Citrus phenylpropanoids and defense against pathogens. Part I: Metabolic profiling in elicited fruits

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Abstract

Penicillium spp. are among the major postharvest pathogens of citrus fruit. Induction of natural resistance in fruits constitutes one of the alternatives to chemical fungicides. Here, we investigated the involvement of the phenylpropanoid pathway in the induction of resistance in Navelate oranges by examining changes in the metabolic profile of upon eliciting citrus fruits. By using both HPLC-PDA-FD and HPLC-PDA-QTOF-MS allowed the identification of several compounds that seem to be relevant for induced resistance. In elicited fruits, a greater diversity of phenolic compounds was observed in the flavedo (outer colored part of the peel) as compared to the albedo (inner white part). Moreover, only small changes were detected in the most abundant citrus flavonoids. The coumarin scoparone was among the compounds with the highest induction upon elicitation. Two other highly induced compounds were identified as citrusnin A and drupanin aldehyde. All three compounds are known to exert antimicrobial activity. Our results suggest that phenylpropanoids and their derivatives play an important role in the induction of resistance in citrus fruit.

Keywords

Citrusnin A; drupanin aldehyde; induced resistance; Penicillium digitatum; scoparone
1. Introduction

The understanding of defense mechanisms related to induced resistance against pathogens attack in fruits and other horticultural crops is important to reduce the use of chemical fungicides. However, most of the knowledge in this research area has been obtained through studies on model plants, including Arabidopsis and tomato (Hammerschmidt, 2009). These studies indicate that induced resistance involves accumulation of phytoalexins, reinforcement of cell walls, synthesis of pathogenesis-related proteins such as chitinases and β-1,3-glucanases (Hammerschmidt, 1999; van Loon, Rep, & Pieterse, 2006). Nevertheless, further research is necessary to understand key processes involved in induced resistance in citrus fruits.

The class of flavonoids comprise at least 6,000 molecules, divided into aurones, isoflavonoids, flavones, flavonols, flavanols, and anthocyanins (Harborne & Williams, 2000). Besides their function as pigments in flowers and fruits to attract pollinators and seed dispersers and their relevance in nutrition, flavonoids are involved in UV scavenging, fertility and disease resistance as phytoalexins and phytoanticipins, (Dixon & Paiva, 1995). Citrus fruits are a rich source of flavanones and many polymethoxylated flavones (PMFs), which are naturally synthesized by the fruit, and which may also been involved in the natural resistance of citrus fruit against pathogens acting as phytoanticipins. The most important PMFs in citrus are tangeretin, sinensetin and heptamethoxyflavone (Nogata, Sakamoto, Shiratsuchi, Ishii, Yano, & Ohta, 2006). Their content is high in the peel but low in the pulp and juice of the fruit (Goulas & Manganaris, 2012; Lafuente, Ballester, Calejero, Zacarías, & González-Candelas, 2011). These PMFs are believed to play a key role in the
defense responses of citrus fruit against pathogens (Ballester, Izquierdo, Lafuente, & González-Candelas, 2010; H. G. Kim, Kim, Lee, Park, Jeong, Kim et al., 2011; Ortuño, Báidez, Gómez, Arcas, Porras, García-Lidón et al., 2006; Ortuño, Díaz, Alvarez, Porras, García-Lidón, & Del Río, 2011).

*Penicillium digitatum* (Pers.:Fr.) Sacc., the causal agent of the citrus green mold rot, is the most destructive postharvest pathogen of citrus fruit in Mediterranean regions, being responsible for important economic losses during postharvest handling. The application of fungicides constitutes the most common method used to control postharvest diseases in citrus fruits. However, due to the development of resistant strains and the growing public concern on the negative effects of fungicides on human health and the environment, there is a trend to develop alternative methods to control postharvest diseases. In citrus fruit, induction of natural resistance constitutes one of these alternatives. Treatments triggering induced resistance in citrus fruit against fungal infections include the application of physical treatments such as heat treatment and ultraviolet light (Arcas, Botía, Ortuño, & Del Río, 2000; Ben Yehoshua, Rodov, Kim, & Carmeli, 1992; Droby, Chalutz, Horev, Cohen, Gaba, Wilson et al., 1993; J. J. Kim, Ben Yehoshua, Shapiro, Henis, & Carmeli, 1991; Rodov, Ben Yehoshua, Kim, Shapiro, & Ittah, 1992), chemicals such as β-amino butyric acid and sodium carbonates (Porat, McCollum, Vinokur, & Droby, 2002; Porat, Vinokur, Holland, McCollum, & Droby, 2001; Venditti, Molinu, Dore, Agabbio, & D’Hallewin, 2005), and microbial antagonists such as *Candida famata* and *Candida oleophila* (Arras, 1996; Fajardo, McCollum, McDonald, & Mayer, 1998). Nevertheless, their efficacy is variable and depends on the maturity of the fruit. In the context of the present work, it is important to point out that the outer colored (flavedo)
and the inner white (albedo) parts of the peel show different susceptibility to *P. digitatum* infection (Ballester, Lafuente, & González-Candelas, 2006; Kavanagh & Wood, 1967). Moreover, both tissues show different ability to activate phenylalanine ammonia-lyase (PAL), a key enzyme at the entry point in the phenylpropanoids pathway, in response to pathogen attack (Ballester, Lafuente, & González-Candelas, 2006), and to other abiotic stimulus in citrus fruits (Cajuste & Lafuente, 2007).

