View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Digital.CSIC

1 LED blue light-induced changes in phenolics and ethylene in citrus fruit:
2 implication in elicited resistance against *Penicillium digitatum* infection
3
4 Ana-Rosa Ballester and María T. Lafuente*
5
6 Instituto de Agroquímica y Tecnología de Alimentos, IATA-CSIC, Calle Agustín
7 Escardino 7, 46980 Paterna, Valencia, Spain
8
9 * Corresponding author. Tel.: +34 900 022; fax: +34 963 636 301
10 E-mail address: ballesterar@iata.csic.es; mtlafuente@iata.csic.es
11
12 Running title: LED blue light-induced changes in phenolics and ethylene in citrus

13 Abstract

14 The objective was to investigate whether LED Blue Light (LBL) induces changes in
15 phenolics and ethylene production of sweet oranges, and whether they participate in
16 LBL–elicited resistance against the most important postharvest pathogen (*Penicillium*
17 *digitatum*) of citrus fruit. The expression of relevant genes of the phenylpropanoid and
18 ethylene biosynthetic pathways during elicitation of resistance was also determined.
19 Different LBL (wavelength 450 nm) quantum fluxes were used within the 60–630 μmol
20 $m^{-2}s^{-1}$ range. The HPLC analysis showed that the most relevant increase in
21 phenylpropanoids occurred in scoparone, which markedly increased 3 days after
22 exposing fruits to a very high quantum flux (630 μmol $m^{-2}s^{-1}$) for 18 h. However,
23 phenylpropanoids, including scoparone, were not critical factors in LBL–induced
24 resistance. The genes involved in ethylene biosynthesis were differentially regulated by
25 LBL. Ethylene is not involved in elicited resistance, although high LBL levels increased
26 ethylene production in only 1 h.

27

28 Keywords: induced resistance, infection, phenylalanine ammonia–lyase,
29 phenylpropanoids, plant hormones, postharvest disease.

30

31 The chemical compounds studied in this article:
32 Narirutin (PubChem CID: 442431), isorhoifolin (PubChem CID: 9851181), eriocitrin
33 (PubChem CID: 83489), diosmin (PubChem CID: 5281613), didymin (PubChem CID:
34 16760075), caffeic acid (PubChem CID: 689043), hesperidine (PubChem CID: 3594),
35 chlorogenic acid (PubChem CID: 1794427), scoparone (PubChem CID: 8417),
36 isosinesetin (PubChem CID: 632135), sinensetin (PubChem CID: 145659), nobiletin
37 (PubChem CID: 72344), and tangeretin (PubChem CID: 68077). Polymethoxylated

38 flavones (PMFs) hexamethyl–O–gossypetin (3',4',3,5,7,8–hexamethoxyflavone),
39 hexamethyl–O–quercetagetin (3',4',3,5,6,7–hexamethoxyflavone), tetramethyl–O–
40 scutellarein (4',5,6,7–tetramethoxyflavone) and heptamethoxyflavone (3',4',3,5,6,7,8–
41 heptmethoxyflavone) were kindly supplied by Dr. J.M. Sendra (IATA–CSIC, Valencia,
42 Spain).
43

1. Introduction

The antimicrobial properties of light is a research area that receives growing interest due, in part, to the development of resistance to standard control methods (Dai et al., 2013; Ondrusch & Kreft, 2011). Lighting based on Light Emitting Diodes (LEDs) is one of the main emerging technologies in agriculture (Folta & Childers, 2008). In the context of the present study, it is remarkable that LED blue light (LBL) may control food-relevant fungi (Schmidt-Heydt, Rüfer, Raupp, Bruchmann, Perrone, & Geisen, 2011) and other harmful pathogens for consumers, such as *Listeria monocytogenes* (Ondrusch & Kreft, 2011).

Green mold rot, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., is the most important postharvest disease of citrus fruit grown under Mediterranean climate conditions. It causes major economic losses, mostly due to pathogen contaminations and the development of strains resistant to synthetic fungicides (Sánchez-Torres & Tuset, 2011). Hence given the growing concern about care of human health and the environment, there is a trend to develop alternative methods to control postharvest diseases and to restrict the use of chemicals in fruits (Ballester, Lafuente, De Vos, Bovy, & González-Candelas, 2013; Droby et al., 1993; Droby, Wisniewski, Macarisin, & Wilson, 2009; Montesinos-Herrero, Smilanick, Tebbets, Walse, & Palou, 2011).

Recently, the potential of LBL has been shown for controlling the growth of different *P. digitatum* and *Penicillium italicum* strains that infect citrus fruits, and that LBL efficacy increases with both treatment duration and the applied light quantum flux (Alferez, Liao, & Burns, 2012; Lafuente & Alférez, 2015; Yamaga, Takahashi, Ishii, Kato, & Kobayashi, 2015b). However, the potential of LBL for inducing resistance against *P. digitatum* in citrus fruits is almost unknown (Liao, Alferez, & Burns, 2013). Only two reports are available on the mechanisms by which LBL may increase

69 resistance against *P. digitatum* in citrus fruits, and both imply lipid signaling (Alferez,
70 Liao, & Burns, 2012; Liao, Alferez, & Burns, 2013).

71 Phenylpropanoids and the plant hormone ethylene are important players in the
72 defense of citrus fruit against *P. digitatum* (Ballester, Lafuente, & González-Candelas,
73 2013; D'Hallewin, Schirra, Manueddu, Piga, & Ben Yehoshua, 1999; Droby et al.,
74 1993; Gonzalez-Candelas, Alamar, Sanchez-Torres, Zacarias, & Marcos, 2010; Marcos,
75 González-Candelas, & Zacarías, 2005). However, whether LBL may induce changes in
76 ethylene and phenolics in this fruit, and whether these changes may be involved in
77 LBL-elicited resistance against *P. digitatum,* remain unknown. In this context, it is
78 remarkable that LBL may induce changes in the ethylene production of fruits like
79 peaches (Gong et al., 2015), **and of plants (Corbineau, Rudnicki, Goszczyńska, &**
80 Come, 1995), and that ethylene production in LBL-irradiated plants may depend on the
81 light fluence. For a long time, it has been known that LBL may increase the activity of
82 the enzyme phenylalanine ammonia-lyase (PAL) (Engelsma, 1974), the initial rate-
83 controlling enzyme in the phenylpropanoid pathway, in plants, and that the hormone
84 stimulates PAL activity and phenylpropanoid metabolism in citrus fruit (Lafuente,
85 Zacarías, Martínez-Téllez, Sánchez-Ballesta, & Dupille, 2001). Therefore, the aim of
86 this work was to investigate whether LBL is able to induce changes in ethylene
87 production and phenolic compounds in citrus fruits, and whether these changes
88 participate in LBL-elicited resistance. To that end, we examined the effect of treating
89 harvested sweet oranges at different LBL intensities. Moreover, we compared the effect
90 of LBL on fruit disease susceptibility with that on ethylene production, total phenolic
91 content and on the phenylpropanoid metabolic profile of the elicited fruits. Light was
92 always applied before inoculating fruit with *P. digitatum*. The expression of the relevant
93 genes of the phenylpropanoid and ethylene biosynthetic pathways was also examined.

94 2. Materials and methods

95 *2.1. Fruit and fungal material*

96 Mature Lane Late sweet oranges (*Citrus sinensis* (L.) Osbeck) were selected from
97 commercial orchards at Lliria (Valencia, Spain) and immediately delivered to the
98 laboratory before applying any commercial postharvest treatment. In each experiment,
99 three samples of 23 fruits per treatment were taken and used to examine the effect of
100 LBL treatments on changes in gene expression, phenolics and ethylene production, and
101 on inducing resistance in citrus fruits against *P. digitatum* infection. Fruits were
102 immediately surface–sterilized with a 5% commercial bleach solution (Ballester,
103 Lafuente, De Vos, Bovy, & González–Candelas, 2013), thoroughly rinsed with tap
104 water, and then randomly divided into 2 groups that were always kept in the dark at 20
105 ºC (control fruits, group 1) or were exposed to the selected light treatment at 20 ºC, as
106 described below (group 2).

