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-

Alamos, Vallejo, Primo-Yúfera, & Vargas-Arispuro, 2003; Arcas, Botía, Ortuño, & Del Río, 2000; Del Río, Gómez, Báidez, Arcas, Botía, & Ortuño, 2004). 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 Lliria (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

167 experiment (Sample NT). Peel tissue discs of 13 mm around the inoculation
168 point were sampled using a cork borer. Flavedo and albedo tissues were
169 separated with a scalpel. Tissue discs obtained from 15 oranges with 8 discs
170 per fruit were immediately frozen in liquid nitrogen, mixed and grounded to a
171 fine powder with a coffee mill and stored at -80 ºC until further analysis.
172

173 *2.3. Penicillium digitatum infection*

174 To determine the effectiveness of the elicitor treatment reducing pathogen
175 infection and the importance of the elapsed time between the treatment and the
176 ulterior infection, disease susceptibility was analyzed at the beginning of the
177 experiment in non-treated Navelate fruits, and at 4, 5 and 7 days in the elicited
178 fruits. Each elicited fruit was punched at a distance of 0.5 cm from the previous
179 wound or in the equatorial axis in the control fruits that had not been previously
180 inoculated. Then, 10 µL of a 10^4 conidia mL^{-1} suspension of *P. digitatum* spores
181 were applied to each wound. After inoculation, fruits were kept at 20 ºC and 90-
182 95% RH. The severity (maceration area, in cm^2) was determined for up to 6
183 days of incubation at 20 ºC. The experimental design consisted of 3 replicates
184 of 5 fruits, with 4 wounds per fruit, for each treatment. To test the effect of the
185 elicitor treatment, a one-way analysis of variance (ANOVA) was performed.
186 Means were separated using the LSD test at $p<0.05$. The analysis was
187 performed with Statgraphics Plus 4.0 Software (Manugistics, Inc.).
188

189 *2.4. Determination of phenolic compounds by High-Performance Liquid*
190 *Chromatography*

191 Phenolic compounds from flavedo and albedo of citrus fruits were analyzed as
192 previously described (Ballester, Izquierdo, Lafuente, & González-Candelas,
193 2010). Briefly, freeze-ground material of flavedo and albedo was extracted twice
194 with 80% methanol. Chromatography was carried out with a Waters HPLC
195 system equipped with a 600 quaternary pump, a 996 photodiode array detector
196 (PDA) and a 474 fluorescence detector (FD), and data were analyzed with the
197 Empower software (Waters). Phenolic compounds were separated at 35 °C
198 using a Luna C18 reverse column (250 x 4.6 mm, 5 µm; Phenomenex) coupled
199 to a µBondapak C18 guard column (10 µm) and using a binary gradient elution
200 of acetonitrile and water (pH 2.5). The flow rate was 0.8 mL min^{-1} and the
201 injection volume, 20 µL. Phenolics were detected by fluorescence at excitation
202 and emission wavelengths of 313 nm and of 405 nm, respectively, and by
203 setting the photodiode array detector to scan from 200 to 400 nm. For each
204 analysis, a Maxplot chromatogram, which plots each phenolic compound peak
205 at its corresponding maximum absorbance wavelength, was obtained. Peaks
206 were integrated and phenolic content was calculated using calibration curves.
207 Detection using HPLC-PDA coupled to a quadrupole time of flight-mass
208 spectrometry (QTOF-MS) was based on the method described in Moco, Bino,
209 Vorst, Verhoeven, de Groot, van Beek et al. (2006), with small modifications.
210 Briefly, phenolic compounds were extracted from the previously homogenized
211 flavedo and albedo frozen materials with 80% methanol. Samples were then
212 centrifuged at 3,000 x *g* for 10 min and the supernatants were filtered. For LC-
213 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 ºC 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 ºC 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 ºC, 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^{-}

239 ^{1} fresh weight. When reference compounds were not available (hexamethyl-O-
240 quercetagetin, citrusnin A, drupanin aldehyde and compound 19), the levels
241 were expressed as the area (mAU s) of the peak in the chromatogram.
242

243 *2.6. Determination of fluorescent compounds in the peel of citrus fruits*
244 To determine the presence of fluorescence compounds in the peel of oranges,
245 a stereoscopic zoom microscope SMZ800 with Epi-fluorescence attachment
246 (Nikon) was used. A transversal cut centered in the inoculation point was made
247 in elicited oranges and the tissue was observed using the microscope coupled
248 with an EX 480 / 40 BA 510 filter.
249

