



LED Blue Light-induced changes in phenolics and ethylene in citrus fruit: Implication in elicited resistance against *Penicillium digitatum* infection



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ABSTRACT

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

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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 et al., 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 et al., 2013; Droby, Wisniewski, Macarasin, & Wilson, 2009; Droby et al., 1993; 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

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

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

2. Materials and methods

2.1. Fruit and fungal material

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

In order to test the efficacy of LBL on reducing disease in citrus fruits, oranges were infected with *P. digitatum* (Pers.:Fr.) Sacc. isolate Pd1 (CECT 20795), deposited in the Spanish Type Culture Collection (CECT), and obtained from oranges with typical green mold collected from different orchards or packinghouses. This strain is highly resistant to the two fungicides used in citrus fruit: thiabendazole and imazalil. The strain was grown for 7 days at 24 °C on Potato Dextrose Agar medium before use. Conidia were rubbed from the agar surface by scraping them with a sterile

spatula, and were transferred to 10 mL of sterile water. The resulting suspensions were filtered and the conidia concentration of the obtained filtrate was titrated with a hemacytometer and adjusted to 10^5 conidia mL⁻¹ with sterile water (Ballester et al., 2013). This suspension was then used to infect fruits to evaluate the efficacy of the LBL treatments to elicit resistance.

2.2. Blue light treatments and induced resistance

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

Different LBL regimens were assayed to select the most effective one to induce resistance against *P. digitatum*, and to determine how this treatment affected the phenolic profiling in the flavedo (outer colored part of the peel) and the ethylene production of citrus fruit. The effect of the selected treatment on changes in expression of the relevant genes of both the phenylpropanoid and ethylene biosynthetic pathways was also examined. In order to test whether ethylene and phenolics play important roles in LBL-induced resistance against *P. digitatum*, we determined the changes in these compounds at different time points during the light treatments and after 3 days (3 dpt, 3 days post-treatment). The experimental design outlined in Fig. 1 summarizes the experimental conditions of the selected treatment as well as sampling days. Samples were always taken from non inoculated fruits. Fruits were infected only to determine the efficacy of the light treatments to elicit resistance. The control and light-treated fruits were always infected immediately after finishing the light treatment (0 dpt) and 3 days after ending it (3 dpt). On these 3 days, both the elicited and control fruits were kept in the dark at 20 °C with 90–95% relative humidity (RH).

2.3. *P. digitatum* infection and decay evaluation

To determine the effectiveness of the LBL elicitor treatment to reduce pathogen infection and the importance of the time that elapsed between the treatment and the ulterior infection, disease susceptibility was evaluated in the fruits infected at 0 and 3 dpt (Fig. 1). Control samples, maintained for the same periods in the dark, were infected like the elicited fruits (Fig. 1). Each elicited and control fruit was pricked on the equatorial axis with a 2 mm (diameter) × 1 mm (depth) sterilized needle, equipped with a stopper to ensure uniformity of wounds. Then 10 μL of a 10^5 conidia mL⁻¹ suspension of *P. digitatum* spores were applied to each wound. After inoculation, fruits were stored at 20 °C with 90–95% RH.

To evaluate how light treatments could affect disease severity, the fruit macerated diameter (cm) was periodically determined