Although several studies deal with global changes in gene expression associated with induced resistance (Ballester, Lafuente, Forment, Gadea, De Vos, Bovy et al., 2011; Hershkovitz, Ben-Dayan, Raphael, Pasmanik-Chor, Liu, Belausov et al., 2011), and with defense response in citrus fruit (Gonzalez-Candelas, Alamar, Sanchez-Torres, Zacarias, & Marcos, 2010), so far only a limited number of metabolites involved in induced resistance have been identified. An increased level of scoparone has been observed in elicited citrus fruit that showed a decreased *P. digitatum* infection (Ballester, Izquierdo, Lafuente, & González-Candelas, 2010; J. J. Kim, Ben Yehoshua, Shapiro, Henis, & Carmeli, 1991). Induction of scoparone and other coumarins such as scopoletin and umbelliferone has also been observed in UV-irradiated fruit (D'Hallewin, Schirra, Manueddu, Piga, & Ben Yehoshua, 1999), or after elicitation by antagonistic yeasts (Arras, 1996; Droby, Vinokur, Weiss, Cohen, Daus, Goldschmidt et al., 2002). On the other hand, *in vitro* studies indicate that umbelliferone has antimicrobial properties against different fungi (Afek, Orenstein, Carmeli, Rodov, & Joseph, 1999), and that PMFs and the flavanone naringenin can reduce the growth of *Phytophthora citrophthora*, *P. digitatum*, and *Colletotrichum gloeosporioides* (Almada-Ruiz, Martínez-Téllez, Hernández-
However, little information exists concerning the involvement of these compounds in the induction of resistance in citrus fruit. It is also important to note that changes in the levels of phenylpropanoids and derivatives related to defense responses and induced resistance have been mainly addressed in the whole peel of citrus fruit. To the best of our knowledge, only a limited number of studies have been reported in the flavedo and/or albedo separately in spite of their different susceptibility to infection. The accumulation of umbelliferone increased in the albedo of grapefruits four days following the inoculation with *P. digitatum* (Afek, Orenstein, Carmeli, Rodov, & Joseph, 1999), and an increase in the levels of scoparone has been observed in the flavedo and albedo of elicited oranges (Ballester, Izquierdo, Lafuente, & González-Candelas, 2010). However, metabolic profiling in both flavedo and albedo of elicited oranges has not been conducted until now. Therefore, in this study, we used a metabolomic approach to determine whether the phenylpropanoids and their derivatives are induced in both tissues of elicited citrus fruit, and to investigate whether differences in the concentration of these metabolites in the flavedo and albedo could be related to their different susceptibility to *P. digitatum* infection.

2. Materials and methods

2.1. Fruit samples and fungal material

Navelate orange fruits (*Citrus sinensis* L. Osbeck) were selected from a commercial orchard in Llíria (Valencia, Spain) and used in the experiments before any commercial postharvest treatment was applied. Fruits were taken in
three independent samplings and used for the induction of resistance treatment. They were immediately surface-sterilized with 5% commercial bleach solution for 5 min, extensively washed with tap water and allowed to dry at room temperature until next day.

Petri dishes containing potato dextrose agar were inoculated with *Penicillium digitatum* (Pers.:Fr.) Sacc. isolate PHI-26 and incubated at 24 ºC for 7 days (López-García, González-Candelas, Pérez-Payá, & Marcos, 2000). Conidia were rubbed from the agar surface by scraping them with a sterile spatula and transferred to sterile water. The conidial suspension was then filtered and the concentration determined with a haemocytometer and adjusted to the desired concentration.

2.2. Induction of resistance treatment

The treatment for eliciting resistance was described previously by Ballester et al. (2011). A schematic diagram indicating tissue sampling and pathogen inoculation for the elicitor treatment is shown in Fig. 1. Briefly, three biological replicates of Navelate fruits were wounded by making punctures (3 mm in depth) with a sterilized nail and inoculated with 10 µL of a *P. digitatum* conidial suspension adjusted to $10^5$ conidia mL$^{-1}$. Treated fruits were placed into plastic boxes and maintained at 90-95% relative humidity (RH) and 20 ºC for 1 day to allow pathogen development. Then, fruits were heat-treated at 37 ºC for 3 days under water-saturated conditions (curing) in order to stop the progress of the pathogen. Elicited samples were taken at 4, 5 and 7 days after the beginning of the experiment (0, 1 and 3 days after the elicitor treatment; samples IC4, IC5 and IC7, respectively). A control sample was obtained the first day of the
experiment (Sample NT). Peel tissue discs of 13 mm around the inoculation point were sampled using a cork borer. Flavedo and albedo tissues were separated with a scalpel. Tissue discs obtained from 15 oranges with 8 discs per fruit were immediately frozen in liquid nitrogen, mixed and grounded to a fine powder with a coffee mill and stored at -80 °C until further analysis.