250 *2.7. Standards*
251 Eriocitrin (eriodictyol-7-O-rutinoside), narirutin (naringenin-7-O-rutinoside),
252 isorhoifolin (apigenin-7-O-rutinoside), diosmin (diosmetin-7-O-rutinoside) and
253 didymin (isosakuranetin-7-O-rutinoside), also known as neoponcirin, were
254 purchased from Extrasynthèse (Genay, France); chlorogenic acid and
255 scoparone (6,7-dimethoxycoumarin) from Aldrich (Spain); and caffeic acid and
256 hesperidin (hesperetin-7-O-rutinoside) from Fluka (Spain). The PMFs
257 isosinensetin (3',4',5,7,8-pentamethoxyflavone), hexamethyl-O-gossypetin
258 (3',4',3,5,7,8-hexamethoxyflavone), sinensetin (3',4',5,6,7-
259 pentamethoxyflavone), hexamethyl-O-quercetagetin (3',4',3,5,6,7,-
260 hexamethoxyflavone), nobiletin (3',4',5,6,7,8-hexamethoxyflavone), tetramethyl-
261 O-scutellarein (4',5,6,7-tetramethoxyflavone), heptamethoxyflavone (3',4',3,
262 5,6,7,8-heptamethoxyflavone), and tangeretin (4',5,6,7,8-pentamethoxyflavone)
263 were kindly supplied by Dr. J.M. Sendra (IATA-CSIC, Valencia, Spain).

264

265 *2.7. Statistics*

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

270

271 **3. Results and discussion**

272 The elicitor treatment increased the resistance of Navelate oranges to a
273 subsequent pathogen infection. Our results showed that the lowest severity of
274 the infection was observed when the pathogen was inoculated 7 days after the
275 beginning of the experiment (severity of 3.7 ± 0.7 cm^2). Elicitor treatment also
276 showed a statistically significant, but lower, reduction in severity when the
277 pathogen was inoculated 4 or 5 days after the beginning of the experiment (7.3
278 ± 0.5 and 6.7 ± 1.1 cm^2, respectively), compared to non-treated oranges (29.7 ±
279 1.8 cm^2). The involvement of the enzyme PAL (Ballester, Izquierdo, Lafuente, &
280 González-Candelas, 2010), and the relevance of phenylpropanoids metabolism
281 in the induction of resistance (Ballester et al., 2011; Hershkovitz et al., 2011),
282 and in the defense of citrus fruit against pathogens (Gonzalez-Candelas,
283 Alamar, Sanchez-Torres, Zacarias, & Marcos, 2010) has been pointed out by
284 using biochemical and transcriptomic approaches. However, in spite of the
285 broad number of phenylpropanoid genes associated with induced resistance,
286 little is known about the role that metabolites from this pathway may play in this
287 process. Therefore, we have examined the metabolic profile of
288 phenylpropanoids and derivatives involved in induced resistance in the flavedo

289 and albedo of citrus fruit. This information would be interesting in order to
290 increase the knowledge of this pathway in citrus and to contribute to the
291 development of new and safer alternatives for controlling postharvest
292 pathogens of citrus fruit.
293

294 *3.1. Differences in the phenylpropanoid metabolic profiles between flavedo and*
295 *albedo peel tissues in non-treated Navelate oranges*
296 The flavedo and the albedo tissues, which show different susceptibility to
297 infection caused by *P. digitatum* (Ballester, Lafuente, & González-Candelas,
298 2006; Kavanagh & Wood, 1967), also showed different phenylpropanoid
299 metabolic profiles. The flavanone hesperidin was the most abundant flavonoid
300 in the flavedo of non-treated Navelate oranges (FNT), followed by
301 phenylpropanoid chlorogenic acid and the PMFs tetramethyl-O-scutellarein,
302 heptamethoxyflavone, sinensetin and tangeretin (Table 1). Other flavanones,
303 such as didymin, narirutin and eriocitrin, and the phenylpropanoid caffeic acid
304 were also abundant in this external peel tissue. However, the coumarin
305 scoparone, which has been related to the defense of citrus fruit against *P.*
306 *digitatum* infection (Ballester, Izquierdo, Lafuente, & González-Candelas, 2010;
307 J. J. Kim, Ben Yehoshua, Shapiro, Henis, & Carmeli, 1991), was not detected in
308 the flavedo of non-treated fruits (FNT) neither in the albedo of non-treated
309 oranges (ANT). The internal tissue contained similar levels of hesperidin but
310 much higher levels of didymin and narirutin, and remarkable lower amounts of
311 chlorogenic acid, PMFs and eriocitrin as compared to the flavedo (Table 2).
312 This is in concordance with previous findings showing that composition and
313 content of the phenolic compounds differ among tissues and citrus varieties

(Goulas & Manganaris, 2012; Lafuente, Ballester, Calejero, Zacarías, & González-Candelas, 2011; Nogata, Sakamoto, Shiratsuchi, Ishii, Yano, & Ohta, 2006). 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.