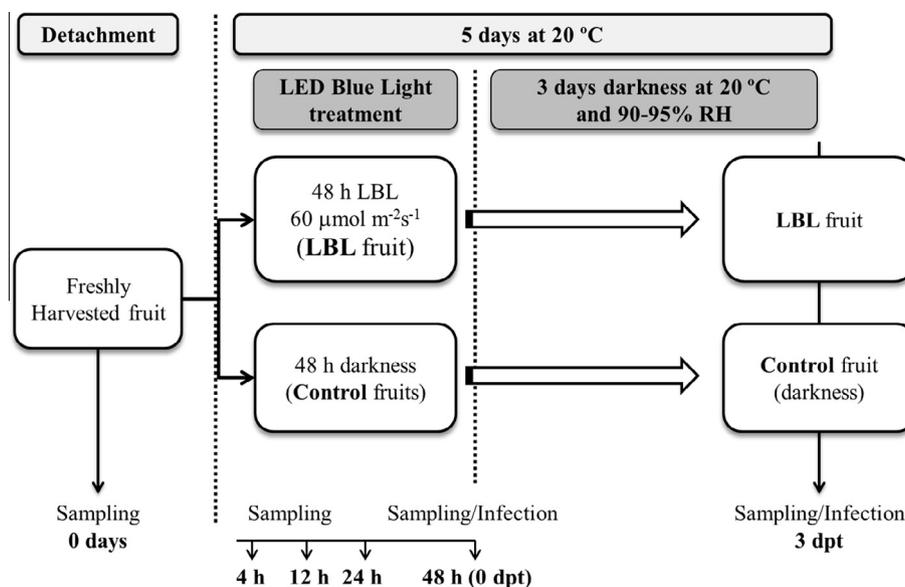


Fig. 1. Schematic diagram of the experimental design. Samples were always taken from fruits that were not inoculated with the fungus. Fruits were infected only to determine the effect of blue light (450 nm) on *P. digitatum* infection and light was always applied prior to infecting fruits.

with a flexible ruler in two directions during fruit incubation at 20 °C. The experimental design consisted of 3 replicates of 15 fruits, with 1 wound per fruit for each treatment. The efficacy of the selected LBL treatments was evaluated at 0 and 3 dpt. Therefore, four groups of fruit were prepared in this experiment; two were used as the control and light-treated samples for the infections done at 0 dpt, and the other two for the infections at 3 dpt. The control samples consisted of inoculated fruits, which were always maintained in the dark at the same temperature. The percentage of growth inhibition was also calculated using the following formula:

$$\text{Percentage of growth inhibition} = 100 \times (GC - GSL) / GC,$$

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

2.4. Analysis of total phenolics

Total phenolic content was determined as reported by Lafuente, Alférez, and Romero (2014). Briefly, 200 mg of the homogenized frozen flavedo were extracted with 1 mL of ethanol using a Mini Beadbeater 8 Cell Disruptor (Biospec Products, Inc.). The extract was centrifuged at 13,000g at 4 °C, and the phenolic content was estimated in the supernatant. Two sample aliquots of 20 μL were diluted with 80 μL ethanol and 400 μL nanopure water, and were incubated at room temperature with 500 μL of 1 N Folin-Ciocalteu and 5 mL of 2% Na_2CO_3 . After centrifugation at 13,000g at 4 °C, absorbance was determined at 724 nm, and total phenolic content was calculated by using a standard curve developed with chlorogenic acid. The results are the means of three replicate samples \pm SEM.

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

The phenolic compounds from flavedo were extracted as previously described (Ballester et al., 2013). Briefly, freeze-ground flavedo was extracted twice with 80% methanol and the chromatographic analyses of the extracts were performed in a Waters HPLC

system. The system was equipped with a 600 quaternary pump and fitted with a 717 autosampler and a 996 photodiode array detector (PDA), operated from 200 to 400 nm, and a fluorescence detector (FD) operated at the excitation and emission wavelengths of 313 nm and 405 nm, respectively. The FD detector better allows the changes in phenolics to be analyzed, which are less abundant than flavonoids, but have been related to the defense of citrus fruit against *P. digitatum* (Ballester et al., 2013). Separation was accomplished in a Luna C18 reverse column (250 \times 4.6 mm, 5 μm ; Phenomenex) coupled to a $\mu\text{Bondapak}$ C18 guard column (10 μm). Elution was performed by using a binary gradient elution of acetonitrile and water (pH 2.5) with a flow rate of 0.8 mL min^{-1} and an injection volume of 20 μL . Compound identification was based on the comparison made between the retention times and the spectrum obtained from the standards (see the section ‘Chemical compounds studied in this article’), and from the chromatographic signals in the samples run under the same experimental conditions. Peaks were integrated and phenolic content was calculated using calibration curves.

