European Journal of Plant Pathology **106:** 617–622, 2000.
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UV irradiation alters the levels of flavonoids involved in the defence mechanism of *Citrus aurantium* fruits against *Penicillium digitatum*

M.C. Arcas, J.M. Botía, A.M. Ortuño and J.A. Del Río*
*Departamento de Fisiología Vegetal (Biología Vegetal), Facultad de Biología, Universidad de Murcia, Campus de Espinardo, 30100, Murcia, Spain; *Author for correspondence (Phone: +34968364944; Fax: +34968363963; E-mail: jadelrio@fcu.um.es)*

Accepted 30 May 2000

Key words: Citrus, flavanone, flavone, fungitoxins, naringin, tangeretin

Abstract

The effect of UV irradiation on the levels of the flavanone, naringin, and the polymethoxyflavone, tangeretin, in the peel of *Citrus aurantium* fruits is described, as changes in the synthesis and/or accumulation of these compounds after infection with *Penicillium digitatum*. The growth of *P. digitatum* on previously irradiated fruit was reduced by up to 45%. Changes in flavonoid levels were detected, associated with inhibition of fungus growth, the naringin content falling by 69% and tangeretin levels increasing by 70%. The possible participation of naringin and tangeretin in the defence mechanism of this *Citrus* species is discussed.

Introduction

In previous studies, the accumulation of the flavanone naringin (Castillo et al., 1992) and of the polymethoxyflavone tangeretin was described (Del Río et al., 1998a,b) in *Citrus aurantium* fruit. As regard the distribution of these compounds in this species and other citrus, they were shown to be present in the surface tissues of the fruit, i.e. in the albedo and flavedo (Machida and Osawa, 1989; Mizuno et al., 1991; Kanes et al., 1992; Ortuño et al., 1999). The highest levels of these compounds were associated with young developing fruits (Castillo et al., 1992; 1993; Benavente-García et al., 1993; Del Río et al., 1995; 1997; 1998a; Ortuño et al., 1995; 1997a; 1999), where they represented up to 6% of the dry weight in the case of naringin and up to 3×10^{-3}% in that of tangeretin, this polymethoxyflavone being a minor component. In addition, the polymethoxyflavones, sinensetin and nobiletin, were accumulated to a lesser degree in the maturation phase of *C. aurantium* fruits (Del Río et al., 1998a). The accumulation of these compounds can be modulated by chemical factors (phytoregulators) (Del Río et al., 1995; García-Puig et al., 1995) and physical factors, including light, temperature, humidity, ultra violet (UV) radiation, etc. (Dixon and Paiva, 1995).

The interest in *Citrus* flavonoids lies in their potential pharmacological and physiological activity. For example, the compounds studied showed anti-carcinogenic activity due to their antioxidant properties and the ability to absorb UV light (Shimoi et al., 1994), with anti-mutagenic and anti-proliferative effects (Stapleton and Walbot, 1994; Manach et al., 1996). Others properties affect capillary fragility (Szent-Gyorgyi, 1938) and erythrocyte aggregation (Robbins, 1976). The above flavonoids also show anti-inflammatory, anti-allergic and analgesic activities (Gabor, 1986; Galati et al., 1994).

On the other hand, the taxonomic interest of these compounds has been widely described (Kamiya et al., 1979; Mizuno et al., 1991; Kanes et al., 1992). The polyphenolic profile has been used to detect the adulteration of juice products (Ooghe et al., 1994; Mouly et al., 1998).

As regards the physiological role of these compounds, some authors have suggested that flavonoids are a plant defence mechanism, with flavone levels

increasing in reponse to high visible light levels, perhaps in order to attenuate the amount of light reaching the photosynthetic cells (Beggs et al., 1987). There are also studies on the role of these compounds in resistance to pathogens (Huet, 1982; Buffar et al., 1996; Weidenbörner et al., 1992) and on the resistance conferred by polymethoxyflavones in Citrus (Ben-Aziz, 1967; Del Río et al., 1998b). Flavonoids, can act as antioxidants and inhibitors of numerous enzymes such as phenolases (Challice and Willians, 1970) and pectinmethyltransferases (De Swardt et al., 1967). These compounds may also influence IAA-oxidase activity (Pradhan and Basu, 1981), regulate the polar transport of IAA (Jacobs and Rubery, 1988) and induce nodule formation after infection with *Rhizobium* by activating *nod* genes (Vierheilig et al., 1998).