107 In order to test the efficacy of LBL on reducing disease in citrus fruits, oranges
108 were infected with *P. digitatum* (Pers.:Fr.) Sacc. isolate Pd1 (CECT 20795), deposited
109 in the Spanish Type Culture Collection (CECT), and obtained from oranges with typical
110 green mold collected from different orchards or packinghouses. This strain is highly
111 resistant to the two fungicides used in citrus fruit: thiabendazole and imazalil. The strain
112 was grown for 7 days at 24 ºC on Potato Dextrose Agar medium before use. Conidia
113 were rubbed from the agar surface by scrapping them with a sterile spatula, and were
114 transferred to 10 mL of sterile water. The resulting suspensions were filtered and the
115 conidia concentration of the obtained filtrate was titrated with a hemacytometer and
116 adjusted to 10^5 conidia mL^{-1} with sterile water (Ballester, Lafuente, & González–
117 Candelas, 2013). This suspension was then used to infect fruits to evaluate the efficacy
118 of the LBL treatments to elicit resistance.

119

120 *2.2. Blue light treatments and induced resistance*

121 To know whether the effect of LBL on ethylene, phenylpropanoids and the elicited
122 resistance against *P. digitatum* may depend on the light quantum flux, and whether there
123 is a link between LBL–induced resistance and the changes in phenolics and ethylene,
124 sweet oranges were exposed to LBL for different periods at quantum fluxes that ranged
125 between 60 and 630 μmol $m^{-2}s^{-1}$. Fruits were always treated with light before being
126 inoculated with the fungus. To ensure a uniform light quantum flux, the light regimes
127 were applied in Mammoth Pro dark growth tents (60 × 60 × 160 cm) (Mammoth Pro 60,
128 Eltac Hidrofarm, Spain), equipped with velcro–sealable ventilation panels (300 mm ×
129 200 mm) and tough fabric lined with 95% reflective mylar (Lafuente & Alférez, 2015).
130 Tents had sufficient capacity for air exchange and were placed in a temperature–
131 controlled room to maintain temperature at 20 °C. The light source was a LumiGrow
132 Pro 650TM LED array (LumiGrow, Novato, CA, USA), which emitted LBL at a center
133 wavelength of 450 nm with a full width at the half–maximum of 20 nm. The light
134 quantum flux was measured and adjusted using a spectroradiometer (GL Spectics,
135 Sttutgart, Germany) (Lafuente & Alférez, 2015).

136 Different LBL regimens were assayed to select the most effective one to induce
137 resistance against *P. digitatum,* and to determine how this treatment affected the
138 phenolic profiling in the flavedo (outer colored part of the peel) and the ethylene
139 production of citrus fruit. The effect of the selected treatment on changes in expression
140 of the relevant genes of both the phenylpropanoid and ethylene biosynthetic pathways
141 was also examined. In order to test whether ethylene and phenolics play important roles
142 in LBL–induced resistance against *P. digitatum*, we determined the changes in these
143 compounds at different time points during the light treatments and after 3 days (3 dpt, 3

144 days post–treatment). The experimental design outlined in Fig. 1 summarizes the
145 experimental conditions of the selected treatment as well as sampling days. Samples
146 were always taken from non inoculated fruits. Fruits were infected only to determine the
147 efficacy of the light treatments to elicit resistance. The control and light–treated fruits
148 were always infected immediately after finishing the light treatment (0 dpt) and 3 days
149 after ending it (3 dpt). On these 3 days, both the elicited and control fruits were kept in
150 the dark at 20 ºC with 90–95% relative humidity (RH).
151
152 *2.3. P. digitatum infection and decay evaluation*
153 To determine the effectiveness of the LBL elicitor treatment to reduce pathogen
154 infection and the importance of the time that elapsed between the treatment and the
155 ulterior infection, disease susceptibility was evaluated in the fruits infected at 0 and 3
156 dpt (Fig. 1). Control samples, maintained for the same periods in the dark, were infected
157 like the elicited fruits (Fig. 1). Each elicited and control fruit was pricked on the
158 equatorial axis with a 2 mm (diameter) × 1 mm (deepness) sterilized needle, equipped
159 with a stopper to ensure uniformity of wounds. Then 10 μL of a 10^5 conidia mL^{-1}
160 suspension of *P. digitatum* spores were applied to each wound. After inoculation, fruits
161 were stored at 20 ºC with 90–95% RH.
162 To evaluate how light treatments could affect disease severity, the fruit
163 macerated diameter (cm) was periodically determined with a flexible ruler in two
164 directions during fruit incubation at 20 ºC. The experimental design consisted of 3
165 replicates of 15 fruits, with 1 wound per fruit for each treatment. The efficacy of the
166 selected LBL treatments was evaluated at 0 and 3 dpt. Therefore, four groups of fruit
167 were prepared in this experiment; two were used as the control and light–treated samples
168 for the infections done at 0 dpt, and the other two for the infections at 3 dpt. The control

169 samples consisted of inoculated fruits, which were always maintained in the dark at the
170 same temperature. The percentage of growth inhibition was also calculated using the
171 following formula:

172 Percentage of growth inhibition = 100 × (GC–GSL)/GC,

173 where GC is growth of the control (continuous darkness) and GSL is growth of the
174 macerated fruit zone of the sample exposed to the light treatment (Fadda et al., 2015).

175

176 *2.4. Analysis of total phenolics*

177 Total phenolic content was determined as reported by Lafuente, Alférez, and Romero
178 (2014). Briefly, 200 mg of the homogenized frozen flavedo were extracted with 1 mL of
179 ethanol using a Mini Beadbeater 8 Cell Disruptor (Biospec Products, Inc.). The extract
180 was centrifuged at 13000 × *g* at 4 ºC, and the phenolic content was estimated in the
181 supernatant. Two sample aliquots of 20 µL were diluted with 80 µL ethanol and 400 µL
182 nanopure water, and were incubated at room temperature with 500 µL of 1 N Folin–
183 Ciocalteau and 5 mL of 2 % Na_2CO_3. After centrifugation at 13000 × *g* at 4 ºC,
184 absorbance was determined at 724 nm, and total phenolic content was calculated by
185 using a standard curve developed with chlorogenic acid. The results are the means of
186 three replicate samples±SEM.

187

188 2.5. *Determination of phenolic compounds by high-performance liquid chromatography*

189 The phenolic compounds from flavedo were extracted as previously described
190 (Ballester, Lafuente, De Vos, Bovy, & González–Candelas, 2013). Briefly, freeze–
191 ground flavedo was extracted twice with 80 % methanol and the chromatographic
192 analyses of the extracts were performed in a Waters HPLC system. The system was
193 equipped with a 600 quaternary pump and fitted with a 717 autosampler and a 996