2.6. Ethylene production measurements

Ethylene production from whole fruits and from the flavedo discs (0.7 cm diameter) was measured periodically by incubating three replicate samples of fruits or discs in 1.5 L sealed glass jars for 3 h (for fruits) or in 8 mL tubes (for flavedo discs) for 1 h at 20 °C. Three oranges or six discs per replicate were used. The samples exposed to light at each sampling point were incubated under the same light quantum flux, while the samples kept in the dark were incubated in darkness. Two replicate samples of 1 mL gas sample were withdrawn from the head space of each container and injected into a gas chromatograph, equipped with an activated alumina column and a flame ionization detector, as previously described (Lafuente et al., 2001). The results are the means of three replicate samples \pm SEM.

2.7. RNA extraction and cDNA synthesis

Total RNA was isolated from flavedo tissue, its concentration was measured spectrophotometrically, and its integrity was

verified by agarose gel electrophoresis and ethidium-bromide staining (Ballester et al., 2013). The quality and concentration of total RNA were analyzed by gel electrophoresis and in a spectrophotometer. DNase treatment and first-strand cDNA synthesis were conducted with the 'Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase' (Thermo Scientific) using 2 µg of total RNA.

2.8. RT-qPCR expression analysis

The gene expression analysis was carried out by following the MIQE guidelines. Gene-specific primer sets were designed for the gene expression analysis with Primer3Plus (Untergasser et al., 2012) (Table S1, Supplementary Material). A LightCycler480 System (Roche) was used with SYBR Green to monitor cDNA amplification. For each primer pair and each sample, PCR efficiency (E) and the quantification cycle (Cq) were assessed using version 2014.2 of the LinRegPCR software (Ruijter et al., 2009). Amplicon specificity was examined by a melting curve analysis. The relative gene expression of the target gene was calculated based on the E and Cq values of the target and the reference genes, according to the following equation: $E_{\text{target}}^{-(Cq_{\text{target}})} / E_{\text{ref}}^{-(Cq_{\text{ref}})}$ (Pfaffl, 2001). The Cq value for the reference normalization factor was calculated by taking the geometric mean of the three *C. sinensis* reference genes: *CsACT*, *CsEF1*, and *CsTUB*. Three independent biological replicates, with at least two technical replicates, were performed for each sample.

2.9. Statistics

A one-way analysis of variance (ANOVA) was performed to test the effect of the elicitor treatment. Means were separated using the LSD test at $p < 0.05$. The analysis was performed with the Statgraphics Plus 4.0 Software (Manugistics, Inc.).

3. Results

3.1. Effect of LBL on phenolic profiling and on ethylene production of citrus fruits

To determine whether LBL may induce changes in phenylpropanoid metabolism in the flavedo of citrus fruit, the effect of increasing LBL doses on phenolics profiling and content was examined. Fruits were treated at the 70, 210 and 630 µmol m⁻² s⁻¹ quantum fluxes for 3 and 18 h. Phenolics were determined at the end of each treatment, and also at 3 dpt to know whether this elapsed time could favor or decrease the synthesis of phenolics, which might affect the efficacy of LBL to elicit resistance against *P. digitatum*.

By using PDA and FD detectors, we found that LBL did not induce relevant changes at either 0 or 3 dpt in the phenolic profiling in the flavedo of fruits when treated for 3 or 18 h with the lowest selected quantum flux (70 µmol m⁻² s⁻¹; data not shown). Treating fruits with the highest quantum flux (630 µmol m⁻² s⁻¹) also had no effect on the phenolic profiling in the samples analyzed immediately after finishing the LBL treatment. However, this treatment modified the profile at 3 dpt (Fig. 2A). At this time point, no differences between the control and the LBL-treated samples were found in the concentration of the most abundant flavonoid in the flavedo, the flavanone hesperidin, or in other abundant flavanones, such as narirutin and didymin, nor in flavones like isorhoifolin and diosmin. The flavedo also contained polymethoxylated flavones (PMFs), including tangeretin, nobiletin, hexamethyl-O-queretagenin, sinensetin, tetramethyl-O-scutellarein and heptamethoxyflavone, which are found almost exclusively in citrus fruit. Some display