364 The rise in the levels of flavonoids in response to the elicitor treatment could be
365 related with a higher resistance of the elicited fruits against an ulterior infection.
366 This is concordance with previous results showing that citrus fruits with higher
367 levels of the flavanones hesperidin and naringenin, the flavanone diosmin and
368 total polymethoxyflavone levels showed lower susceptibility to *P. digitatum*
369 infection (Ortuño, Díaz, Alvarez, Porras, García-Lidón, & Del Río, 2011), and
370 that some of these flavonoids show *in vitro* antifungal activity against for
371 instance *Penicillium* sp., *Phytophthora* sp. and *Geotrichum* sp. (Del Río, Arcas,
372 Benavente-García, & Ortuño, 1998; Ortuño et al., 2006). In addition with
373 flavonoid content changes in response to an elicitor treatment, a transitory
374 increase in the flavonoid concentration has been observed in response to *P.*
375 *digitatum* infection (Ballester, Lafuente, & González-Candelas, Submitted; H. G.
376 Kim et al., 2011). However, the induction of flavonoid content was, in general,
377 higher in response to the elicitor treatment than in response to pathogen
378 infection.
379 As indicated above, the increases observed in some phenolics were transient,
380 which agrees with the fact that the induction of genes involved in
381 phenylpropanoid biosynthesis may be transient in elicited citrus fruit (Ballester
382 et al., 2011). It is also noticing that results of the present paper showing the
383 transient increase of such phenolics are in concordance with other reports
384 showing that increases in phenolics, and also in the expression of genes
385 involved in phenylpropanoid biosynthesis, occurring in citrus fruit exposed to
386 abiotic stress or to treatments that increase the fruit tolerance to such stress
387 may be transient (Lafuente, Ballester, Calejero, Zacarías, & González-

388 Candelas, 2011; Sánchez-Ballesta, Lluch, Gosalbes, Zacarías, Granell, &
389 Lafuente, 2003).
390

391 *3.3. Identification and quantification of new phenolic compounds in elicited fruits*
392 HPLC-PDA results show that the highest increase observed in elicited fruits for
393 any flavonoid is lower than 2-fold, whereas we have found 4 fluorescent
394 compounds with much larger increases in response to the elicitor treatment
395 (Fig. 2A). Therefore, a qualitative and quantitative analysis of these compounds
396 was further performed by using a HPLC-PDA-QTOF-MS system. As shown in
397 Fig. 2A, the levels of 4 fluorescence compounds (nos. 8, 18, 19 and 20) peaked
398 at 5 or 7 days after the beginning of the experiment, being the levels of them
399 higher in the flavedo (Table 1) than in the albedo (Table 2). By comparing the
400 HPLC retention times, UV absorbance spectra (Fig. 2B) and accurate mass
401 signals (Fig. 2C) with those of authentic standards, fluorescent compound 8
402 was identified as scoparone (6, 7-dimethoxycoumarin; Fig. 2D). Scoparone was
403 not detected in the flavedo or albedo of non-treated fruits, while substantial
404 amounts of this compound were detected in the flavedo (90.5 and 54.0 μg g^{-1}
405 fresh weight at 5 and 7 days, respectively) and lower amounts in the internal
406 tissue (12.2 and 24.7 μg g^{-1} fresh weight at 5 and 7 days, respectively) of
407 elicited fruits. It is noteworthy that these levels were substantially higher than
408 those detected in response to *P. digitatum* infection, with maximum levels of
409 14.8 and 5.3 μg g^{-1} fresh weight in the flavedo and albedo, respectively, 72 h
410 post-inoculation (Ballester, Lafuente, & González-Candelas, Submitted) in spite
411 of the lack of infection in the elicited samples. Likewise, in the context of the
412 present work it is important to note that even the lower scoparone level detected

413 in the albedo of elicited fruits was close to the median effective dose for the
414 inhibition of germ tube elongation of *P. digitatum* (J. J. Kim, Ben Yehoshua,
415 Shapiro, Henis, & Carmeli, 1991). Therefore, this coumarin may play a role in
416 the higher resistance observed in elicited fruits at 7 days after the beginning of
417 the experiment. This is in concordance with previous data indicating that
418 scoparone is associated with the defense of citrus fruit against different stresses
419 such as UV light and pathogen infection (Afek, Orenstein, Carmeli, Rodov, &
420 Joseph, 1999; Ballester, Lafuente, & González-Candelas, Submitted;
421 D'Hallewin, Schirra, Manueddu, Piga, & Ben Yehoshua, 1999; Kuniga,
422 Tsumura, Matsuo, & Matsumoto, 2006). Other authors have associated the
423 coumarins umbelliferone (7-hydroxycoumarin) and scopoletin (6-methoxy, 7-
424 hidroxycoumarin), which are probable precursors of scoparone, with a higher
425 resistance of citrus fruits to *P. digitatum* infection (Afek, Orenstein, Carmeli,
426 Rodov, & Joseph, 1999; Droby et al., 2002; Nafussi, Ben Yehoshua, Rodov,
427 Peretz, Ozer, & D'Hallewin, 2001). However, none of these 2 compounds were
428 detected in either non treated or elicited Navelate oranges.
429 We have recently shown that the combination of pathogen inoculation followed
430 by a curing treatment reduced the incidence of a subsequent *P. digitatum*
431 infection in oranges and triggered relevant changes in the expression of a broad
432 number of phenylpropanoid genes, being noteworthy the increase in expression
433 levels of several O-methyltransferases (OMTs) encoding genes (Ballester et al.,
434 2011). Previous reports have shown that OMTs and various cytochrome P450
435 enzymes are involved in the formation of phenolic compounds, including
436 coumarins and PMFs (Bourgaud, Hehn, Larbat, Doerper, Gontier, Kellner et al.,
437 2006; Ibrahim, Bruneau, & Bantignies, 1998). This, together with the fact that