194 photodiode array detector (PDA), operated from 200 to 400 nm, and a fluorescence
195 detector (FD) operated at the excitation and emission wavelengths of 313 nm and 405
196 nm, respectively. The FD detector better allows the changes in phenolics to be analyzed,
197 which are less abundant than flavonoids, but have been related to the defense of citrus
198 fruit against *P. digitatum* (Ballester, Lafuente, De Vos, Bovy, & González-Candelas,
199 2013). Separation was accomplished in a Luna C18 reverse column (250 × 4.6 mm, 5
200 **µm; Phenomenex) coupled to a µBondapak C18 guard column (10 µm).** Elution was
201 performed by using a binary gradient elution of acetonitrile and water (pH 2.5) with a
202 flow rate of 0.8 mL min^{-1} and an **injection volume of 20 µL. Compound identification**
203 was based on the comparison made between the retention times and the spectrum
204 **obtained from the standards (see the section 'Chemical com**pounds studied in this
205 **article')**, and from the chromatographic signals in the samples run under the same
206 experimental conditions. Peaks were integrated and phenolic content was calculated
207 using calibration curves.
208
209 *2.6. Ethylene production measurements*
210 Ethylene production from whole fruits and from the flavedo discs (0.7 cm diameter)
211 was measured periodically by incubating three replicate samples of fruits or discs in 1.5
212 L sealed glass jars for 3 h (for fruits) or in 8 mL tubes (for flavedo discs) for 1 h at 20
213 ºC. Three oranges or six discs per replicate were used. The samples exposed to light at
214 each sampling point were incubated under the same light quantum flux, while the
215 samples kept in the dark were incubated in darkness. Two replicate samples of 1 mL gas
216 sample were withdrawn from the head space of each container and injected into a gas
217 chromatograph, equipped with an activated alumina column and a flame ionization

218 detector, as previously described (Lafuente, Zacarías, Martínez–Téllez, Sánchez–
219 Ballesta, & Dupille, 2001). The results are the means of three replicate samples±SEM.
220

221 2.7. *RNA extraction and cDNA synthesis*

222 Total RNA was isolated from flavedo tissue, its concentration was measured
223 spectrophotometrically, and its integrity was verified by agarose gel electrophoresis and
224 ethidium–bromide staining (Ballester, Lafuente, & González–Candelas, 2013). The
225 quality and concentration of total RNA were analyzed by gel electrophoresis and in a
226 spectrophotometer. DNase treatment and first–strand cDNA synthesis were conducted
227 **with the 'Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase' (Thermo
228 Scientific) using 2 μg of total RNA.**
229

230 *2.8. RT-qPCR expression analysis*

231 The gene expression analysis was carried out by following the MIQE guidelines. Gene–
232 specific primer sets were designed for the gene expression analysis with Primer3Plus
233 (Untergasser et al., 2012) (Table S1, Supplementary Material). A LightCycler480
234 System (Roche) was used with SYBR Green to monitor cDNA amplification. For each
235 primer pair and each sample, PCR efficiency (E) and the quantification cycle (Cq) were
236 assessed using version 2014.2 of the LinRegPCR software (Ruijter et al., 2009).
237 Amplicon specificity was examined by a melting curve analysis. The relative gene
238 expression of the target gene was calculated based on the E and Cq values of the target
239 and the reference genes, according to **the following equation: Etarget^(−Cqtarget)
240 /Eref^(−Cqref) (Pfaffl, 2001). The Cq value for the reference normalization factor was**
241 calculated by taking the geometric mean of the three *C. sinensis* reference genes:

242 *CsACT, CsEF1, and CsTUB*. Three independent biological replicates, with at least two
243 technical replicates, were performed for each sample.
244
245 *2.9. Statistics*
246 A one-way analysis of variance (ANOVA) was performed to test the effect of the
247 elicitor treatment. Means were separated using the LSD test at $p < 0.05$. The analysis
248 was performed with the Statgraphics Plus 4.0 Software (Manugistics, Inc.).
249
250 3. Results
251 *3.1. Effect of LBL on phenolic profiling and on ethylene production of citrus fruits*
252 To determine whether LBL may induce changes in phenylpropanoid metabolism in the
253 flavedo of citrus fruit, the effect of increasing LBL doses on phenolics profiling and
254 content was examined. Fruits were treated at the 70, 210 and 630 μmol $m^{-2}s^{-1}$ quantum
255 fluxes for 3 and 18 h. Phenolics were determined at the end of each treatment, and also
256 at 3 dpt to know whether this elapsed time could favor or decrease the synthesis of
257 phenolics, which might affect the efficacy of LBL to elicit resistance against *P.*
258 *digitatum.*
259 By using PDA and FD detectors, we found that LBL did not induce relevant
260 changes at either 0 or 3 dpt in the phenolic profiling in the flavedo of fruits when treated
261 for 3 or 18 h with the lowest selected quantum flux (70 μmol $m^{-2}s^{-1}$; data not shown).
262 Treating fruits with the highest quantum flux (630 μmol $m^{-2}s^{-1}$) also had no effect on the
263 phenolic profiling in the samples analyzed immediately after finishing the LBL
264 treatment. However, this treatment modified the profile at 3 dpt (Fig. 2A). At this time
265 point, no differences between the control and the LBL-treated samples were found in
266 the concentration of the most abundant flavonoid in the flavedo, the flavanone

267 hesperidin, or in other abundant flavanones, such as narirutin and didymin, nor in
268 flavones like isorhoifolin and diosmin. The flavedo also contained polymethoxylated
269 flavones (PMFs), including tangeretin, nobiletin, hexamethyl-O-quercetagetin,
270 sinensetin, tetramethyl-O-scutellarein and heptamethoxyflavone, which are found
271 almost exclusively in citrus fruit. Some display antifungal activity against fungi that are
272 able to infect citrus fruit (Ortuño et al., 2006), but the concentration of PMFs did not
273 change in response to this light treatment. In contrast, LBL induced an important
274 increase in the scoparone concentration (Fig. 2B), which has been related to resistance
275 to postharvest decay in citrus fruit (D'Hallewin, Schirra, Manueddu, Piga, & Ben
276 Yehoshua, 1999). This compound was identified by being compared with the spectra
277 and retention time of the commercial standard. Its qualitative identification in the
278 flavedo was previously performed in our group under the same HPLC experimental
279 conditions and with a HPLC-PDA-QTOF-MS system (Ballester, Lafuente, De Vos,
280 Bovy, & González-Candelas, 2013). The comparison of the phenolic profiling, using
281 PDA and FD (Fig. 2A), and the determination of the concentration of each separated
282 phenolic compound, indicated that this was the only phenolic compound to be
283 significantly induced by the treatment. This coumarin did not abound in the flavedo of
284 the fruits kept in the dark for 3 or 18 h, but increased by about 8-fold at 3 dpt in the
285 fruits treated for 18 h with the highest LBL quantum flux, compared to their control
286 sample maintained continuously in darkness (Fig. 2B). The scoparone concentration
287 also increased at 3 dpt when fruits were previously exposed for 18 h to the medium
288 LBL quantum flux. This increase was much less marked (c.a. 2-fold increase) (Fig. 2B)
289 than that induced by the highest quantum flux. Changes in phenolics were also analyzed
290 in the fruits treated with the same quantum fluxes for 3 h to ensure that no initial

291 transient increase occurred in response to light. The results showed that no significant
292 change was induced at either 0 or 3 dpt (data not shown).
293 The effect of different LBL quantum fluxes on ethylene production was
294 examined in the flavedo. As shown in Fig. 3, the medium and highest LBL quantum
295 fluxes were effective enough to significantly increase ethylene production. However, no
296 increase was induced by the lowest quantum flux.
297
298 *3.2. Induction of resistance in citrus fruit against P. digitatum by LBL*
299 Previous reports have shown that by applying 40 μmol $m^{-2}s^{-1}$ of LBL to citrus fruits
300 infected with *P. digitatum* reduces infection in fruits, although this quantum flux had
301 little effect on the mycelium growth and sporulation of the fungus *in vitro* (Liao,
302 Alferez, & Burns, 2013), and that the efficacy of LBL to control the *in vitro* growth of
303 different *P. digitatum* strains increases with the light quantum flux and treatment
304 duration (Lafuente & Alférez, 2015). The results of Yamaga, Takahashi, Ishii, Kato, and
305 Kobayashi (2015a) also suggest that LBL may induce resistance against *P. italicum* in
306 mandarins. However, no study has been performed in fruits treated with LBL before
307 being inoculated with *P. digitatum*. Therefore, in order to understand the mechanism
308 that underlies elicitation of resistance by LBL, the effect of different LBL regimes on
309 the resistance of citrus fruits against *P. digitatum* was tested by treating fruits with LBL
310 before inoculating fruits.
311 Different light regimes were assayed to assess whether the elicitor treatment
312 could be shortened by increasing the LBL quantum flux, and whether the elapsed time
313 between the LBL treatment and the ulterior infection was important in the elicited
314 resistance. The preliminary experiments suggested that, for the same light regime,
315 elicitation of resistance was higher at 3 than at 0 dpt (data not shown). Therefore, the