2.3. *Penicillium digitatum* infection

To determine the effectiveness of the elicitor treatment reducing pathogen infection and the importance of the elapsed time between the treatment and the ulterior infection, disease susceptibility was analyzed at the beginning of the experiment in non-treated Navelate fruits, and at 4, 5 and 7 days in the elicited fruits. Each elicited fruit was punched at a distance of 0.5 cm from the previous wound or in the equatorial axis in the control fruits that had not been previously inoculated. Then, 10 µL of a $10^4$ conidia mL$^{-1}$ suspension of *P. digitatum* spores were applied to each wound. After inoculation, fruits were kept at 20 °C and 90-95% RH. The severity (maceration area, in cm$^2$) was determined for up to 6 days of incubation at 20 °C. The experimental design consisted of 3 replicates of 5 fruits, with 4 wounds per fruit, for each treatment. To test the effect of the elicitor treatment, a one-way analysis of variance (ANOVA) was performed. Means were separated using the LSD test at $p<0.05$. The analysis was performed with Statgraphics Plus 4.0 Software (Manugistics, Inc.).
2.4. Determination of phenolic compounds by High-Performance Liquid Chromatography

Phenolic compounds from flavedo and albedo of citrus fruits were analyzed as previously described (Ballester, Izquierdo, Lafuente, & González-Candelas, 2010). Briefly, freeze-ground material of flavedo and albedo was extracted twice with 80% methanol. Chromatography was carried out with a Waters HPLC system equipped with a 600 quaternary pump, a 996 photodiode array detector (PDA) and a 474 fluorescence detector (FD), and data were analyzed with the Empower software (Waters). Phenolic compounds were separated at 35 °C using a Luna C18 reverse column (250 x 4.6 mm, 5 µm; Phenomenex) coupled to a µBondapak C18 guard column (10 µm) and using a binary gradient elution of acetonitrile and water (pH 2.5). The flow rate was 0.8 mL min⁻¹ and the injection volume, 20 µL. Phenolics were detected by fluorescence at excitation and emission wavelengths of 313 nm and of 405 nm, respectively, and by setting the photodiode array detector to scan from 200 to 400 nm. For each analysis, a Maxplot chromatogram, which plots each phenolic compound peak at its corresponding maximum absorbance wavelength, was obtained. Peaks were integrated and phenolic content was calculated using calibration curves. Detection using HPLC-PDA coupled to a quadrupole time of flight-mass spectrometry (QTOF-MS) was based on the method described in Moco, Bino, Vorst, Verhoeven, de Groot, van Beek et al. (2006), with small modifications. Briefly, phenolic compounds were extracted from the previously homogenized flavedo and albedo frozen materials with 80% methanol. Samples were then centrifuged at 3,000 x g for 10 min and the supernatants were filtered. For LC-PDA-QTOF-MS analysis, 5 µl of the methanolic extract were injected and
separated using a Waters Alliance 2795 HT system equipped with a Luna C18 reversed phase column (150 x 2.1 mm, 3 μm; Phenomenex) at 40 °C using a binary gradient of water and acetonitrile. Eluted compounds were detected online first at 210-600 nm using a 2996 PDA detector (Waters Corporation), and then by a QTOF Ultima V4.00.00 accurate mass spectrometer (Waters Corporation). The following settings were applied during the LC-MS runs: desolvation temperature of 250 °C with a nitrogen gas flow of 600 L h⁻¹, cone gas flow of 50 L h⁻¹, capillary spray at 2.75 kV, source temperature of 120 °C, cone voltage at 35 eV with 50 L h⁻¹ nitrogen gas flow, collision energy at 5 eV (ESI positive mode) or 10 eV (ESI negative mode). Ions in the m/z range 100-1,500 were detected using a scan time of 0.9 s and an interscan delay of 0.1 s. Before each series of analysis, the mass spectrometer was calibrated using 0.05% phosphoric acid in 50% acetonitrile, and leucine enkaphalin was used as the lock mass for on-line accurate mass correction. Masslynx software version 4.1 (Waters) was used to control all instruments and calculate accurate masses.

2.5. Quantification of individual phenolic compounds by HPLC-PDA-FD

Individual phenolic compounds were quantified using calibration curves of the respective reference compounds. For this purpose, stock solutions (1000 μg mL⁻¹) were diluted to concentrations of 0.5-100 μg mL⁻¹ (chlorogenic acid, isosinensetin, tetramethyl-O-scutellarein, heptamethoxyflavone, scoparone), 1-400 μg mL⁻¹ (hesperidin), 0.5-50 μg mL⁻¹ (narirutin, didymin, caffeic acid, isorhoifolin, diosmin, sinensetin, tangeretin), 0.1-5 μg mL⁻¹ (hexamethyl-O-gossypetin, nobiletin), 5-25 μg mL⁻¹ (eriocitrin), and the solutions were analyzed as described in Section 2.4. Metabolite concentrations were expressed as μg g⁻¹.
1 fresh weight. When reference compounds were not available (hexamethyl-O-
quercetagetin, citrusnin A, drupanin aldehyde and compound 19), the levels
were expressed as the area (mAU s) of the peak in the chromatogram.