antifungal activity against fungi that are able to infect citrus fruit (Ortuño et al., 2006), but the concentration of PMFs did not change in response to this light treatment. In contrast, LBL induced an important increase in the scoparone concentration (Fig. 2B), which has been related to resistance to postharvest decay in citrus fruit (D'Hallewin et al., 1999). This compound was identified by being compared with the spectra and retention time of the commercial standard. Its qualitative identification in the flavedo was previously performed in our group under the same HPLC experimental conditions and with a HPLC-PDA-QTOF-MS system (Ballester et al., 2013). The comparison of the phenolic profiling, using PDA and FD (Fig. 2A), and the determination of the concentration of each separated phenolic compound, indicated that this was the only phenolic compound to be significantly induced by the treatment. This coumarin did not abound in the flavedo of the fruits kept in the dark for 3 or 18 h, but increased by about 8-fold at 3 dpt in the fruits treated for 18 h with the highest LBL quantum flux, compared to their control sample maintained continuously in darkness (Fig. 2B). The scoparone concentration also increased at 3 dpt when fruits were previously exposed for 18 h to the medium LBL quantum flux. This increase was much less marked (c.a. 2-fold increase) (Fig. 2B) than that induced by the highest quantum flux. Changes in phenolics were also analyzed in the fruits treated with the same quantum fluxes for 3 h to ensure that no initial transient increase occurred in response to light. The results showed that no significant change was induced at either 0 or 3 dpt (data not shown).

The effect of different LBL quantum fluxes on ethylene production was examined in the flavedo. As shown in Fig. 3, the medium and highest LBL quantum fluxes were effective enough to significantly increase ethylene production. However, no increase was induced by the lowest quantum flux.

3.2. Induction of resistance in citrus fruit against *P. digitatum* by LBL

Previous reports have shown that by applying 40 µmol m⁻² s⁻¹ of LBL to citrus fruits infected with *P. digitatum* reduces infection in fruits, although this quantum flux had little effect on the mycelium growth and sporulation of the fungus *in vitro* (Liao et al., 2013), and that the efficacy of LBL to control the *in vitro* growth of different *P. digitatum* strains increases with the light quantum flux and treatment duration (Lafuente & Alferez, 2015). The results of Yamaga, Takahashi, Ishii, Kato, and Kobayashi (2015a) also suggest that LBL may induce resistance against *P. italicum* in mandarins. However, no study has been performed in fruits treated with LBL before being inoculated with *P. digitatum*. Therefore, in order to understand the mechanism that underlies elicitation of resistance by LBL, the effect of different LBL regimes on the resistance of citrus fruits against *P. digitatum* was tested by treating fruits with LBL before inoculating fruits.

Different light regimes were assayed to assess whether the elicitor treatment could be shortened by increasing the LBL quantum flux, and whether the elapsed time between the LBL treatment and the ulterior infection was important in the elicited resistance. The preliminary experiments suggested that, for the same light regime, elicitation of resistance was higher at 3 than at 0 dpt (data not shown). Therefore, the effect of the lowest and highest LBL quantum fluxes, applied for 3 h and 18 h, on inhibiting fungal growth in sweet oranges inoculated with the fungus at 3 dpt, was first compared. Treating fruits with the highest quantum flux may induce resistance against *P. digitatum* in only 3 h, but the efficacy of this treatment was poor (Table S2, Supplementary Material). Low inhibition (31%) was achieved at 7 dpi (days post-inoculation), but no effect was observed at 4 dpi when the macerated zone started to become evident. Increasing treatment duration until 18 h inhibited fungal growth by about a 47% at 4 dpi, although fungal growth inhibition was very low by day 7