316 effect of the lowest and highest LBL quantum fluxes, applied for 3 h and 18 h, on
317 inhibiting fungal growth in sweet oranges inoculated with the fungus at 3 dpt, was first
318 compared. Treating fruits with the highest quantum flux may induce resistance against
319 *P. digitatum* in only 3 h, but the efficacy of this treatment was poor (Table S2,
320 Supplementary Material). Low inhibition (31%) was achieved at 7 dpi (days post–
321 inoculation), but no effect was observed at 4 dpi when the macerated zone started to
322 become evident. Increasing treatment duration until 18 h inhibited fungal growth by
323 about a 47% at 4 dpi, although fungal growth inhibition was very low by day 7 (21%).
324 As expected, treating fruits only for 3 h with the lowest LBL did not induce resistance.
325 However, elicitation of resistance was achieved when the LBL application was extended
326 to 3 days. Thus treating fruits for 3 days with 70 μmol $m^{-2}s^{-1}$ caused 90 % and 60 %
327 inhibition at 4 and 7 dpi, respectively (Table S2, Supplementary Material).

328 In a subsequent experiment, we also found that when treating fruits for 2 days
329 with 60 μmol $m^{-2}s^{-1}$, LBL was able to elicitate resistance. As shown in Fig. 4, the
330 treatment significantly reduced disease severity when fruits were inoculated
331 immediately after the treatment finished (0 dpt) and, as expected, this reduction was
332 even greater when fruits were inoculated at 3 dpt. Therefore, the flavedo samples from
333 the fruits treated in this experiment were taken and frozen, following the experimental
334 design shown in Fig. 1, to further study the potential involvement of ethylene and
335 phenolics in the elicited resistance.

336

337 3.3. Effect of the LBL elicitor treatment on ethylene and phenolics

338 To determine whether the beneficial effect of the LBL elicitor treatment was related to
339 phenolics and ethylene, we first determined changes in the expression of key genes
340 required for the synthesis of phenylpropanoids and ethylene in the frozen flavedo

341 samples, and also changes in the total phenolics and in the composition and
342 concentration of these compounds.

343 The results showed that LBL induced a sharp and transient initial increase in the
344 expression of the *CsPAL* gene (Fig. 5A). However, no differences were found between
345 the control and LBL–treated fruits by the end of the light treatment, nor after
346 transferring fruits to the dark. Compared to the control fruits kept in the dark, total
347 phenolic content was only significantly higher in the fruits treated for 12 h with LBL.
348 However, these differences were small and did not continue until the end of the light
349 treatment (0 dpt) or at 3 dpt (Fig. S1, Supplementary Material). This result agrees with
350 the fact that no relevant differences were found between the phenylpropanoid metabolic
351 profile of the control and the LBL–elicited fruits, as determined by PDA and FD (data
352 not shown).

353 The genes involved in ethylene biosynthesis that encode ACC (1–
354 aminocyclopropane–1–carboxylic acid) synthase (ACS), the immediate precursor of
355 ethylene, and ACC oxidase (ACO), which oxidizes ACC to ethylene, were differentially
356 regulated by LBL (Fig. 5). Light delayed the initial decline in the expression of *CsACO*,
357 which occurred by 4 h, but no relevant differences between the control and light–treated
358 samples were found thereafter (Fig. 5B). In contrast, LBL accelerated the decline in the
359 expression of *CsACS2* and did not affect *CsACS1* (Fig. 5C–D). After transferring fruits
360 to darkness (3 dpt, 120 h in Fig. 5), major differences between the LBL–treated and
361 control fruits were found in the expression of *CsACS1*.

362 Based on these results, changes in ethylene production were examined during
363 the LBL treatment, and after transferring the LBL–treated fruits for 3 days to darkness
364 (3 dpt) in two subsequent experiments. First, ethylene production of the fruits exposed
365 to the elicitor treatment was determined (Fig. 6A). The light had an initial effect on

366 delaying the drop in ethylene production, which occurred in the control fruits in only 4
367 h. Thereafter, the differences found between the elicited and the control fruits were
368 lost.Moreover, the ethylene production of the flavedo discs taken from both the LBL–
369 treated fruits and control fruits kept in the dark was compared when a major difference
370 in fruit ethylene production was found (4 h). As shown in the insert panel of Fig. 6A,
371 the ethylene production of the flavedo of the LBL–treated fruits was also higher than
372 that of the control fruits. Conversely in a subsequent experiment, no significant
373 difference was found between the ethylene production of the flavedo of the LBL–treated
374 and the control fruits (Fig. 6B). Although the initial effect of light on ethylene
375 production differed in both experiments, the LBL treatment was always effective at
376 eliciting resistance (data not shown). So even though hormone levels may increase in
377 response to LBL in citrus fruit, it appears that ethylene does not play an important role
378 in LBL–induced resistance against *P. digitatum*.
379

380 4. Discussion

381 Given the beneficial effects of phenolics on several human diseases, interest in studying
382 these compounds on plants and fruits has increased (Tripoli, Guardia, Giammanco,
383 Majo, & Giammanco, 2007). These compounds are also relevant in eliciting resistance
384 against pathogenic fungi in citrus fruits (Ballester, Lafuente, De Vos, Bovy, &
385 González–Candelas, 2013). Studies that characterize how phenolic composition is
386 affected by pre– and postharvest conditions in horticultural crops, including citrus fruits,
387 have been conducted (Del Caro, Piga, Vacca, & Agabbio, 2004; Ballester, Lafuente, De
388 Vos, Bovy, & González–Candelas, 2013). Yet despite previous knowledge having
389 suggested that LBL may elicit resistance against *P. digitatum* and *P. italicum* in citrus
390 fruits (Liao, Alferez, & Burns, 2013; Yamaga, Takahashi, Ishii, Kato, & Kobayashi,

391 2015b), and showing that LBL may increase PAL activity in plants (Engelsma, 1974),
392 the effect of LBL on both phenolic compounds and the possible involvement of
393 metabolites from this pathway in LBL–elicited resistance in this fruit crop remains
394 unknown.

395 The results presented herein indicate that the concentration of the phytoalexin
396 scoparone increases with the LBL light quantum flux applied (Fig. 2B). This increase
397 was observed in the flavedo when the medium (210 μmol $m^{-2}s^{-1}$) and the highest (630
398 μmol $m^{-2}s^{-1}$) quantum fluxes were applied for at least 18 h, but only at 3 dpt. Therefore,
399 blue light is able to activate phenylpropanoid metabolism in citrus fruit peel, but a 3–
400 days period after light treatment may be necessary to increase the concentration of this
401 metabolite. No increase in total phenolics, flavonoids, which are the most abundant
402 phenolic compounds in the flavedo of blond sweet oranges (Ballester, Lafuente, &
403 González–Candelas, 2013), or in scoparone, was induced by exposing fruits for at least
404 2 days to a lower LBL quantum flux (60 μmol $m^{-2}s^{-1}$) in spite of the initial (4 h)
405 transient induction in the *CsPAL* gene expression (Fig. 5A). This result suggests that
406 such a transient response does not suffice to increase the concentration of relevant
407 metabolites from the phenylpropanoid pathway under conditions that elicit resistance
408 against *P. digitatum* in citrus fruit. Likewise, our results indicate that, although the
409 enzyme PAL and scoparone have been linked to the elicitation of resistance in citrus
410 fruit peel against *P. digitaum* (Ballester, Lafuente, De Vos, Bovy, & González–
411 Candelas, 2013), they are not critical factors in LBL–induced resistance. In fact the
412 selected elicitor treatment did not increase scoparone levels. However, treating fruits for
413 18 h at the highest LBL quantum flux was less effective at eliciting resistance, and
414 increased the phytoalexin concentration by about 8–fold. In contrast, both PAL and
415 scoparone have been related to UV–C–induced resistance in this fruit crop (D'Hallewin,

