UV absorption (UVA) were carried out using an Optronic 742 spectroradiometer which measured the irradianic at 1 nm intervals. Total UVA (320–380 nm) was obtained by integrating the spectroradiometric reading over this range.

### 3.4. Bioassays

Bioassays were carried out for umbelliferone. The $EC_{50}$ values for various fungi were determined by adding increasing concentrations of umbelliferone to cooled molten potato-dextrose agar (PDA) immediately before pouring into Petri plates. Plates were inoculated with conidia of *P. digitatum*, *P. italicum*, *B. cinerea* and *A. alternata* or with blended mycelium of *P. citrophthora* and then incubated in darkness. The $EC_{50}$ values for colony area growth in these plates were calculated from the regression lines obtained by plotting the percent inhi-

bition of growth against the log concentration of the compounds.

### 3.5. Statistical analysis

All experiments were conducted in a completely randomized design with 5 replicates for each treatment. Data were analyzed by ANOVA procedures using the Statistical Analysis System (SAS) package (Cary, NC). Experiments were conducted three times.

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## References

Afek, U., & Sztejnberg, A. (1988). *Phytopathology*, *78*, 1678.
Afek, U., Sztejnberg, A., & Carmely, S. (1986). *Phytochemistry*, *25*, 1855.
Aritmo, Y., Homma, Y., & Ohsawa, T. (1986). *Ann. Phytopathol. Soc. Jpn.*, *52*, 620.
Ben-Yehoshua, S., Rodov, V., Kim, J. J., & Carmeli, S. (1992). *J. Agric. Food Chem.*, *40*, 1217.
Ben-Yehoshua, S., Shapiro, B., Kim, J. J., Sharoni, J., Carmeli, S., & Kashman, Y. (1988). *Proc. 6th Int. Citrus Congr.* (Vol. 3, p. 1371).
Brown, S. A. (1985). *Can. J. Biochem. Cell Biol.*, *63*, 292.
De Lange, J. H., Vincent, A. P., Du Plessis, L. M., Van Wyk, P. J., & Ackerman, L. G. J. (1976). *Phytophylactica*, *8*, 83.
Eckert, J. W. (1975). In N. F. Haard & D. K. Salunkhe (Eds.), *Post-harvest biology and handling of fruits and vegetables* (ch. 9, p. 81). Westport, CT: Avi Publishing Co.
Eckert, J. W. & Eaks, I. L. (1989). In *The Citrus Industry* (Vol. 5, p. 179). Riverside, CA: University of California Press.
El-Modafar, C., Clerivet, A., Fleuriet, A., & Macheix, J. J. (1993). *Phytochemistry*, *34*, 1271.
Hamerski, D., Schmitt, D., & Matern, U. (1990). *Phytochemistry*, *29*, 1131.
Khan, A. J., Kunesch, G., Chuilon, S., & Ravise, A. (1985). *Fruits*, *40*, 807.
Kim, J. J., Ben-Yehoshua, S., Shapiro, B., Henis, Y., & Cameli, S. (1991). *Plant. Physiol.*, *97*, 880.
Prusky, D. (1996). *Ann. Rev. Phytopathol.*, *34*, 413.
Rodov, V., Ben-Yehoshua, S., Kim, J. J., Shapiro, B., & Ittah, Y. (1992). *J. Am. Soc. Hort. Sci.*, *117*, 788.
Rodov, V., Burns, P., Ben-Yehoshua, S., Fluhr, R. & Ben-Shalom, N. (1996). *Proc. Int. Soc. Citriculture* (Vol. 2, p. 1101).
Vernenghi, A., Ramiandrasoa, F., Chuilon, S., & Ravise, A. (1987). *Fruits*, *42*, 103.