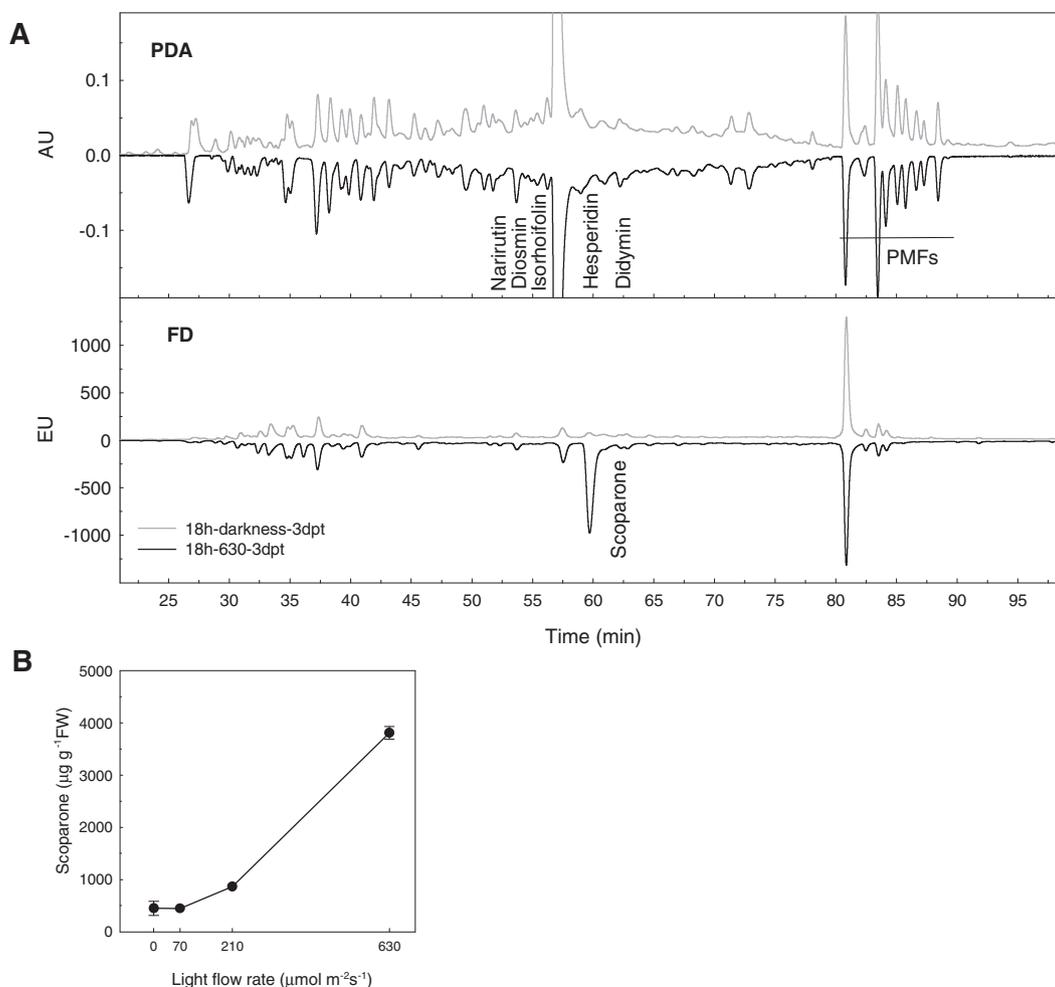


Fig. 2. Phenolic profiling in the flavedo of the fruits kept at 20 °C and treated for 18 h with 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$ LBL and then transferred to darkness for 3 d (A); changes in scoparone in the flavedo of the fruits treated with different quantum fluxes for 18 h and then transferred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

(21%). As expected, treating fruits only for 3 h with the lowest LBL did not induce resistance. However, elicitation of resistance was