416 Schirra, Manueddu, Piga, & Ben Yehoshua, 1999). We might think that this difference
417 is related to the fact that UV is more energetic than blue light given its shorter
418 wavelength. However, differences in the sensitivity and responses of distinct plants or
419 fungi species to light of distinct wavelengths have also been related to the different
420 sensitivity of light receptors (Ensminger & Schäfer, 1992). As scoparone increased
421 mainly in response to the very high LBL intensity applied for 18 h (Fig. 2B), and only at
422 3 dpt, we cannot rule out the idea that the increase in scoparone may reflect oxidative
423 stress in citrus fruit peel exposed to excess light. It is well-known that: 1) excess light
424 may cause oxidative stress and affect the mitochondrial electron transport chain system
425 (Li, Wakao, Fischer, & Niyogi, 2009); 2) at very high intensities, blue light can
426 photochemically destroy photopigments and some other molecules, which then act as
427 free radicals and can cause oxidative damage (Jourdan et al., 2015); 3) scoparone has a
428 suppressive effect on reactive oxygen species and protects the mitochondrial electron
429 transport chain system (Lee & Jang, 2015). Hence these results suggest that although
430 LBL is able to induce scoparone in citrus fruit, this coumarin does not play a critical
431 role in LBL-induced resistance against *P. digitatum* in citrus fruits. They also indicate
432 that flavonoids and other phenolics are not relevant in this process.
433 In line with this idea, our findings show that ethylene production rapidly
434 increases in citrus fruit peel in response to the strongest LBL intensity (Fig. 3), but
435 might not increase while eliciting resistance when applying a lower quantum flux (Fig.
436 6B). Therefore, the rise in ethylene could be a stress response, at least in part. Along
437 these lines, previous work by our group have indicated that ethylene production
438 increases in response to abiotic stresses in non climacteric citrus fruit, and revealed the
439 link between the rise in ethylene production and oxidative stress in this fruit crop

440 (Establés–Ortiz, Romero, Ballester, González–Candelas, & Lafuente, 2016; Lafuente,
441 Zacarías, Martínez–Téllez, Sánchez–Ballesta, & Dupille, 2001).

442 The results of the present work also show that the key genes involved in
443 ethylene biosynthesis (*CsACS1, CsACS2* and *CsACO*) are differentially regulated by
444 LBL during resistance elicitation (Fig. 5) and that the LBL quantum flux selected for
445 elicitation may delay the decline in ethylene production that occurs after harvesting fruit
446 (Fig. 6A). A comparison of the results is shown in Fig. 5 and 6A, and indicates that this
447 effect on ethylene might be related mostly to changes in the expression of the *CsACO*
448 gene, whose expression was much higher than that of the *CsACS1* and *CsACS2* genes.
449 No increase in ethylene was observed after transferring fruits to darkness for 3 days and
450 despite the rise in the *CsACS1* gene expression. Nevertheless, the expression of this
451 gene was very low. Our results also reveal that the low LBL quantum flux used in the
452 selected elicitor treatment induces few changes in ethylene production (Fig. 6 A and B),
453 and that the initial differences found between the light–treated and control fruits may not
454 occur in spite of the efficacy of the LBL–treatment. Such differences in the ethylene
455 production pattern (Fig. 6A and 6B) might be related to the influence of pre–harvest
456 factors. Therefore, high LBL levels may increase ethylene production in citrus fruits,
457 but we should rule out the possibility that this hormone plays a key role in triggering the
458 defense responses involved in the LBL–induced resistance against *P. digitatum* in citrus
459 fruit.

460 By way of conclusion, LBL is able to increase the scoparone concentration and
461 ethylene production in the flavedo of citrus fruits. However, ethylene and
462 phenylpropanoids, including scoparone, are not critical factors in the LBL–elicited
463 response.

464

465 Acknowledgements

466 Special thanks are due to Dr. L. González–Candelas for providing the *Penicillium*
467 *digitatum* strain and to Dr. Alférez for helpful comments. The technical assistance of M.
468 Lledi is gratefully acknowledged. This work was supported by the Spanish Ministry of
469 Economy and Competitiveness (Research Grants AGL2013–41734–R and AGL2014–
470 55802–R) and the Generalitat Valenciana, Spain (Grant PROMETEOII/2014/027).

471

472 Appendix. Supplementary Material

473 Figure S1. Changes in the total phenolics in the flavedo of fruits treated up to 2 days
474 with 60 µmol $m^{-2}s^{-1}$ LBL (0 dpt) and then transferred for 3 days to darkness (3 dpt) (O).
475 Control samples (●) continuously remained in the dark. Values are the means of three
476 replicates±SEM. Asterisks indicate a significant difference ($p \leq 0.05$) for the same
477 analysis day.

478 Table S1. Primers designed for the gene expression analyses by RT–qPCR.

479

480 Table S2. Effect of LBL on the inhibition of fungal growth in oranges inoculated at 3
481 dpt. Values were recorded at 4 and 7 days post–inoculation (dpi).

482

483 References

484 Alferez, F., Liao, H.-L., & Burns, J. K. (2012). Blue light alters infection by *Penicillium*
485 *digitatum* in tangerines. *Postharvest Biology and Technology, 63*(1), 11–15.
486 Ballester, A. R., Lafuente, M. T., De Vos, C. H. R., Bovy, A., & González-Candelas, L.
487 (2013). Citrus phenylpropanoids and defense against pathogens. Part I:
488 Metabolic profiling in elicited fruits. *Food Chemistry, 136*, 178–185.
489 Ballester, A. R., Lafuente, M. T., & González-Candelas, L. (2013). Citrus
490 phenylpropanoids and defense against pathogens. Part II: Gene expression and
491 metabolite accumulation in the response of fruits to *Penicillium digitatum*
492 infection. *Food Chemistry, 136*(1), 285–291.
493 Corbineau, F., Rudnicki, R., Goszczyńska, D., & Come, D. (1995). The effect of light
494 quality on ethylene production in leaves of oat seedlings (*Avena sativa* L.).
495 *Environmental and Experimental Botany, 35*(2), 227–233.
496 D'Hallewin, G., Schirra, M., Manueddu, E., Piga, A., & Ben Yehoshua, S. (1999).
497 Scoparone and scopoletin accumulation and ultraviolet-C induced resistance to
498 postharvest decay in oranges as influenced by harvest date. *Journal of the*
499 *American Society for Horticultural Science, 124*(6), 702–707.
500 Dai, T., Gupta, A., Huang, Y.-Y., Yin, R., Murray, C. K., Vrahas, M. S., et al. (2013).
501 Blue light rescues mice from potentially fatal *Pseudomonas aeruginosa* burn
502 infection: efficacy, safety, and mechanism of action. *Antimicrobial Agents and*
503 *Chemotherapy, 57*(3), 1238–1245.
504 Del Caro, A., Piga, A., Vacca, V., & Agabbio, M. (2004). Changes of flavonoids,
505 vitamin C and antioxidant capacity in minimally processed citrus segments and
506 juices during storage. *Food Chemistry, 84*(1), 99–105.
507 Droby, S., Chalutz, E., Horev, B., Cohen, L., Gaba, V., Wilson, C. L., et al. (1993).
508 Factors affecting UV-induced resistance in grapefruit against the green mold
509 decay caused by *Penicillium digitatum. Plant Pathology, 42*(3), 418–424.
510 Droby, S., Wisniewski, M., Macarisin, D., & Wilson, C. (2009). Twenty years of
511 postharvest biocontrol research: is it time for a new paradigm? *Postharvest*
512 *Biology and Technology, 52*(2), 137–145.
513 Engelsma, G. (1974). On the mechanism of the changes in phenylalanine ammonia-
514 lyase activity induced by ultraviolet and blue light in gherkin hypocotyls. *Plant*
515 *Physiology, 54*(5), 702–705.
516 Ensminger, P. A., & Schäfer, E. (1992). Blue and ultraviolet B light photoreceptors in
517 parsley cells. *Photochemistry and Photobiology, 55*(3), 437–447.
518 Establés-Ortiz, B., Romero, P., Ballester, A.-R., González-Candelas, L., & Lafuente, M.
519 T. (2016). Inhibiting ethylene perception with 1-methylcyclopropene triggers
520 molecular responses aimed to cope with cell toxicity and increased respiration in
521 citrus fruits. *Plant Physiology and Biochemistry, 103*, 154–166.
522 Fadda, A., Barberis, A., D'Aquino, S., Palma, A., Angioni, A., Lai, F., et al. (2015).
523 Residue levels and performance of potassium sorbate and thiabendazole and
524 their co-application against blue mold of apples when applied as water dip
525 treatments at 20 or 53° C. *Postharvest Biology and Technology, 106*, 33–43.
526 Folta, K. M., & Childers, K. S. (2008). Light as a growth regulator: controlling plant
527 biology with narrow-bandwidth solid-state lighting systems. *Hortscience, 43*(7),
528 1957–1964.
529 Gong, D., Cao, S., Sheng, T., Shao, J., Song, C., Wo, F., et al. (2015). Effect of blue
530 light on ethylene biosynthesis, signalling and fruit ripening in postharvest
531 peaches. *Scientia Horticulturae, 197*, 657–664.