438 scoparone and PMFs are methylated compounds, raises the possibility that
439 induced OMTs play a role in their synthesis, although a conclusive relationship
440 between any of them and scoparone or PMFs still remains to be elucidated.
441 Three other yet unknown compounds increased substantially in response to the
442 elicitor treatment (Fig. 2A, compounds 18, 19 and 20). Low levels of these
443 compounds were detected in the flavedo of non-treated fruits, while they were
444 undetectable in the internal tissue of non-treated fruits. In both tissues the
445 relative levels of compounds 18 and 20 increased substantially in response to
446 the elicitor treatment, peaking at day 7, whereas compound 19 reached the
447 highest level at day 5. Thus, in the flavedo, 100-, 20- and 200-fold increases
448 were found by 7 days for compounds 18, 19 and 20, respectively. These
449 proportions could not be estimated in the albedo since these compounds were
450 not detected in the non-treated fruits, but final levels were at least 4-fold lower in
451 this tissue than in the flavedo. To identify these 3 compounds, samples were
452 subjected to accurate mass spectrometry (LC-PDA-QTOF-MS) using both
453 negative and positive electrospray ionization (ESI) modes (Fig. 2B, 2C).
454 Compound 19, with λ_{max} of 215.57 and 263.57 nm, could not be identified
455 because its accurate mass is still unknown due to its low ionization efficiency in
456 both positive and negative ESI modes.
457 Compound 18 had a UV spectrum with λ_{max} of 267.6 nm and an observed
458 accurate mass of *m*/*z* 231.0996 $[M-H]^-$, corresponding to a molecular formula of
459 $C_{14}H_{16}O_3$. Using different databases, such as KNApSAcK (Sinbo, Nakamura,
460 Altaf-Ul-Amin, Asahi, Kurokawa, Arita et al., 2006) and Dictionary of Natural
461 Products (CHEMnetBASE), this compound was putatively identified as citrusnin
462 A (Fig. 2D). Citrusnin A has been isolated from leaves of *Citrus natsudaidai*

463 inoculated with a *Pseudomonas sp*. antagonistic to *Xanthomonas campestri* pv.
464 *citri* (Watanabe, Myiyakado, Ohno, Ota, & Nonaka, 1985). The physicochemical
465 properties of this compound, such as MS *m/z* and UV λ_{max} nm, matched
466 perfectly with the ones observed in elicited oranges. The antibacterial effect of
467 this compound was also tested *in vitro*, being effective against different
468 pathogenic bacteria (Watanabe, Myiyakado, Ohno, Ota, & Nonaka, 1985).
469 However, this is the first report linking citrusnin A with the resistance of citrus
470 fruit to infection caused by *P. digitatum*. Furthermore, as far as we know, this
471 compound has not been yet related to the resistance of citrus or other fruits to
472 pathogens causing postharvest losses.
473 Compound 20 showed a similar λ_{max} at 267.6 nm, but an accurate mass of *m*/*z*
474 215.1076 $[M-H]^{\cdot}$, corresponding to a molecular formula of $C_{14}H_{16}O_2$. Based on
475 comparison with different metabolite databases, this compound was putatively
476 identified as drupanin aldehyde (i.e. 3-[4-hydroxy,3-(3-methyl-2-butenyl)-
477 phenyl]-2-(*E*)-propenal or 4-hydroxy-3-prenylcinnamaldehyde) (Fig. 2D). This
478 compound was previously isolated from the peel of wounded grapefruits (*Citrus*
479 *paradise*) and oranges (*C. sinensis*) (Stange, Midland, Eckert, & Sims, 1993). It
480 is also known that drupanin itself, isolated from *Baccharis* sp., has antifungal
481 and antibacterial activity (Bisogno, Mascoti, Sanchez, Garibotto, Giannini,
482 Kurina-Sanz et al., 2007; Feresin, Tapia, Gimenez, Ravelo, Zacchino, Sortino et
483 al., 2003). However, its involvement in the resistance of citrus fruits to
484 pathogenic fungi has not been reported until now. Moreover, it has to be noted
485 that although citrusnin A and drupanin aldehyde levels increased in response to
486 the elicitor treatment, none of these compounds were detected in response to
487 *P. digitatum* infection (Ballester, Lafuente, & González-Candelas, Submitted). In