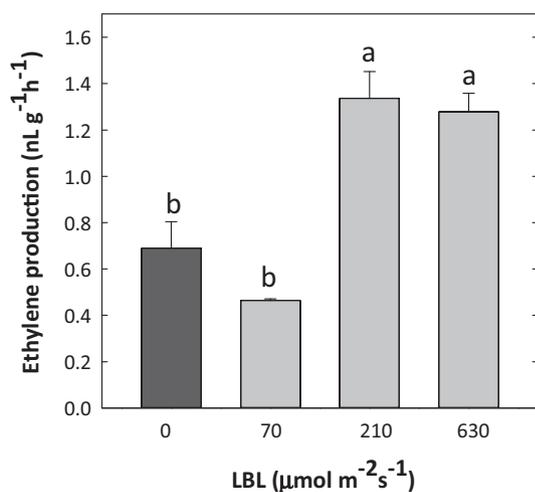


Fig. 3. Ethylene production of the flavedo discs treated at 20 °C with 70, 210 and 630 $\mu\text{mol m}^{-2}\text{s}^{-1}$ LBL for 1 h (gray bars) compared to the control fruits (0 $\mu\text{mol m}^{-2}\text{s}^{-1}$) that remained continuously in the dark at the same temperature (black bar). Values are the means of three replicates \pm SEM. Different letters mean a significant difference at $p \leq 0.05$.

achieved when the LBL application was extended to 3 days. Thus treating fruits for 3 days with 70 $\mu\text{mol m}^{-2}\text{s}^{-1}$ caused 90% and 60% inhibition at 4 and 7 dpi, respectively (Table S2, Supplementary Material).

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

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

To determine whether the beneficial effect of the LBL elicitor treatment was related to phenolics and ethylene, we first determined changes in the expression of key genes required for the synthesis of phenylpropanoids and ethylene in the frozen flavedo samples, and also changes in the total phenolics and in the composition and concentration of these compounds.

The results showed that LBL induced a sharp and transient initial increase in the expression of the *CsPAL* gene (Fig. 5A).

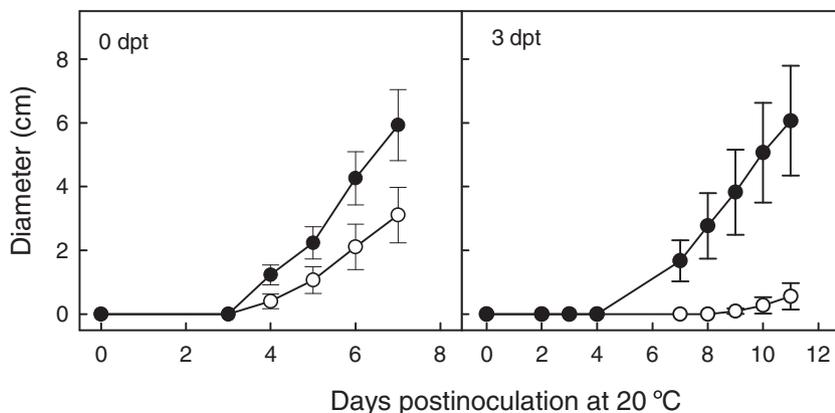


Fig. 4. Changes in the diameter of the macerated area of the fruits treated for 2 days with $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ LBL (0 dpt) (○) and then transferred for 3 more days to darkness (3 dpt). Control fruits (●) remained continuously in the darkness. Fruits were infected at both 0 and 3 dpt. Values are the means of three replicates \pm SEM. Significant differences ($p \leq 0.05$) between the light-treated and control fruits for the same analysis day were found from day 4.

However, no differences were found between the control and LBL-treated fruits by the end of the light treatment, nor after transferring fruits to the dark. Compared to the control fruits kept in the dark, total phenolic content was only significantly higher in the fruits treated for 12 h with LBL. However, these differences were small and did not continue until the end of the light treatment (0 dpt) or at 3 dpt (Fig. S1, Supplementary Material). This result agrees with the fact that no relevant differences were found between the phenylpropanoid metabolic profile of the control and the LBL-elicited fruits, as determined by PDA and FD (data not shown).

The genes involved in ethylene biosynthesis that encode ACC (1-aminocyclopropane-1-carboxylic acid) synthase (ACS), the

immediate precursor of ethylene, and ACC oxidase (ACO), which oxidizes ACC to ethylene, were differentially regulated by LBL (Fig. 5). Light delayed the initial decline in the expression of *CsACO*, which occurred by 4 h, but no relevant differences between the control and light-treated samples were found thereafter (Fig. 5B). In contrast, LBL accelerated the decline in the expression of *CsACS2* and did not affect *CsACS1* (Fig. 5C and D). After transferring fruits to darkness (3 dpt, 120 h in Fig. 5), major differences between the LBL-treated and control fruits were found in the expression of *CsACS1*.