532 Gonzalez-Candelas, L., Alamar, S., Sanchez-Torres, P., Zacarias, L., & Marcos, J.
533 (2010). A transcriptomic approach highlights induction of secondary metabolism
534 in citrus fruit in response to *Penicillium digitatum* infection. *BMC Plant Biology,*
535 *10*(1), 194–211.
536 Jourdan, N., F Martino, C., El-Esawi, M., Witczak, J., Bouchet, P.-E., d'Harlingue, A.,
537 et al. (2015). Blue-light dependent ROS formation by Arabidopsis
538 cryptochrome-2 may contribute toward its signaling role. *Plant Signaling &*
539 *Behavior, 10*(8), e1042647.
540 Lafuente, M. T., & Alférez, F. (2015). Effect of LED Blue light on *Penicillium*
541 *digitatum* and *Penicillium italicum* strains. *Photochemistry and Photobiology*.
542 Lafuente, M. T., Alférez, F., & Romero, P. (2014). Postharvest ethylene conditioning as
543 a tool to reduce quality loss of stored mature sweet oranges. *Postharvest Biology*
544 *and Technology, 94*, 104–111.
545 Lafuente, M. T., Zacarías, L., Martínez-Téllez, M. A., Sánchez-Ballesta, M. T., &
546 Dupille, E. (2001). Phenylalanine ammonia-lyase as related to ethylene in the
547 development of chilling symptoms during cold storage of citrus fruits. *Journal*
548 *of Agricultural and Food Chemistry, 49*(12), 6020–6025.
549 Lee, S.-H., & Jang, H.-D. (2015). Scoparone attenuates RANKL-induced osteoclastic
550 differentiation through controlling reactive oxygen species production and
551 scavenging. *Experimental Cell Research, 331*(2), 267–277.
552 Li, Z., Wakao, S., Fischer, B. B., & Niyogi, K. K. (2009). Sensing and responding to
553 excess light. *Annual Review of Plant Biology, 60*, 239–260.
554 Liao, H.-L., Alferez, F., & Burns, J. K. (2013). Assessment of blue light treatments on
555 citrus postharvest diseases. *Postharvest Biology and Technology, 81*(0), 81–88.
556 Marcos, J. F., González-Candelas, L., & Zacarías, L. (2005). Involvement of ethylene
557 biosynthesis and perception in the susceptibility of citrus fruit to *Penicillium*
558 *digitatum* infection and the accumulation of defense-related mRNAs. *Journal of*
559 *Experimental Botany, 56*(148), 2183–2193.
560 Montesinos-Herrero, C., Smilanick, J. L., Tebbets, J. S., Walse, S., & Palou, L. (2011).
561 Control of citrus postharvest decay by ammonia gas fumigation and its influence
562 on the efficacy of the fungicide imazalil. *Postharvest Biology and Technology,*
563 *59*(1), 85–93.
564 Ondrusch, N., & Kreft, J. (2011). Blue and red light modulates SigB-dependent gene
565 transcription, swimming motility and invasiveness in *Listeria monocytogenes*.
566 *PLoS ONE, 6*(1), e16151.
567 Ortuño, A., Báidez, A., Gómez, P., Arcas, M. C., Porras, I., García-Lidón, A., et al.
568 (2006). *Citrus paradisi* and *Citrus sinensis* flavonoids: Their influence in the
569 defence mechanism against *Penicillium digitatum*. *Food Chemistry, 98*, 351–
570 358.
571 Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time
572 RT-PCR. *Nucleic Acids Research, 29*(9), e45.
573 Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den
574 Hoff, M. J. B., et al. (2009). Amplification efficiency: linking baseline and bias
575 in the analysis of quantitative PCR data. *Nucleic Acids Research, 37*(6), e45.
576 Sánchez-Torres, P., & Tuset, J. J. (2011). Molecular insights into fungicide resistance in
577 sensitive and resistant *Penicillium digitatum* strains infecting citrus. *Postharvest*
578 *Biology and Technology, 59*(2), 159–165.
579 Schmidt-Heydt, M., Rüfer, C., Raupp, F., Bruchmann, A., Perrone, G., & Geisen, R.
580 (2011). Influence of light on food relevant fungi with emphasis on ochratoxin

581 producing species. *International Journal of Food Microbiology, 145*(1), 229–
582 237.
583 Tripoli, E., Guardia, M. L., Giammanco, S., Majo, D. D., & Giammanco, M. (2007).
584 Citrus flavonoids: Molecular structure, biological activity and nutritional
585 properties: A review. *Food Chemistry, 104*(2), 466–479.
586 Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al.
587 (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Research,*
588 *40*(15), e115.
589 Yamaga, I., Takahashi, T., Ishii, K., Kato, M., & Kobayashi, Y. (2015a). Antifungal
590 effect of blue LED irradiation on the blue mold, *Penicillium italicum*, in satsuma
591 mandarin fruits. *Horticultural Research (Japan), 14*(1), 83–87.
592 Yamaga, I., Takahashi, T., Ishii, K., Kato, M., & Kobayashi, Y. (2015b). Suppression of
593 blue mold symptom development in satsuma mandarin fruits treated by low–
594 intensity blue LED irradiation. *Food Science and Technology Research, 21*(3),
595 347–351.
596

597 Figure Captions

598 Figure 1. Schematic diagram of the experimental design. Samples were always taken
599 from fruits that were not inoculated with the fungus. Fruits were infected only to
600 determine the effect of blue light (450 nm) on *P. digitatum* infection and light was
601 always applied prior to infecting fruits.