2.6. Determination of fluorescent compounds in the peel of citrus fruits
To determine the presence of fluorescence compounds in the peel of oranges,
a stereoscopic zoom microscope SMZ800 with Epi-fluorescence attachment
(Nikon) was used. A transversal cut centered in the inoculation point was made
in elicited oranges and the tissue was observed using the microscope coupled
with an EX 480 / 40 BA 510 filter.

2.7. Standards
Eriocitrin (eriodictyol-7-O-rutinoside), narirutin (naringenin-7-O-rutinoside),
isorhoifolin (apigenin-7-O-rutinoside), diosmin (diosmetin-7-O-rutinoside) and
didymin (isosakuranetin-7-O-rutinoside), also known as neoponcirin, were
purchased from Extrasynthèse (Genay, France); chlorogenic acid and
scoparone (6,7-dimethoxycoumarin) from Aldrich (Spain); and caffeic acid and
hesperidin (hesperetin-7-O-rutinoside) from Fluka (Spain). The PMFs
isosinensetin (3',4',5,7,8-pentamethoxyflavone), hexamethyl-O-gossypetin
(3',4',3,5,7,8-hexamethoxyflavone), sinensetin (3',4',5,6,7-
pentamethoxyflavone), hexamethyl-O-quercetagetin (3',4',3,5,6,7-
hexamethoxyflavone), nobiletin (3',4',5,6,7,8-hexamethoxyflavone), tetramethyl-
O-scutellarein (4',5,6,7-tetramethoxyflavone), heptamethoxyflavone (3',4',3,
5,6,7,8-heptamethoxyflavone), and tangeretin (4',5,6,7,8-pentamethoxyflavone)
were kindly supplied by Dr. J.M. Sendra (IATA-CSIC, Valencia, Spain).
2.7. Statistics

The values are the means of three replicate samples ± standard deviation (SD). Data were evaluated using Statgraphics. Plus 4.0 Software (Manugistics, Inc.) and LSD test was performed to identify significant differences between samples at $p \leq 0.05$.

3. Results and discussion

The elicitor treatment increased the resistance of Navelate oranges to a subsequent pathogen infection. Our results showed that the lowest severity of the infection was observed when the pathogen was inoculated 7 days after the beginning of the experiment (severity of $3.7 \pm 0.7 \text{ cm}^2$). Elicitor treatment also showed a statistically significant, but lower, reduction in severity when the pathogen was inoculated 4 or 5 days after the beginning of the experiment ($7.3 \pm 0.5$ and $6.7 \pm 1.1 \text{ cm}^2$, respectively), compared to non-treated oranges ($29.7 \pm 1.8 \text{ cm}^2$). The involvement of the enzyme PAL (Ballester, Izquierdo, Lafuente, & González-Candelas, 2010), and the relevance of phenylpropanoids metabolism in the induction of resistance (Ballester et al., 2011; Hershkovitz et al., 2011), and in the defense of citrus fruit against pathogens (Gonzalez-Candelas, Alamar, Sanchez-Torres, Zacarias, & Marcos, 2010) has been pointed out by using biochemical and transcriptomic approaches. However, in spite of the broad number of phenylpropanoid genes associated with induced resistance, little is known about the role that metabolites from this pathway may play in this process. Therefore, we have examined the metabolic profile of phenylpropanoids and derivatives involved in induced resistance in the flavedo
and albedo of citrus fruit. This information would be interesting in order to increase the knowledge of this pathway in citrus and to contribute to the development of new and safer alternatives for controlling postharvest pathogens of citrus fruit.

3.1. Differences in the phenylpropanoid metabolic profiles between flavedo and albedo peel tissues in non-treated Navelate oranges