Green mold (caused by *Penicillium digitatum*) is one of the most important post-harvest diseases in Citrus fruits (Holmes and Eckert, 1995). To prevent development of this pathogen and to limit losses in commercial fruit shipments, treatment with chemical fungicides is a widely used procedure. However, such treatment may produce serious problems with residues on the fruit (Cabras et al., 1999) and fungicide-resistant strains of *P. digitatum* (Ben-Yehoshua et al., 1994). In this paper, the effect of UV irradiation on the levels of the flavanone naringin, and the polymethoxyflavone tangeretin was studied in the peel of *C. aurantium* fruits before and after infection with *P. digitatum*, in order to correlate a possible role in the defence against the fungus.

Materials and methods

Chemicals

Naringin was obtained from Sigma Chemical Co. (USA) and tangeretin was purchased from Extrasynthèse S.A. (France).

Fungal cultures and estimation of IC_{50}

An isolate of the fungus *P. digitatum* obtained from the Spanish Collection of Type Culture (Valencia, Spain), was cultured on potato dextrose agar (PDA) medium at 25 °C to serve as inoculum. The antifungal activity of naringin and tangeretin was determined by '*in vitro*' assays with *P. digitatum* (Del Río et al., 1998b). The inhibition index (IC_{50}) was expressed as the concentration (g/L) of these compounds required to provide 50% inhibition of radial growth at 100 h. The IC_{50} was determined by linear regression.

Plant materials and UV treatment

Fruit from *C. aurantium* trees located in an experimental field of Murcia University were used. After harvesting, the mature fruits were washed in water before being placed under UV-C light (254 nm) using a germicidal G30T8 lamp (supplied by OSRAM SYLVANIA Products Inc., USA) for 1 or 2 h. The intact fruits were placed 65 cm from the UV-C source (0.1 W/m^2). Non-irradiated fruits were used as control. The control (non-irradiated) and UV treated fruits were stored for 2 days in a thermostatted chamber at 25 °C and 45% relative humidity in the dark. These fruits were used for the inoculation assays with the fungus *P. digitatum* and for the corresponding controls (non-inoculated).

Extraction of flavonoids and chromatographic analysis

Four fruits were used in each experiment. The peel (flavedo and albedo) was taken from around the wound area (control) or from the lesion of inoculated fruit (both irradiated and non-irradiated) and immediately dried at 50 °C to constant weight. The dried peel was ground and shaken with dimethylsulphoxide for 24 h in a proportion of 40 mg of dry weight/ml in the case of the polymethoxylated flavone extraction and 60 mg of dry weight/ml for the flavanone glycosides. The resulting extracts were filtered through a 0.45 μm nylon membrane before analysis.

A Hewlett Packard Liquid Chromatograph (mod. HP1050, USA), with a diode array detector (range scanned: 220–500 nm) was used to quantify the naringin and tangeretin present in the extracts. The stationary phase was a C_{18} column (250 mm × 4 mm i.d.) with a particle size of 5 μm thermostatted at 30 °C. For naringin isocratic separation a mixture of water : methanol : acetonitrile : acetic acid (15 : 2 : 2 : 1) was used as solvent, while for tangeretin the stationary phase was the same and as solvent we used (A) tetrahydrofurane: (B) water: (C) acetonitrile (Ooghe et al., 1994), optimized for our particular work conditions with a gradient profile of 12% (A), 68% (B) and 20% (C) in 20 min, and then 18% (B) and 70% (C) in 20 min. At 45 min the mixture began to change to its initial composition, a process that lasted 15 min. Eluent flow was 1 ml/min in both cases.

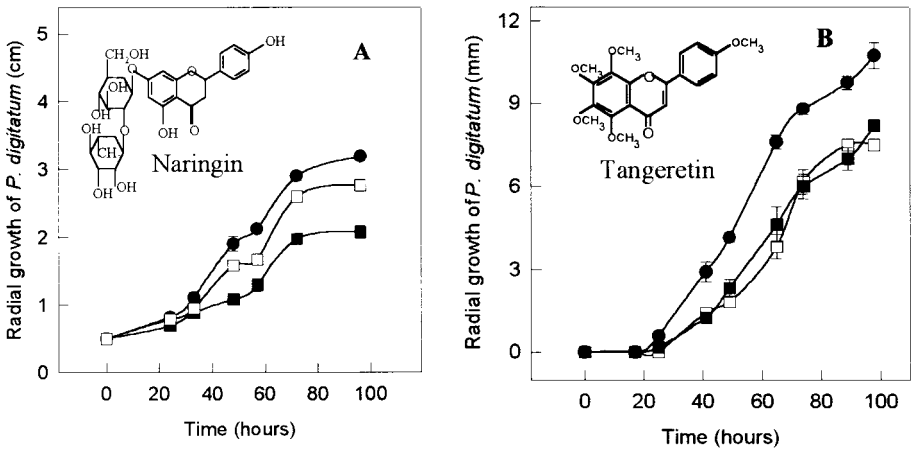

Figure 1. Effects of naringin (A) at 1 g/L (□) and 5 g/L (■), and tangeretin (B) at 1 g/L (□) and 0.1 g/L (■) on the growth of *P. digitatum*. The controls are represented by (●). The data correspond to mean values of mycelial diameters in cm (A) and mm (B). Verticals bars denote ±SE when larger than symbols.

