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Comparative study of flavonoid and scoparone accumulation in different Citrus species and their susceptibility to *Penicillium digitatum*

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ABSTRACT

Fungi of the genus *Penicillium* are responsible for substantial post-harvest losses in *Citrus* fruits. The results obtained following artificial inoculation of *Citrus* fruits with *Penicillium digitatum* showed that the degree of fungal development depended on the *Citrus* species. Thus, the mature fruit of *Citrus paradisi* were more susceptible to this fungus than the mature fruit of *Citrus limon, Citrus sinensis, Citrus clementina* Hort. ex Tan., and *Citrus unshiu* (mak) Marc. The results point to an inverse correlation between the degree of susceptibility of *Citrus* species to this fungus and the flavanone content – hesperidin in *C. sinensis*, *C. clementina* Hort. ex Tan. and *C. unshiu* (mak) Marc.; naringin in *C. paradisi* and the flavanone hesperidin and the flavone diosmin in *C. limon*. Thus, in *C. sinensis, C. clementina* Hort. ex Tan and *C. unshiu* (mak) Marc. the highest levels of the polymethoxyflavones, sinensetin, tangeretin, heptamethoxyflavone and nobiletin, were observed in the least susceptible varieties and viceversa. In the case of *C. paradisi*, no significant differences were detected in the polymethoxyflavone levels between varieties, while in *C. limon*, polymethoxyflavones were hardly detectable. The production of scoparone was observed in all the species and varieties studied after inoculation with the fungus, especially in *C. limon* fruits. Based on the evidence, it seems that flavanones, flavones, polymethoxyflavones (phytoanticipins) and scoparone (a phytoalexin) may well be involved in the defence mechanisms of *Citrus* fruits against *P. digitatum*. Depending on the *Citrus* species in question, the relative participation of one group of secondary compounds or another may vary.

1. Introduction

Green mould, caused by *Penicillium digitatum*, is one of the most damaging post-harvest diseases in *Citrus* fruits (Holmes & Eckert, 1995). Several authors reported that some species of *Citrus* when infected by phytopathogenic fungi accumulate coumarins such as scoparone (6,7-dimethoxycoumarin), which is considered as the main phytoalexin involved in the induced defence mechanism of *Citrus* against pathogens such as *Phytophthora citrophthora* (Afek & Sztejnberg, 1988; Afek, Sztejnberg, & Carmely, 1986), *Phytophthora parasitica* (Ortuño et al., 1997), *Guignardia citricarpa* Kiely (De Lange, Vincent, Du Plessis, Van Wyk, & Ackerman, 1976) P. *digitatum* Sacc. (Kim, Ben-Yehoshua, Shapiro, Henis, & Carmeli, 1991), *Diaporthe citri* (Wolf) (Aritmo, Homma, & Ohsawa 1986), and *Botrytis cinerea* (Kuniga & Matsumoto 2006). The nature and rate of phytoalexin production/accumulation have been described as depending on the host and pathogen genotypes (Baudoin & Eckert, 1985; Kim et al., 1991; Purkayastha, 1994).

It is known that the *Citrus* genus accumulates a series of species-specific flavanones, flavones and polymethoxyflavanones, including hesperidin, naringin, diosmin, sinensetin (5,6,7,3′,4′-pentamethoxyflavone), tangeretin (5,6,7,8,4′-pentamethoxyflavone), 3,5,6,7,8,3′,4′-heptamethoxyflavone and nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) (Albach & Redman, 1969; Del Río, Arcas, Benavente-García, Sabater, & Ortuño, 1998; Del Río & Ortuño, 1994; Del Río et al., 2004; Jourdan, McIntosh, & Mansell, 1985; Ooghe, Ooghe, Detavernier, & Huyghebaert, 1994; Ortuño et al., 1995; Ortuño et al., 1997), which are naturally synthesised by the plant before infection of the tissues of immature fruits, and which may also be involved in the natural resistance of *Citrus* against pathogens acting as phytoanticipins (Arcas, Botía, Ortuño, & Del Río, 2000; Ben-Aziz, 1967; Del Río, Arcas, Benavente-García, & Ortuño, 1998; Del Río & Ortuño, 2004; Del Río et al., 2004; Ortuño, Arcas, Botía, Fuster, & Del Río, 2002; Ortuño et al., 2006), although the participation of other secondary compounds of terpenic nature or derivates in this complex signalling pathway cannot be discarded (Ben-Yehoshua et al., 2008; Rodov, Ben-Yehoshua, Fang, Kim, & Ashkenazi, 1995).

Some studies have pointed to differences in the susceptibility of different *Citrus* species to certain pathogenic fungi, such as Alternaria (Gardner, Kono, & Chandler, 1986; Peever, Olsen, Ibáñez, & Timmer, 2000; Pegg, 1966; Reis, Almeida, Stuchi, & Goes, 2007;

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Solel & Kimchi, 1997; Timmer, Solel, & Orozco-Santos, 2000) and Phytophthora (Afek & Sztejnberg, 1988). Such differences in susceptibility could be attributed to the levels of ethylene in the first case, so that ethylene may well be considered as a possible marker of *Citrus* sp. susceptibility to *A. alternata* (Ortuño et al., 2008), or scoparone in the latter (Afek & Sztejnberg, 1988).

In previous studies we showed that some citric flavonoids may act as antifungal agents against *P. digitatum*, polymethoxyflavones being more active than the flavanones in this respect (Arcas et al., 2000; Ortuño et al., 2006). However, little information exists concerning the susceptibility of the *Citrus* sp. to this fungus and the possible secondary compounds involved in these changes.

The objective of this work was to investigate the susceptibility of different *Citrus* sp. against *P. digitatum* and to establish a possible relation between these changes in susceptibility and possible differences in the flavonoid/scoparone expression associated with the species in question.