The flavedo and the albedo tissues, which show different susceptibility to infection caused by *P. digitatum* (Ballester, Lafuente, & González-Candelas, 2006; Kavanagh & Wood, 1967), also showed different phenylpropanoid metabolic profiles. The flavanone hesperidin was the most abundant flavonoid in the flavedo of non-treated Navelate oranges (FNT), followed by phenylpropanoid chlorogenic acid and the PMFs tetramethyl-O-scutellarein, heptamethoxyflavone, sinensetin and tangeretin (Table 1). Other flavanones, such as didymin, narirutin and eriocitrin, and the phenylpropanoid caffeic acid were also abundant in this external peel tissue. However, the coumarin scoparone, which has been related to the defense of citrus fruit against *P. digitatum* infection (Ballester, Izquierdo, Lafuente, & González-Candelas, 2010; J. J. Kim, Ben Yehoshua, Shapiro, Henis, & Carmeli, 1991), was not detected in the flavedo of non-treated fruits (FNT) neither in the albedo of non-treated oranges (ANT). The internal tissue contained similar levels of hesperidin but much higher levels of didymin and narirutin, and remarkable lower amounts of chlorogenic acid, PMFs and eriocitrin as compared to the flavedo (Table 2). This is in concordance with previous findings showing that composition and content of the phenolic compounds differ among tissues and citrus varieties.
Moreover, this data reveal the higher abundance of PMFs and chlorogenic acid, which may reduce the growth of fruit pathogenic fungi (Ortuño et al., 2006; Ruelas, Tiznado-Hernández, Sánchez-Estrada, Robles-Burgueño, & Troncoso-Rojas, 2006), in the flavedo of Navelate oranges. This external tissue constitutes the first natural barrier in the defense against pathogen attack and is less susceptible to infection than the albedo (Ballester, Lafuente, & González-Candelas, 2006). In the context of the present work, it is also interesting to note that the levels of phenylpropanoids and derivatives in Navelate oranges were lower than those observed in the same tissues of Navelina oranges (Ballester, Lafuente, & González-Candelas, Submitted).

3.2. Effect of the elicitor treatment on the phenylpropanoid metabolic profiles in the flavedo and albedo peel tissues

Most of the phenolic compounds identified did not show major changes due in response to the elicitor treatment. However, some of them showed marked differences, which in some instances were tissue-specific. The amounts of the phenylpropanoids chlorogenic acid and caffeic acid did not change significantly in response to the elicitor treatment in the flavedo (Table 1), although they may have antifungal activity (Ruelas, Tiznado-Hernández, Sánchez-Estrada, Robles-Burgueño, & Troncoso-Rojas, 2006). Results also showed that only a slight but significant increase in hesperidin occurred in the elicited flavedo by days 4 and 5 after the beginning of the experiment (FIC4 and FIC5, respectively), and that the slight increase in didymin was only statistically significant by day 7 (FIC7).
As shown in Table 2, the concentration of these compounds barely changed in the albedo in any examined condition. Although the concentration of chlorogenic acid increased by days 4 and 5, no significant difference was found between the albedo of non-treated oranges (ANT) and the albedo of elicited fruits by day 7 (AIC7), which showed the lowest infection severity. Moreover, caffeic acid was detected neither in the non-treated nor in the elicited albedo samples.

Our results also showed that the levels of the PMFs hexamethyl-O-quercetagetin, nobiletin, heptamethoxyflavone and tangeretin increased in both tissues in elicited fruits. In the flavedo, such increases were statistically significant by day 5 (FIC5) for all of them, and also by day 7 (FIC7) for hexamethyl-O-quercetagetin and tangeretin (Table 1). In the albedo, the levels of all detected PMFs, except tetramethyl-O-scutellarein, increased significantly by day 5 (AIC5) and for 3 of them the high level was maintained by day 7 (AIC7) (Table 2). Although the lowest susceptibility to *P. digitatum* infection occurred by day 7, infection was also reduced by day 5. Therefore, the participation of PMFs in the elicitation of disease resistance cannot be ruled out. However, and in spite of their proven efficacy reducing *P. digitatum* growth (Ortuño et al., 2006), other compounds should participate in this process. The different pattern of accumulation of these compounds in both tissues might be associated with the fact that PAL activity was lower in the albedo (Ballester, Lafuente, & González-Candelas, 2006). Genes or proteins involved in the synthesis of flavonoids in citrus fruits, including PMFs, have not been identified yet and, therefore, results from the present work, together with previously obtained results (Ballester et al., 2011) encourage new investigations in such direction.
The rise in the levels of flavonoids in response to the elicitor treatment could be related with a higher resistance of the elicited fruits against an ulterior infection. This is concordance with previous results showing that citrus fruits with higher levels of the flavanones hesperidin and naringenin, the flavanone diosmin and total polymethoxyflavone levels showed lower susceptibility to *P. digitatum* infection (Ortuño, Díaz, Alvarez, Porras, García-Lidón, & Del Río, 2011), and that some of these flavonoids show *in vitro* antifungal activity against for instance *Penicillium* sp., *Phytophthora* sp. and *Geotrichum* sp. (Del Río, Arcas, Benavente-García, & Ortuño, 1998; Ortuño et al., 2006). In addition with flavonoid content changes in response to an elicitor treatment, a transitory increase in the flavonoid concentration has been observed in response to *P. digitatum* infection (Ballester, Lafuente, & González-Candelas, Submitted; H. G. Kim et al., 2011). However, the induction of flavonoid content was, in general, higher in response to the elicitor treatment than in response to pathogen infection.