The absorbance changes were recorded in V/UV diode-array detector at 280 nm for the flavanones and 340 nm for the flavones. The amounts of flavonoids were determined from the area given by the integrator using the response factor of the corresponding standards.

Inoculation and evaluation of fruit resistance

Two 6 mm diameter sections of flavedo (from opposite points in the equatorial zone) were removed from each fruit to be inoculated by means of a glass capillary and sterile scalpel. A disk of similar diameter of culture medium with mycelium of *P. digitatum* and another of fungus-free culture medium were then placed one in each wound to represent infected and control fruit, respectively. Inoculation was carried out immediately after wounding and the wounds were sealed by adhesive plastic strip as were the controls. The respective fruits were kept in a chamber at 20 °C and 85% relative humidity. At different times after inoculation (2, 3, 4 and 5 days), the resistance of fruit to infection was determined by measuring the diameter of the lesion.

Results

Effect of naringin and tangeretin on 'in vitro' *growth of* P. digitatum

The presence of naringin (1 and 5 g/L) and tangeretin (0.1 and 1 g/L) in the culture medium inhibited the radial growth of *P. digitatum* (Figure 1A and B, respectively). The IC_{50} for each of these compounds reveals that naringin is considerably less active than tangeretin (10.4 ± 0.2 and 2.4 ± 0.2 g/L, respectively), although in both cases the flavonoids acted as fungistatic agents at the concentration assayed. Examination of the hyphal morphology showed lower spore production in the fungus grown in the presence of tangeretin (data not shown).

Changes in naringin and tangeretin levels as a result of treatment with UV light and inoculation with P. digitatum

The concentrations of naringin and tangeretin detected 7 days after irradiation in the peel of control fruit and fruit subjected to UV irradiation for 1 or 2 h are shown in Figure 2. It can be seen that the control fruit and the fruit irradiated for 1 h showed similar naringin and tangeretin concentrations, while the levels of both flavonoids increased by 7% and 55% respectively after irradiation for 2 h (Figure 2A and B). These findings suggest that UV irradiation may physically modulate the synthesis and/or accumulation of these phenolic compounds in the plant material studied, as has been described by other authors for other flavonic compounds in other plant species (Lois, 1994; Lois and Buchanan, 1994; Dixon and Paiva, 1995).

When these fruit were inoculated with *P. digitatum* substantial changes in the naringin and tangeretin levels were observed. At the end of the experimental period

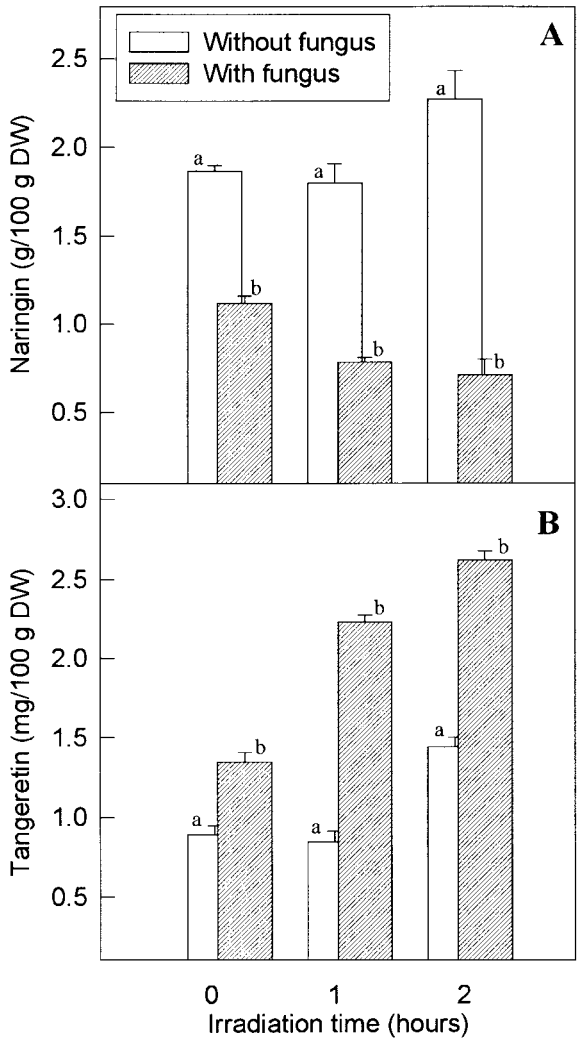

Figure 2. Effect of UV irradiation and inoculation after irradiation with *P. digitatum* on naringin (A) and tangeretin (B) levels. Flavanone and polymethoxyflavone levels were recorded 5 days after inoculation (7 days after irradiation) in control and inoculated fruit. The vertical bars denote ±SE ($n = 4$). Values corresponding to uninoculated and inoculated fruits were compared by Duncan's multiple range test ($p = 0.01$).