488 light of their structures both citrusnin A and drupanin could be biochemically
489 derived from precursors in the first part of the phenylpropanoid pathway, but the
490 genes and enzymes involved in their synthesis are unknown yet. The study of
491 the possible antifungal activity of these compounds against *P. digitatum* has not
492 been undertaken because they are not commercially available and their
493 concentration in the peel of citrus fruits is very low. However, the results
494 presented in this work encourage further research in this direction.
495 Since the HPLC-FD analysis of phenolic metabolites revealed the induction of
496 fluorescent compounds in the peel of elicited fruits, we checked the presence of
497 fluorescence in elicited oranges using a stereoscopic zoom microscope
498 SMZ800 with Epi-fluorescence attachment (Nikon) (Fig. 3). The amount of
499 fluorescence in the transversal cut of peel oranges was higher in elicited fruits
500 than in non-treated fruits. The fluorescence was concentric around the
501 inoculation point, which reinforces the idea that the elicitor treatment induced
502 only local disease resistance and that the effect is limited to only a small area
503 around the origin of infection (1-4 mm distance from the inoculation site).
504 Metabolic profiling results of this study strongly suggest an implication of
505 phenylpropanoids, flavonoids and their derivatives in the induction of resistance
506 in citrus fruit, being especially relevant the induction of scoparone and three
507 other fluorescent phenolic compounds that have not been previously related to
508 the resistance of citrus fruit against disease caused by *P. digitatum*. Two of
509 them, citrusnin A and drupanin aldehyde, were putatively identified and showed
510 very relevant increases in elicited fruits. Therefore, their implication in citrus fruit
511 responses deserves further investigation. Finally, our results indicate that the
512 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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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 1. Phenylpropanoid and flavonoid concentration (µg g^{-1} fresh weight) in the flavedo of non-treated (FNT) and elicited Navelate oranges 4, 5 and 7 days after the beginning of the experiment (FIC4, FIC5 and FIC7, respectively). 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 papper submitted to Food Chemistry).

			FNT		FIC4		FIC5		FIC7	
No.	**Compound**	**Family**	**Conc.**	**SD**	**Conc.**	**SD**	**Conc.**	**SD**	**Conc.**	**SD**
3	Eriocitrin	Flavanone	34.8	0.3 a	17.1	0.3 c	18.2	1.5 c	24.6	0.1 b
4	Narirutin	Flavanone	33.1	2.6 a	nd		nd		14.9	17.5 a
7	Hesperidin	Flavanone	1840.9	74.8 b	2103.9	118.9 a	2179.1	41.4 a	1979.0	83.2 ab
9	Didymin	Flavanone	56.8	9.3 b	67.1	4.0 ab	67.3	8.8 ab	78.6	11.8 a
1	Chlorogenic acid	Cinnamic acid	161.0	26.4 a	134.0	3.3 a	149.8	3.5 a	152.9	15.9 a
2	Caffeic acid	Cinnamic acid	68.8	16.7 a	64.3	14.0 a	57.5	2.3 a	60.5	7.2 a
5	Isorhoifolin	Flavone	65.8	9.0 b	130.7	29.6 a	59.5	7.1 b	64.8	1.1 b
6	Diosmin	Flavone	26.5	0.9 a	22.2	3.2 a	26.4	1.0 a	24.1	9.6 a
10	Isosinensetin	PMF	3.5	1.1 a	2.7	0.9 a	3.6	0.4 a	4.0	0.4 a
11	Hexamethyl-O-gossypetin	PMF	1.0	0.4 ab	0.7	0.2 b	1.7	0.2 a	1.3	0.6 ab
12	Sinensetin	PMF	100.5	6.4 b	119.7	10.0 a	102.4	0.3 b	80.8	3.6 c
13	Hexamethyl-O-quercetagetin*	PMF	420.7	11.1 b	428.7	58.8 b	579.5	3.9 a	532.3	56.3 a
14	Nobiletin	PMF	29.7	1.4 b	29.0	1.6 b	35.9	1.2 a	30.9	2.3 b
15	Tetramethyl-O-scutellarein	PMF	140.8	21.6 a	153.2	9.2 a	141.8	3.5 a	128.6	10.9 a
16	Heptamethoxyflavone	PMF	125.4	11.0 b	132.2	2.6 ab	142.8	3.3 a	126.2	2.9 ab
17	Tangeretin	PMF	88.7	14.1 c	110.1	4.6 bc	138.2	4.4 a	123.5	9.1 ab
8	Scoparone (FD)	Coumarin	nd		29.7	14.9 b	90.5	7.7 a	54.0	2.7 b
18	Citrusnin A (FD)*		28.4	2.3 c	755.4	51.6 bc	1121.2	282.7 b	3249.7	707.8 a
19	Compound 19 (FD)*		14.2	5.5 c	189.2	68.2 b	408.4	25.0 a	325.4	32.6 a
20	Drupanin aldehyde (FD)*		178.4	17.4 c	930.0	78.6 b	1538.5	33.3 b	3310.5	653.0 a