As indicated above, the increases observed in some phenolics were transient, which agrees with the fact that the induction of genes involved in phenylpropanoid biosynthesis may be transient in elicited citrus fruit (Ballester et al., 2011). It is also noticing that results of the present paper showing the transient increase of such phenolics are in concordance with other reports showing that increases in phenolics, and also in the expression of genes involved in phenylpropanoid biosynthesis, occurring in citrus fruit exposed to abiotic stress or to treatments that increase the fruit tolerance to such stress may be transient (Lafuente, Ballester, Calejero, Zacarías, & González-
3.3. Identification and quantification of new phenolic compounds in elicited fruits

HPLC-PDA results show that the highest increase observed in elicited fruits for any flavonoid is lower than 2-fold, whereas we have found 4 fluorescent compounds with much larger increases in response to the elicitor treatment (Fig. 2A). Therefore, a qualitative and quantitative analysis of these compounds was further performed by using a HPLC-PDA-QTOF-MS system. As shown in Fig. 2A, the levels of 4 fluorescence compounds (nos. 8, 18, 19 and 20) peaked at 5 or 7 days after the beginning of the experiment, being the levels of them higher in the flavedo (Table 1) than in the albedo (Table 2). By comparing the HPLC retention times, UV absorbance spectra (Fig. 2B) and accurate mass signals (Fig. 2C) with those of authentic standards, fluorescent compound 8 was identified as scoparone (6, 7-dimethoxycoumarin; Fig. 2D). Scoparone was not detected in the flavedo or albedo of non-treated fruits, while substantial amounts of this compound were detected in the flavedo (90.5 and 54.0 µg g⁻¹ fresh weight at 5 and 7 days, respectively) and lower amounts in the internal tissue (12.2 and 24.7 µg g⁻¹ fresh weight at 5 and 7 days, respectively) of elicited fruits. It is noteworthy that these levels were substantially higher than those detected in response to *P. digitatum* infection, with maximum levels of 14.8 and 5.3 µg g⁻¹ fresh weight in the flavedo and albedo, respectively, 72 h post-inoculation (Ballester, Lafuente, & González-Candelas, Submitted) in spite of the lack of infection in the elicited samples. Likewise, in the context of the present work it is important to note that even the lower scoparone level detected
in the albedo of elicited fruits was close to the median effective dose for the
inhibition of germ tube elongation of *P. digitatum* (J. J. Kim, Ben Yehoshua,
Shapiro, Henis, & Carmeli, 1991). Therefore, this coumarin may play a role in
the higher resistance observed in elicited fruits at 7 days after the beginning of
the experiment. This is in concordance with previous data indicating that
scoparone is associated with the defense of citrus fruit against different stresses
such as UV light and pathogen infection (Afek, Orenstein, Carmeli, Rodov, &
Joseph, 1999; Ballester, Lafuente, & González-Candela, Submitted;
D'Hallewin, Schirra, Manueddu, Piga, & Ben Yehoshua, 1999; Kuniga,
Tsumura, Matsuo, & Matsumoto, 2006). Other authors have associated the
coumarins umbelliferone (7-hydroxycoumarin) and scopoletin (6-methoxy, 7-
hidroxycoumarin), which are probable precursors of scoparone, with a higher
resistance of citrus fruits to *P. digitatum* infection (Afek, Orenstein, Carmeli,
Rodov, & Joseph, 1999; Droby et al., 2002; Nafussi, Ben Yehoshua, Rodov,
Peretz, Ozer, & D'Hallewin, 2001). However, none of these 2 compounds were
detected in either non treated or elicited Navelate oranges.

We have recently shown that the combination of pathogen inoculation followed
by a curing treatment reduced the incidence of a subsequent *P. digitatum*
infection in oranges and triggered relevant changes in the expression of a broad
number of phenylpropanoid genes, being noteworthy the increase in expression
levels of several O-methyltransferases (OMTs) encoding genes (Ballester et al.,
2011). Previous reports have shown that OMTs and various cytochrome P450
enzymes are involved in the formation of phenolic compounds, including
coumarins and PMFs (Bourgaud, Hehn, Larbat, Doerper, Gontier, Kellner et al.,
2006; Ibrahim, Bruneau, & Bantignies, 1998). This, together with the fact that
scoparone and PMFs are methylated compounds, raises the possibility that induced OMTs play a role in their synthesis, although a conclusive relationship between any of them and scoparone or PMFs still remains to be elucidated. Three other yet unknown compounds increased substantially in response to the elicitor treatment (Fig. 2A, compounds 18, 19 and 20). Low levels of these compounds were detected in the flavedo of non-treated fruits, while they were undetectable in the internal tissue of non-treated fruits. In both tissues the relative levels of compounds 18 and 20 increased substantially in response to the elicitor treatment, peaking at day 7, whereas compound 19 reached the highest level at day 5. Thus, in the flavedo, 100-, 20- and 200-fold increases were found by 7 days for compounds 18, 19 and 20, respectively. These proportions could not be estimated in the albedo since these compounds were not detected in the non-treated fruits, but final levels were at least 4-fold lower in this tissue than in the flavedo. To identify these 3 compounds, samples were subjected to accurate mass spectrometry (LC-PDA-QTOF-MS) using both negative and positive electrospray ionization (ESI) modes (Fig. 2B, 2C). Compound 19, with \( \lambda_{\text{max}} \) of 215.57 and 263.57 nm, could not be identified because its accurate mass is still unknown due to its low ionization efficiency in both positive and negative ESI modes. Compound 18 had a UV spectrum with \( \lambda_{\text{max}} \) of 267.6 nm and an observed accurate mass of \( m/z \) 231.0996 [M-H], corresponding to a molecular formula of \( \text{C}_{14}\text{H}_{16}\text{O}_{3} \). Using different databases, such as KNApSAcK (Sinbo, Nakamura, Altaf-Ul-Amin, Asahi, Kurokawa, Arita et al., 2006) and Dictionary of Natural Products (CHEMnetBASE), this compound was putatively identified as citrusnin A (Fig. 2D). Citrusnin A has been isolated from leaves of Citrus natsudaidai.
inoculated with a *Pseudomonas* sp. antagonistic to *Xanthomonas campestris* pv. *citri* (Watanabe, Miyiyakado, Ohno, Ota, & Nonaka, 1985). The physicochemical properties of this compound, such as MS $m/z$ and UV $\lambda_{\text{max}}$ nm, matched perfectly with the ones observed in elicited oranges. The antibacterial effect of this compound was also tested *in vitro*, being effective against different pathogenic bacteria (Watanabe, Miyiyakado, Ohno, Ota, & Nonaka, 1985). However, this is the first report linking citrusnin A with the resistance of citrus fruit to infection caused by *P. digitatum*. Furthermore, as far as we know, this compound has not been yet related to the resistance of citrus or other fruits to pathogens causing postharvest losses.