(5 days after inoculation) the naringin levels observed were significantly lower in inoculated fruits than in uninoculated fruits (Figure 2A): 1.1 g/100 g DW compared with 1.83 g/100 g DW in non-irradiated fruit; 0.78 compared with 1.80 g/100 g DW in fruit irradiated for one hour; and 0.71 compared with 2.27 g/100 g DW after 2 h irradiation. The drop in naringin levels in inoculated fruit is likely to be at least in part

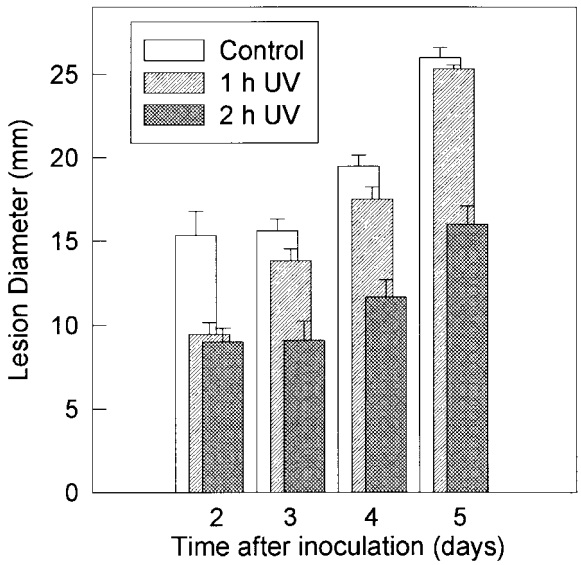

Figure 3. UV irradiation effect on *P. digitatum* growth '*in vivo*'. The vertical bars denote ±SE ($n = 4$).

due to the hydrolysing action of the fungus, which breaks down the naringin molecule into naringenin, rhamnose and glucose. Naringenin was measured in the way described in a previous paper (Castillo et al., 1994) (data not shown). On the other hand, tangeretin levels in fruits at 5 days after inoculation were significantly higher than in non-inoculated fruits (Figure 2B): 1.45 compared to 0.99 mg/100 g DW in non-irradiated fruits; 2.24 compared to 0.95 mg/100 g DW in fruits irradiated for one hour; and 2.62 compared to 1.54 mg/100 g DW in fruits exposed to 2 h irradiation.

'In vivo' *growth of* P. digitatum *on UV irradiated* C. aurantium *fruits*

Figure 3 shows the growth of *P. digitatum* on peel from non-irradiated fruit (control), and from fruit irradiated for 1 and 2 h. It can be seen how the diameter of the lesion grows less in irradiated fruit than in the control (non-irradiated) fruit. Two days after inoculation, inhibition is 40% in the fruit irradiated for 1 h and 43% in fruit irradiated for 2 h. Three days after inoculation the inhibition levels are 16% and 45%, repectively, for the same irradiation times. These levels remain similar (17% and 45%) at four days and fall to 3% and 38% at 5 days.

Discussion

Based on the results obtained, it is suggested that the two constitutive secondary metabolites of *C. aurantium* studied (naringin and tangeretin) may act as fungitoxins in the resistance mechanism against fungal attack, acting as first and second defence barriers, respectively, since polymethoxylated flavones (tangeretin) are mainly localised in the outermost tissue of the fruit, the flavedo (Kanes et al., 1992), while flavanones (naringin) are located in the albedo which is immediately below the flavedo (Kanes et al., 1992). However, this finding does not discount the fact that other secondary compounds induced after infection, such as coumarins, may also act in the defence mechanism of this plant material, as has been described in other *Citrus* species (Angioni et al., 1998; Ortuño et al., 1997b).

Bearing in mind that bitter orange (*C. aurantium*) and other Citrus species are used for the extraction of essential oils and the production of jams and juices, it is important to control the residue levels of chemical fungicides (Friar and Reynolds, 1997). One alternative to the use of chemical fungicides might be to stimulate the defense mechanisms of fruit by stimulating the biosynthesis of the involved secondary compounds (including phenolics) by pre-harvest hormonal treatment (Del Río et al., 1995; Ortuño et al., 1997b) or post-harvest UV irradiation and heat treatment (Ben-Yehoshua et al., 1992; 1995).

Acknowledgements

M.C. Arcas has a grant from the 'Fundación Séneca' in collaboration with the INFO (Murcia).

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