602

603 Figure 2. Phenolic profiling in the flavedo of the fruits kept at 20 ºC and treated for 18
604 h with 630 µmol $m^{-2}s^{-1}$ LBL and then transferred to darkness for 3 d (A); changes in
605 scoparone in the flavedo of the fruits treated with different quantum fluxes for 18 h and
606 then transferred to darkness for 3 d (B). Phenolic profiling was determined by using
607 PDA and FD detectors and scoparone quantified with the FD. Values are the means of
608 three replicates±SEM.

609

610 Figure 3. Ethylene production of the flavedo discs treated at 20 ºC with 70, 210 and 630
611 µmol $m^{-2}s^{-1}$ LBL for 1 h (gray bars) compared to the control fruits (0 µmol $m^{-2}s^{-1}$) that
612 remained continuously in the dark at the same temperature (black bar). Values are the
613 means of three replicates±SEM. Different letters mean a significant difference at $p \leq$
614 0.05.

615

616 Figure 4. Changes in the diameter of the macerated area of the fruits treated for 2 days
617 with 60 µmol $m^{-2}s^{-1}$ LBL (0 dpt) (O) and then transferred for 3 more days to darkness
618 (3 dpt). Control fruits (●) remained continuously in the darkness. Fruits were infected at
619 both 0 and 3 dpt. Values are the means of three replicates±SEM. Significant differences
620 ($p \leq 0.05$) between the light–treated and control fruits for the same analysis day were
621 found from day 4.

622

623 Figure 5. Changes in the expression of the *CsPAL*, *CsACO*, *CsACS1*, and *CsACS2* 624 genes in the flavedo of the fruits treated for 2 days with 60 µmol $m^{-2}s^{-1}$ LBL (0 dpt) and 625 then transferred to darkness for 3 days (3 dpt) (O). The control fruits (●) were 626 continuously kept in the dark. Values are the means of three replicates±SEM. The 627 asterisks for the same analysis day mean a significant difference at $p \leq 0.05$.

628

629 Figure 6. Changes in the ethylene production of both fruits (A) and flavedo discs (B) of 630 the fruits treated for 2 days with 60 µmol $m^{-2}s^{-1}$ LBL and then transferred to darkness 631 for 3 days (O). The control samples (●) were continuously kept in the dark. The data of 632 Fig. 6A and 6B correspond to independent experiments. The insert panel represents the 633 ethylene production of the flavedo taken from the same fruits and exposed to light or 634 darkness for 3 h. Values are the means of three replicates±SEM. Asterisks indicate a 635 significant difference ($p \leq 0.05$) for the same analysis day.

636

Figure 1
Click here to download Figure(s): Fig. 1.docx

Fig. 1

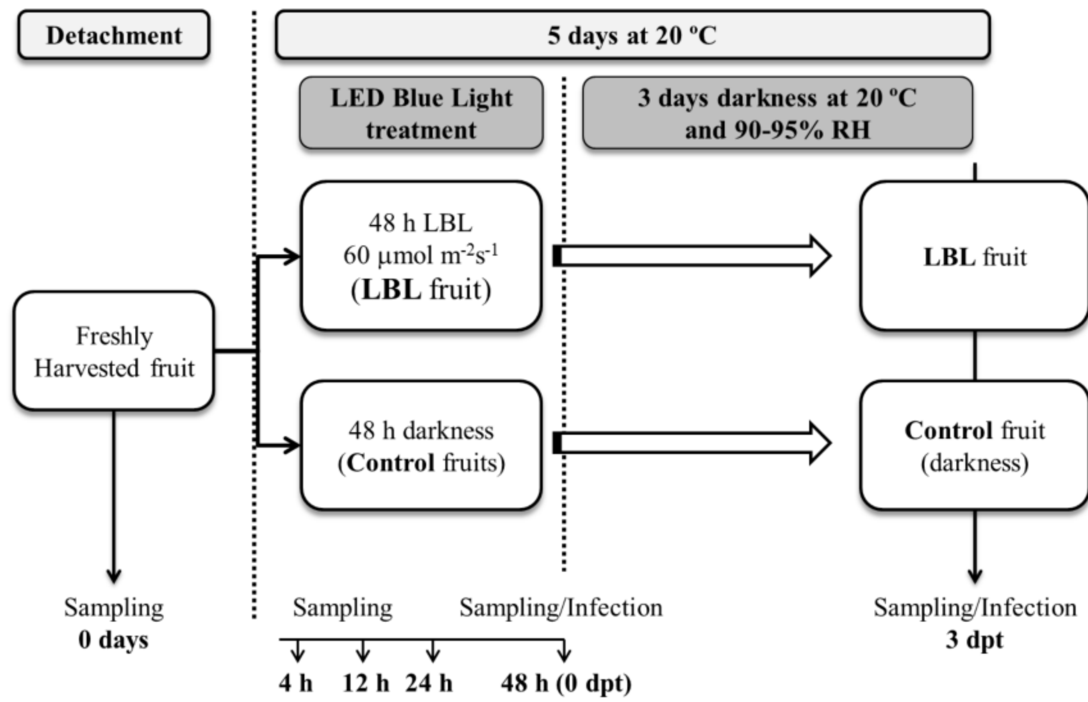
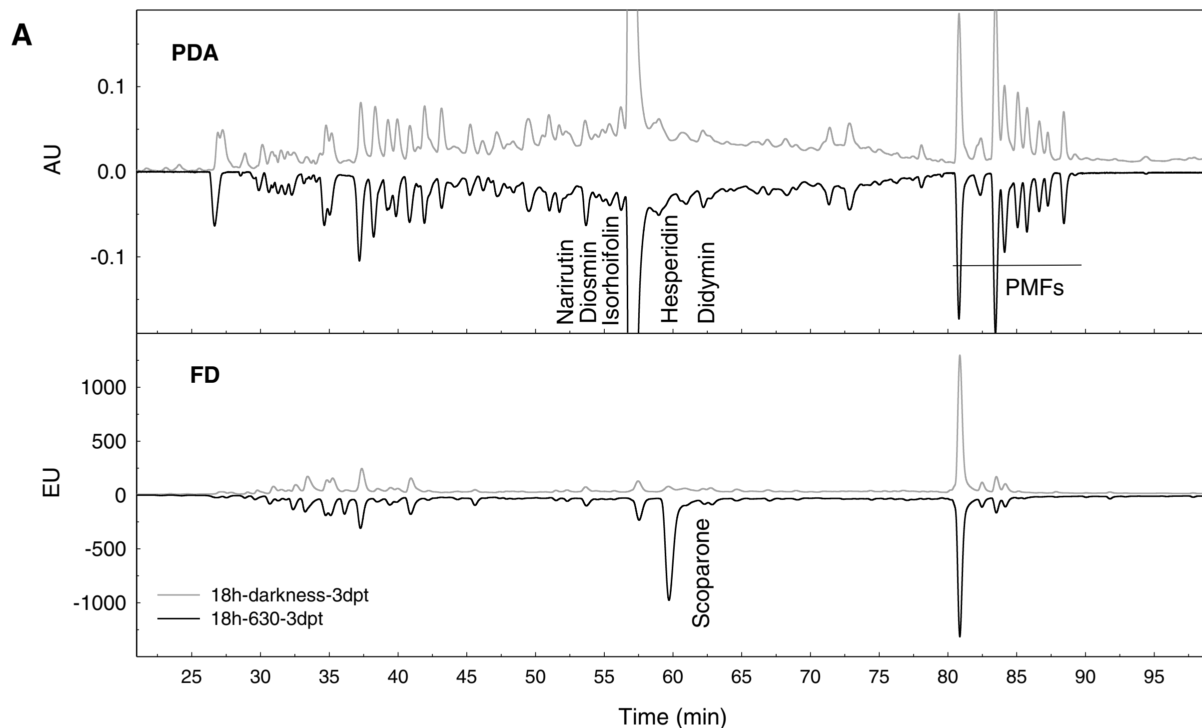