2. Materials and methods

2.1. Plant material

The following varieties of *C. limon* (L.) Burm f. were used in the different experiments: Laphitos, Santa Teresa, Fino-47 and Villafranca, grafted onto *C. macrophylla* and grown in La Alberca (Murcia, Spain).

The varieties of *C. sinensis* were Washington Navel, Salustiana, New Hall and Barberina, grafted onto Citrange Carrizo and grown in Torre Pacheco (Murcia, Spain).

Within the group of mandarins, the varieties of *C. clementina* Hort. ex Tan. were Hernandina, Oronules and Clementina. The variety of *C. unshiu* (mak) Marc. was, Clausellina. In all cases grafted onto Citrange Carrizo and grown in Torre Pacheco (Murcia, Spain).

The varieties of *C. paradisi* were Marsh, Red Blush, Henderson and Star Ruby, grafted onto Cleopatra mandarin orange and grown in La Alberca (Murcia, Spain).

Mature fruits of the above species and varieties were used in all the assays.

2.2. Fungal cultures, fruits inoculation, and measurement of growth

An isolate of the fungus *P. digitatum* obtained from the Spanish Collection of Type Culture (Valencia, Spain) (CECT 2954) was cultured on potato dextrose agar (PDA) medium at 25 °C to serve as inoculum.

To study the *in vivo* growth of the fungus, the fruit of the different citrics studied were sprayed with 90% ethanol and placed on trays to be inoculated. Eighteen similar fruit were used in each of the inoculation assays described below, in which mycelium of *P. digitatum* was deposited on unwounded fruit, which was sealed by adhesive tape. The inoculated fruits were kept in a growth chamber at 20 °C and 85% relative humidity and examined at different times post-inoculation to measure fungal growth as diameter of the lesion in mm.

To determine the initial growth rate of the fungus on the fruit (mm/day), the lesion diameter (mm) four days post-inoculation was used in the case of *C. sinensis*, *C. paradisi*, *C. clementina* Hort. ex Tan. and *C. unshiu* (mak) Marc., and five days post-inoculation in the case of *C. limon*.

2.3. Extraction and measurement of flavonoids and scoparone in different Citrus fruits

Non-inoculated *Citrus* fruit of different species and varieties were used, along with samples of the same fruit 4 days after inoculation with *P. digitatum*. Eighteen fruits, divided into three lots of six fruit each, were used in each assay. The fruit peels of each lot were dried at 50 °C to constant weight. The dried fruits were ground, and shaken with dimethylsulphoxide (DMSO) (Castillo, Benavente-García, & Del Río, 1992) for 2 h in a proportion of 20 mg of dry weight/ml.

The resulting extracts were filtered through a 0.45-μm nylon membrane before analysis in a Jasco liquid Chromatography system equipped with a Jasco quaternary pump (model PU-2089 Plus), a Jasco photodiode array detector (model MD-2010 Plus), and a Jasco autosampler (model AS-2055 Plus). The stationary phase was a LiChroCARTR C18 (Agilent, USA) analytical column with an average particle size of 5 μm (250 × 4 mm i.d.) at 30 °C. The flavonoids and coumarin were separated using a binary gradient of water: methanol: acetonitrile: acetic acid (15:2:2:1) as solvent (A) and acetonitrile as solvent (B). The initial solvent composition consisted of 100% (A) for 40 min; then, the solvent composition changed in a linear gradient to 20% (A) during 30 min. Between 70 and 80 min, the composition was maintained and then the solvent composition changed in a linear gradient to 100% (A). Eluent flow was 1 ml/min. Changes in absorbance were recorded in the UV/Vis diode array detector at 280 nm for the flavanone glycosides and 340 nm for the flavones and coumarin. The quantities of these secondary compounds were determined from the area given by the integrator using the response factor of the corresponding standards. Identification of these compounds was carried out by HPLC–MS with a Agilent model VL ion trap mass spectrometer equipped with ESI interface acoplate with a HPLC Agilent 1100. A 5 μm (250 × 4 mm i.d.) C_{18} Kromasil 100 (Tecnokroma) column was used for the separation, which was performed by means of similar elution gradient to that described above for quantify flavonoids and coumarin. The column was maintained at 30 °C. ESI mass spectra were acquired in both positive and negative ion modes by scanning over the 50–1000 mass range. The ESI parameters were: source voltage 3.5 kV, dry temperature 350 °C, nebullizer 60 psi, and dry gas 9 l/min.

2.4. Chemicals

Sinensetin and tangeretin were purchased from Extrasynthèse S.A. (Genay, Francia). Heptamethoxyflavone and nobiletin were isolated by semipreparative HPLC and identified by MS (Del Río, Arcas, Benavente-García, et al., 1998). Hesperidin, hesperetin, naringin, naringenin, diosmin, diosmetin and scoparone were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.5. Statistical analysis

Values are given as means ± SD. Data were analysed by a General Lineal Model (GLM). Duncan's Multiple Range Test (DMRT) was used for the analysis of the variance in the data of Figs. 1–4. All statistical analyses were made using Statgraphics 5.0 software.

3. Results and discussion

3.1. Evaluation of the susceptibility of Citrus fruit to inoculation with P. digitatum

The results obtained following artificial inoculation of the *Citrus* fruits with *P. digitatum* showed that the kinetics of fungal development depended on the *Citrus* species (Figs. 1–4).

In the case of lemon (*C. limon*), the growth of *P. digitatum* showed a lag phase (up to 4 days after inoculation), followed by an exponential phase in all the varieties studied (Fig. 1). The greatest degree of susceptibility was shown by Laphytos, followed by