Based on these results, changes in ethylene production were examined during the LBL treatment, and after transferring the LBL-treated fruits for 3 days to darkness (3 dpt) in two subsequent

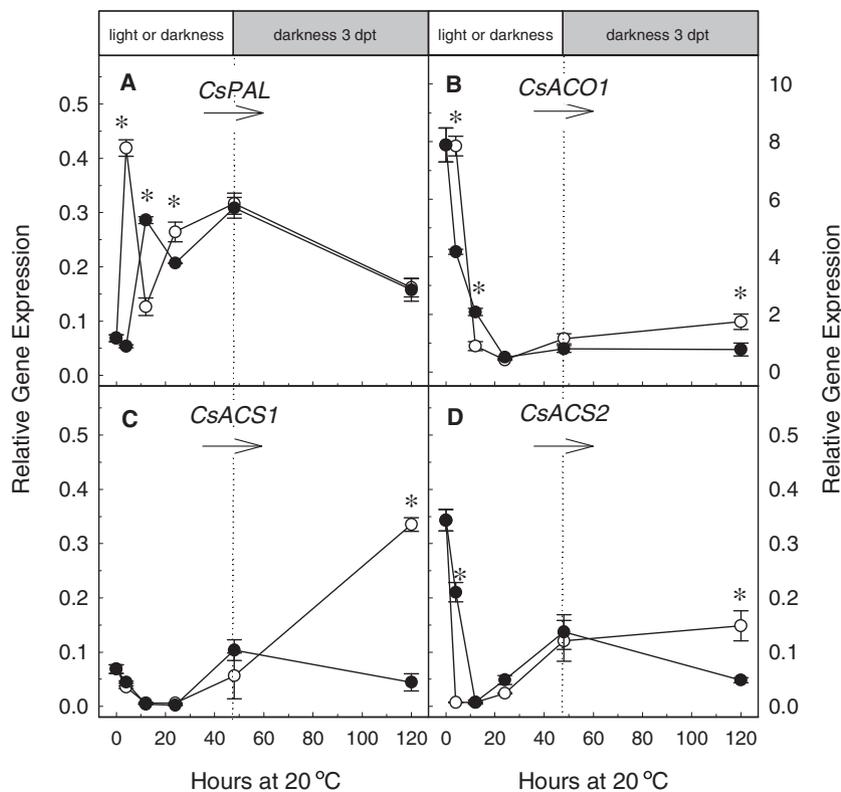


Fig. 5. Changes in the expression of the *CsPAL*, *CsACO*, *CsACS1*, and *CsACS2* genes in the flavedo of the fruits treated for 2 days with $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ LBL (0 dpt) and then transferred to darkness for 3 days (3 dpt) (○). The control fruits (●) were continuously kept in the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