Figure 2
Click here to download Figure(s): Fig. 2.docx



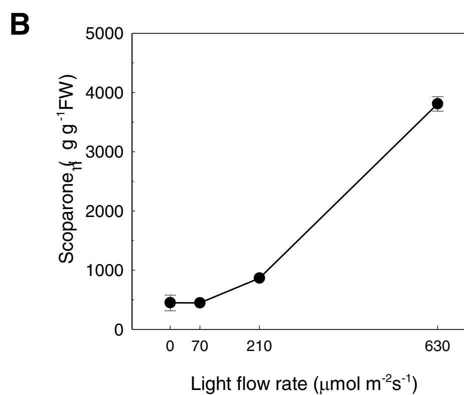


Fig. 2. Phenolic profiling in the flavedo of fruits held at 20 ºC and treated for 18 h with 630 µmol $m^{-2}seg^{-1}$ LBL and then transferred 3 d to darkness (A); and changes in scoparone in the flavedo of fruits treated with different quantum fluxes for 18 h and then transferred 3 d to darkness (B). Phenolic profiling was determined by using PDA and FD detectors and scoparone quantified using the FD. Values are means of three replicates ± SEM

Figure 3

Click here to download Figure(s): Fig. 3.docx

Fig. 3

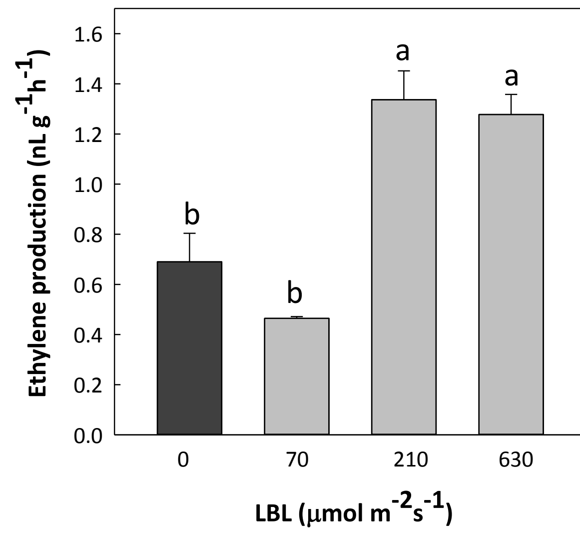


Figure 4
Click here to download Figure(s): Fig. 4.docx

Fig. 4

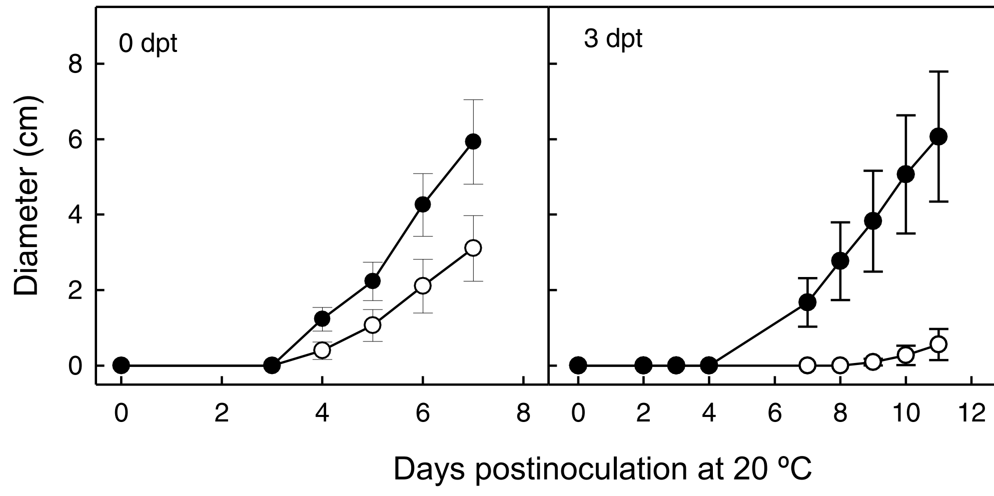


Figure 5

Click here to download Figure(s): Fig. 5.docx

Fig. 5

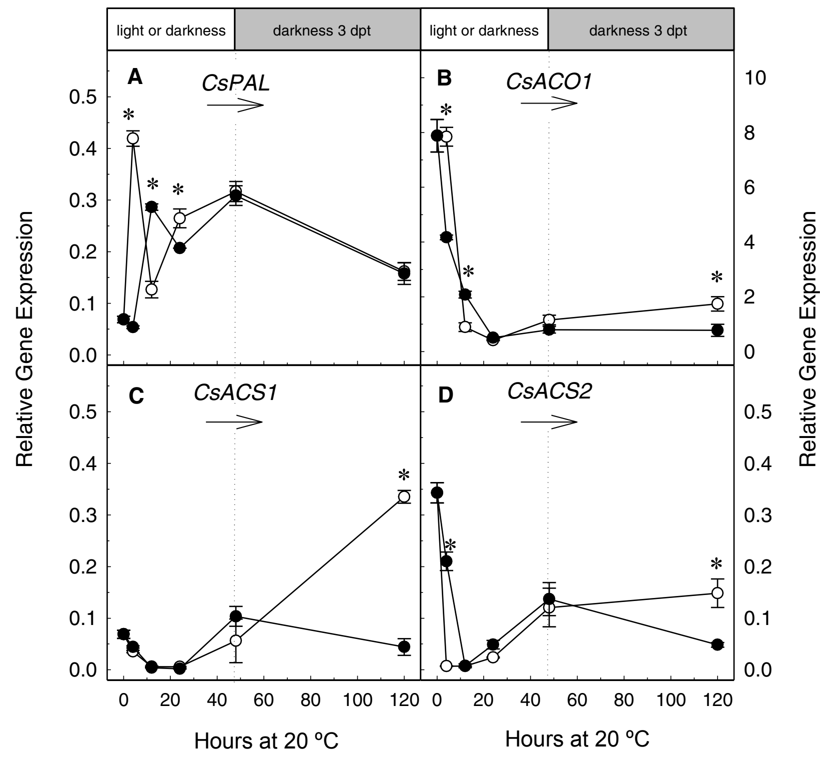
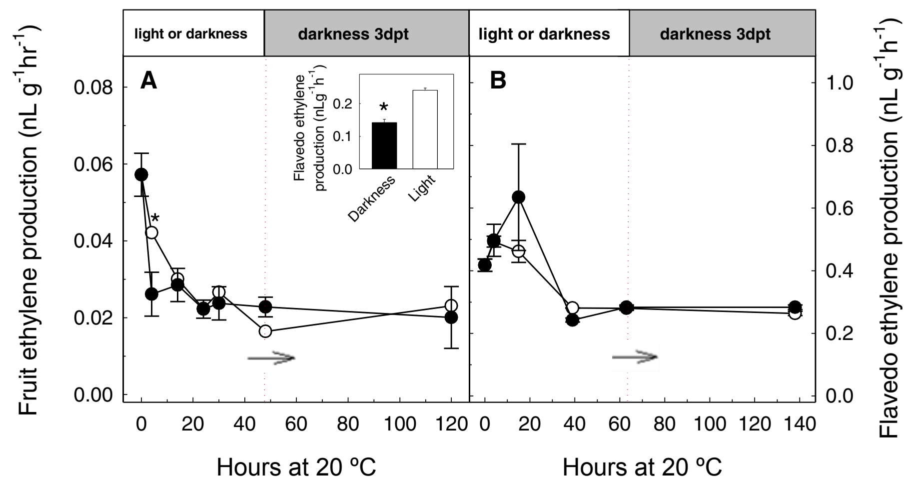


Figure 6

Click here to download Figure(s): Fig .6.docx

Fig. 6



TableS1

Click here to download Supplementary Material: Table S1 vert.docx

TableS2

Click here to download Supplementary Material: TableS2-inhibition growth.docx