Compound 20 showed a similar $\lambda_{\text{max}}$ at 267.6 nm, but an accurate mass of $m/z$ 215.1076 [M-H]$^-$ corresponding to a molecular formula of C$_{14}$H$_{16}$O$_2$. Based on comparison with different metabolite databases, this compound was putatively identified as drupanin aldehyde (i.e. 3-[4-hydroxy,3-(3-methyl-2-butenyl)-phenyl]-2-(E)-propenal or 4-hydroxy-3-prenylcinnamaldehyde) (Fig. 2D). This compound was previously isolated from the peel of wounded grapefruits (*Citrus paradisi*) and oranges (*C. sinensis*) (Stange, Midland, Eckert, & Sims, 1993). It is also known that drupanin itself, isolated from *Baccharis* sp., has antifungal and antibacterial activity (Bisogno, Mascoti, Sanchez, Garibotto, Giannini, Kurina-Sanz et al., 2007; Feresin, Tapia, Gimenez, Ravelo, Zacchino, Sortino et al., 2003). However, its involvement in the resistance of citrus fruits to pathogenic fungi has not been reported until now. Moreover, it has to be noted that although citrusnin A and drupanin aldehyde levels increased in response to the elicitor treatment, none of these compounds were detected in response to *P. digitatum* infection (Ballester, Lafuente, & González-Candelas, Submitted). In
light of their structures both citrusnin A and drupanin could be biochemically
derived from precursors in the first part of the phenylpropanoid pathway, but the
genes and enzymes involved in their synthesis are unknown yet. The study of
the possible antifungal activity of these compounds against \textit{P. digitatum} has not
been undertaken because they are not commercially available and their
concentration in the peel of citrus fruits is very low. However, the results
presented in this work encourage further research in this direction.

Since the HPLC-FD analysis of phenolic metabolites revealed the induction of
fluorescent compounds in the peel of elicited fruits, we checked the presence of
fluorescence in elicited oranges using a stereoscopic zoom microscope
SMZ800 with Epi-fluorescence attachment (Nikon) (Fig. 3). The amount of
fluorescence in the transversal cut of peel oranges was higher in elicited fruits
than in non-treated fruits. The fluorescence was concentric around the
inoculation point, which reinforces the idea that the elicitor treatment induced
only local disease resistance and that the effect is limited to only a small area
around the origin of infection (1-4 mm distance from the inoculation site).

Metabolic profiling results of this study strongly suggest an implication of
phenylpropanoids, flavonoids and their derivatives in the induction of resistance
in citrus fruit, being especially relevant the induction of scoparone and three
other fluorescent phenolic compounds that have not been previously related to
the resistance of citrus fruit against disease caused by \textit{P. digitatum}. Two of
them, citrusnin A and drupanin aldehyde, were putatively identified and showed
very relevant increases in elicited fruits. Therefore, their implication in citrus fruit
responses deserves further investigation. Finally, our results indicate that the
highest inductions in phenylpropanoids were found in the albedo, whereas the
highest metabolite concentrations were detected in the external tissue. These results reinforce the idea that the internal tissue is more susceptible to *P. digitatum* infection and it is the one that should increase to a greater extent the defensive barriers in order to avoid the progression of the fungus.

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**References**


Ballester, A. R., Lafuente, M. T., & González-Candelas, L. (Submitted). Citrus phenylpropanoids and defense against pathogens. Part II: Gene expression and metabolite accumulation in the response of fruits to Penicillium digitatum infection. Accompanying manuscript submitted to Food Chemistry.


fruits against *Phytophthora citrophthora*. *Journal of Agricultural and Food Chemistry*, 52(7), 1913-1917.


digitatum by liquid chromatography coupled with tandem mass spectrometry. *Food Chemistry, 128*(1), 49-54.