* 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

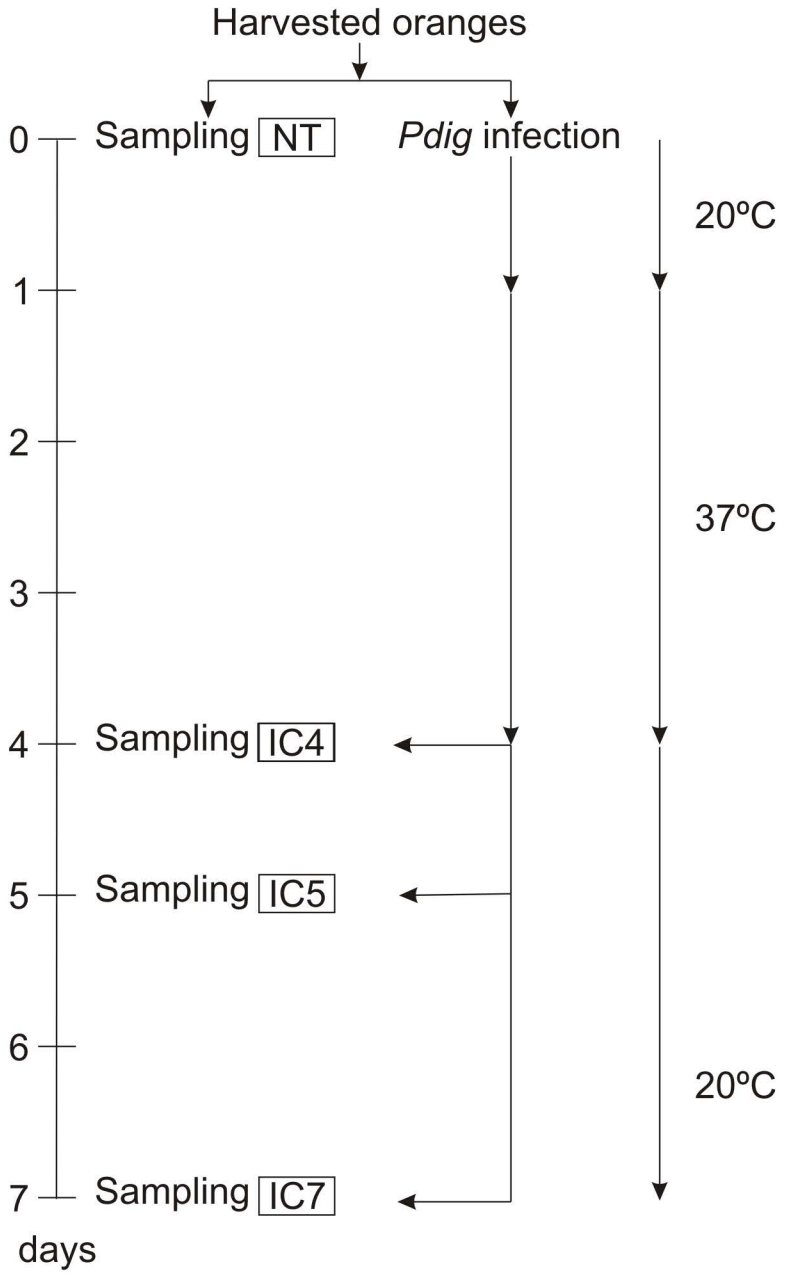
Table 2. Phenylpropanoid and flavonoid concentration (µg g^{-1} fresh weight) in the albedo of non-treated (ANT) and elicited Navelate oranges 4, 5 and 7 days after the beginning of the experiment (AIC4, AIC5 and AIC7, respectively) detected by HPLC-PDA-FD. Results represent the mean of at least two 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 papper submitted to Food Chemistry).

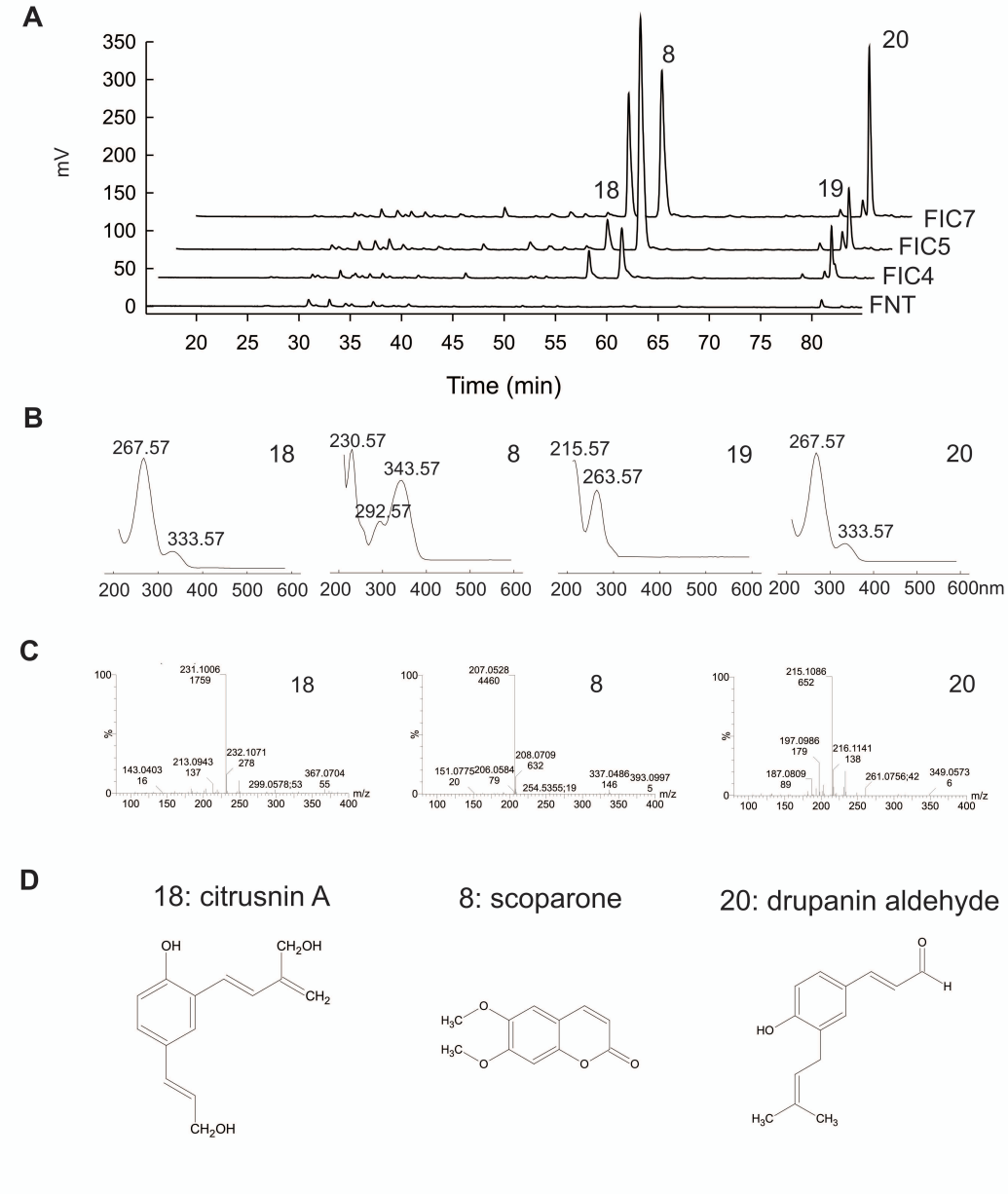
			ANT			AIC4			AIC5			AIC7		
No.	**Compound**	**Family**	**Conc.**	**SD**		**Conc.**	**SD**		**Conc.**	**SD**		**Conc.**	**SD**	
3	Eriocitrin	Flavanone	15.3 ±	6.1	a	14.3 ±	2.2	a	19.0 ±	0.1	a	11.8 ±	0.3	a
4	Narirutin	Flavanone	434.3 ±	35.3	a	308.2 ±	65.9	b	404.7 ±	9.6	ab	373.2 ±	15.1	ab
7	Hesperidin	Flavanone	2,027.1 ±	117.3	a	1,518.3 ±	107.8	b	1,818.1 ±	107.3	a	2,061.2 ±	67.0	a
9	Didymin	Flavanone	348.6 ±	30.8	a	254.3 ±	47.3	b	327.7 ±	0.9	ab	307.1 ±	17.9	ab
1	Chlorogenic acid	Cinnamic acid	14.2 ±	3.0	b	25.4 ±	5.0	a	24.7 ±	0.5	a	10.6 ±	1.4	b
12	Sinensetin	PMF	3.9 ±	0.6	bc	6.7 ±	0.9	ab	8.9 ±	1.1	a	3.7 ±	1.6	c
13	Hexamethyl-O-quercetagetin*	PMF	39.3 ±	3.9	b	58.8 ±	3.4	a	64.1 ±	1.6	a	63.0 ±	4.6	a
14	Nobiletin	PMF	1.4 ±	0.0	b	2.8 ±	0.9	a	3.4 ±	0.2	a	2.6 ±	0.2	ab
15	Tetramethyl-O-scutellarein	PMF	12.5 ±	2.5	a	13.0 ±	1.2	a	11.1 ±	1.6	a	13.7 ±	0.3	a
16	Heptamethoxyflavone	PMF	20.4 ±	3.1	b	32.3 ±	3.2	a	37.1 ±	1.6	a	33.8 ±	2.8	a
17	Tangeretin	PMF	5.9 ±	0.5	c	11.2 ±	2.1	b	16.2 ±	0.0	a	14.2 ±	2.3	ab
8	Scoparone (FD)	Coumarin	nd			6.2 ±	1.4	b	12.2 ±	0.7	ab	24.7 ±	7.6	a
18	Citrusnin A (FD)*		nd			81.9 ±	39.8	b	177.8 ±	58.3	b	520.9 ±	34.3	a
19	Compound 19 (FD)*		nd			120.6 ±	49.9	a	141.6 ±	35.4	a	147.4 ±	74.5	a
20	Drupanin aldehyde (FD)*		nd			396.6 ±	74.9	b	518.1 ±	97.3	b	970.6 ±	127.7	a