**Figure Captions**

**Fig. 1.** Flow chart of the experimental design. Solid vertical arrows indicate the temperature and duration of the incubation period. The induction of resistance treatment consisted of fruit inoculated with *P. digitatum* (indicated in the chart as *Pdig*) and then incubated for 1 day at 20 ºC before being transferred at 37 ºC for 3 day to stop pathogen progress. At the end of this heat treatment, fruit were maintained at 20 ºC. Tissue samples were taken from 15 fruits at 4, 5 and 7 d after the beginning of the experiment (IC4, IC5 and IC7, respectively), and other 15 oranges, with 4 wounds per fruit, were inoculated with *P. digitatum* to assess the effectiveness of the treatment. Infection was allowed to progress for 6 d, when disease severity was determined. Control non-treated fruits (NT) were sampled at the beginning of the experiment.

**Fig. 2.** Metabolic profiling of elicited citrus-fruits. (A) Chromatogram of flavedo (F) from non-treated (NT) an infected-cured oranges at 4 (IC4), 5 (IC5) and 7 (IC7) days after the beginning of the experiment obtained by HPLC-FD. (B) UV spectra of induced compounds. (C) Mass spectra of compounds 18, 8 and 20. (D) Chemical structure of compounds (18) citrusnin A, (8) scoparone, and (20) drupanin aldehyde.

**Fig. 3.** Transversal cuts of the peel of citrus fruits using stereoscopy microscope equipped with a fluorescence system. Photographs of non-treated (A, C) and *P. digitatum* infected and cured (B, D) fruits using white light (A, B) and fluorescence (C, D). Transversal cuts were made 7 days after the beginning of the experiment.
<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Family</th>
<th>FNT Conc. ± SD</th>
<th>FIC4 Conc. ± SD</th>
<th>FIC5 Conc. ± SD</th>
<th>FIC7 Conc. ± SD</th>
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<td>Flavanone</td>
<td>33.1 ± 2.6 a</td>
<td>nd</td>
<td>nd</td>
<td>14.9 ± 17.5 a</td>
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<tr>
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<td>Hesperidin</td>
<td>Flavanone</td>
<td>1840.9 ± 74.8 b</td>
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<td>1979.0 ± 83.2 ab</td>
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<td>67.1 ± 4.0 ab</td>
<td>67.3 ± 8.8 ab</td>
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<td>Cinnamic acid</td>
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<td>149.8 ± 3.5 a</td>
<td>152.9 ± 15.9 a</td>
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<tr>
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<td>Cinnamic acid</td>
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<td>Diosmin</td>
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<td>4.0 ± 0.4 a</td>
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<td>1.7 ± 0.2 a</td>
<td>1.3 ± 0.6 ab</td>
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<td>100.5 ± 6.4 b</td>
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<td>PMF</td>
<td>140.8 ± 21.6 a</td>
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<td>142.8 ± 3.3 a</td>
<td>126.2 ± 2.9 ab</td>
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<td>54.0 ± 2.7 b</td>
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<td>189.2 ± 68.2 b</td>
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<td>930.0 ± 78.6 b</td>
<td>1538.5 ± 33.3 b</td>
<td>3310.5 ± 653.0 a</td>
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</table>

* values represent the area (mAu s) of the peak in the chromatogram
(FD) indicates that those values were obtained with the fluorescent detector.
nd. non-detected compound
Table 2. Phenylpropanoid and flavonoid concentration (µg g\(^{-1}\) fresh weight) in the albedo of non-treated (ANT) and elicited Navelate oranges 4, 5 and 7 days after the beginning of the experiment (AIC4, AIC5 and AIC7, respectively) detected by HPLC-PDA-FD. Results represent the mean of at least two biological replicates ± standard deviation (SD). Different letters among treatments indicate statistically significant differences according to the LSD test \((p<0.05)\). Compound order based on families and retention time (Ballester, Lafuente, & González-Candelas, accompanying paper submitted to Food Chemistry).

<table>
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<th>No.</th>
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<th>SD</th>
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<th>Conc.</th>
<th>SD</th>
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<th>SD</th>
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<td>Flavanone</td>
<td>15.3 ± 6.1 a</td>
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<td>19.0 ± 0.1 a</td>
<td>11.8 ± 0.3 a</td>
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<tr>
<td>4</td>
<td>Narirutin</td>
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<td>434.3 ± 35.3 a</td>
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<td>373.2 ± 15.1 ab</td>
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<td>2,027.1 ± 117.3 a</td>
<td>1,518.3 ± 107.8 b</td>
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<td>Scoparone (FD)</td>
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<td>18</td>
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<td>520.9 ± 34.3 a</td>
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<tr>
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<td>Compound 19 (FD)*</td>
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* values represent the area (mAU s) of the peak in the chromatogram

(FD) indicates that those values are obtained from the fluorescent detector.

nd. non-detected compound