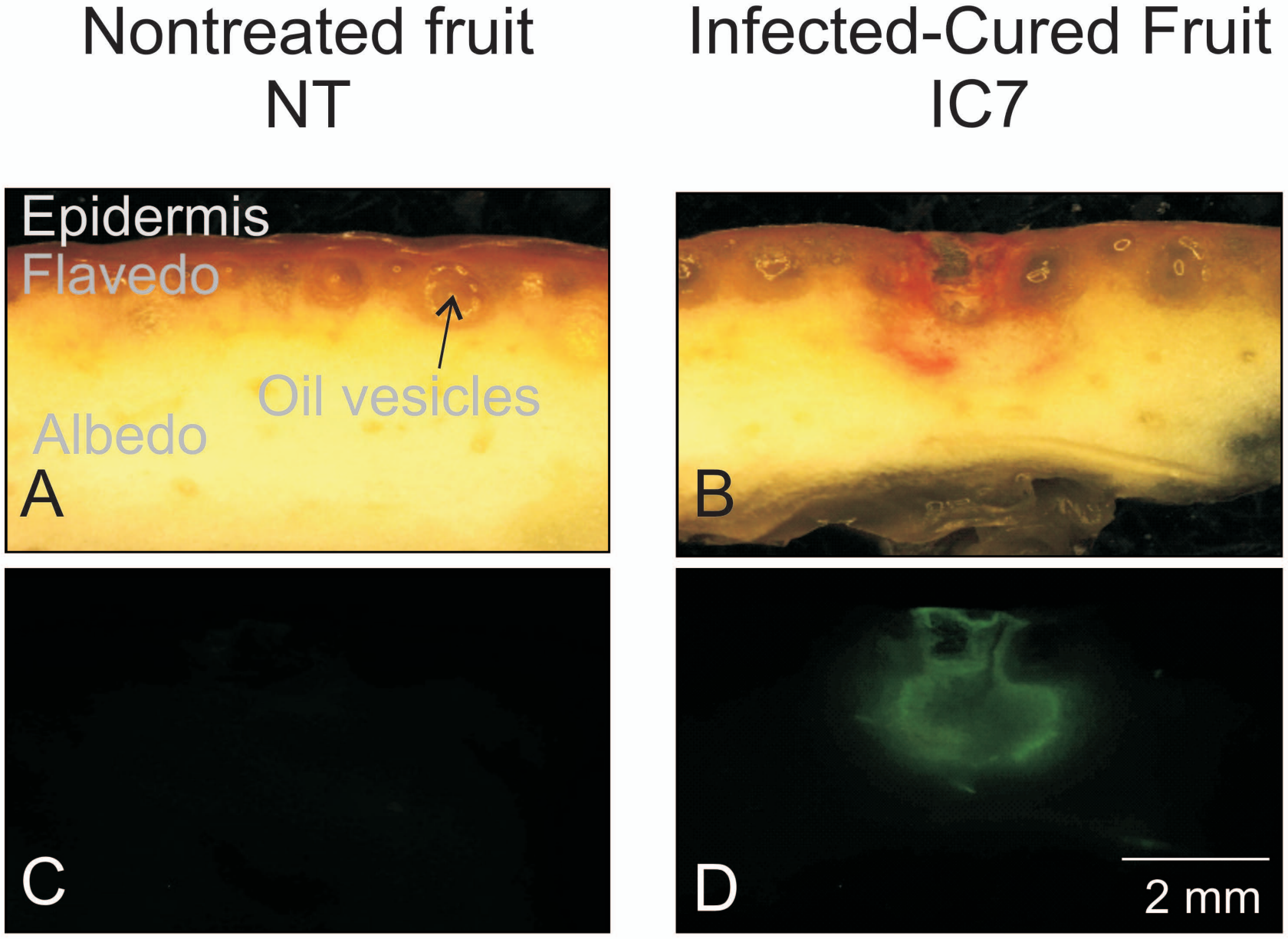
* values represent the area (mAU s) of the peak in the chromatogram

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

nd. non-detected compound


Harvested oranges
0
Sampling NT
Pdig infection
20ºC
1
2
37ºC
3
4
Sampling IC4
5
Sampling IC5
6
20ºC
7
Sampling IC7
days


A
mV
350
300
250
200
150
100
50
0
18
8
19
20
FIC7
FIC5
FIC4
FNT
20 25 30 35 40 45 50 55 60 65 70 75 80
Time (min)
B
267.57
333.57
18
230.57
343.57
292.57
8
215.57
263.57
19
267.57
333.57
20
200 300 400 500 600nm
C
231.1006
1759
232.1071
278
213.0943
137
143.0403
16
367.0704
55
299.0578;53
18
207.0528
4460
208.0709
632
206.0584
79
151.0775
20
337.0486
146
393.0997
5
254.5355;19
8
215.1086
652
216.1141
138
197.0986
179
187.0809
89
349.0573
6
261.0756;42
20
m/z
D
18: citrusnin A
8: scoparone
20: drupanin aldehyde
OH
CH2OH
CH2
CH2OH
H3C
O
O
O
HO
H
H3C
CH3


Nontreated fruit
NT
Infected-Cured Fruit
IC7
Epidermis
Flavedo
Oil vesicles
Albedo
A
B
C
D
2 mm