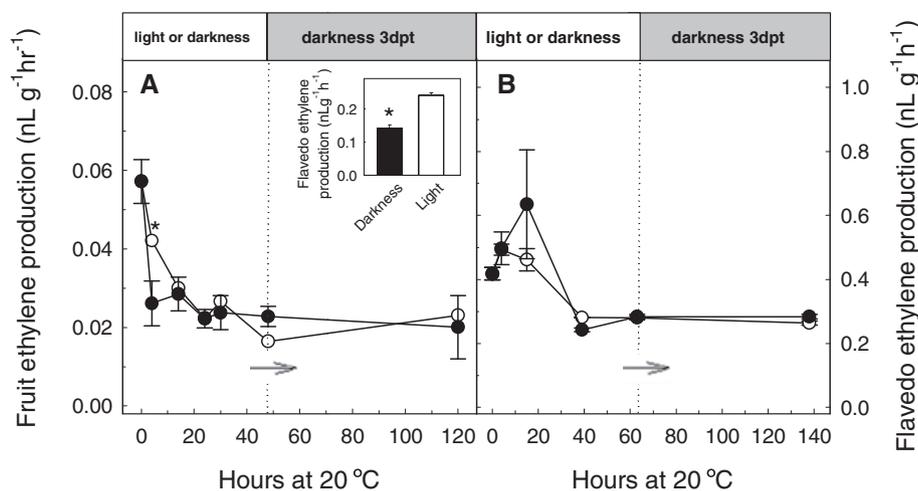


Fig. 6. Changes in the ethylene production of both fruits (A) and flavedo discs (B) of the fruits treated for 2 days with $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ LBL and then transferred to darkness for 3 days (○). The control samples (●) were continuously kept in the dark. The data of figure A and B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

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

4. Discussion

Given the beneficial effects of phenolics on several human diseases, interest in studying these compounds on plants and fruits has increased (Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007). These compounds are also relevant in eliciting resistance against pathogenic fungi in citrus fruits (Ballester et al., 2013). Studies that characterize how phenolic composition is affected by pre- and postharvest conditions in horticultural crops, including citrus fruits, have been conducted (Ballester et al., 2013; Del Caro, Piga, Vacca, & Agabbio, 2004). Yet despite previous knowledge having suggested that LBL may elicit resistance against *P. digitatum* and *P. italicum* in citrus fruits (Liao et al., 2013; Yamaga et al., 2015b), and showing that LBL may increase PAL activity in plants (Engelsma, 1974), the effect of LBL on both phenolic compounds and the possible involvement of metabolites from this pathway in LBL-elicited resistance in this fruit crop remains unknown.

The results presented herein indicate that the concentration of the phytoalexin scoparone increases with the LBL light quantum flux applied (Fig. 2B). This increase was observed in the flavedo when the medium ($210 \mu\text{mol m}^{-2} \text{s}^{-1}$) and the highest

($630 \mu\text{mol m}^{-2} \text{s}^{-1}$) quantum fluxes were applied for at least 18 h, but only at 3 dpt. Therefore, blue light is able to activate phenylpropanoid metabolism in citrus fruit peel, but a 3-days period after light treatment may be necessary to increase the concentration of this metabolite. No increase in total phenolics, flavonoids, which are the most abundant phenolic compounds in the flavedo of blond sweet oranges (Ballester et al., 2013), or in scoparone, was induced by exposing fruits for at least 2 days to a lower LBL quantum flux ($60 \mu\text{mol m}^{-2} \text{s}^{-1}$) in spite of the initial (4 h) transient induction in the *CsPAL* gene expression (Fig. 5A). This result suggests that such a transient response does not suffice to increase the concentration of relevant metabolites from the phenylpropanoid pathway under conditions that elicit resistance against *P. digitatum* in citrus fruit. Likewise, our results indicate that, although the enzyme PAL and scoparone have been linked to the elicitation of resistance in citrus fruit peel against *P. digitatum* (Ballester et al., 2013), they are not critical factors in LBL-induced resistance. In fact the selected elicitor treatment did not increase scoparone levels. However, treating fruits for 18 h at the highest LBL quantum flux was less effective at eliciting resistance, and increased the phytoalexin concentration by about 8-fold. In contrast, both PAL and scoparone have been related to UV-C-induced resistance in this fruit crop (D'Hallewin et al., 1999). We might think that this difference is related to the fact that UV is more energetic than blue light given its shorter wavelength. However, differences in the sensitivity and responses of distinct plants or fungi species to light of distinct wavelengths have also been related to the different sensitivity of light receptors (Ensminger & Schäfer, 1992). As scoparone increased mainly in response to the very high LBL intensity applied for 18 h (Fig. 2B), and only at 3 dpt, we cannot rule out the idea that the increase in scoparone may reflect oxidative stress in citrus fruit peel exposed to excess light. It is well-known that: 1) excess light may cause oxidative stress and affect the mitochondrial electron transport chain system (Li, Wakao, Fischer, & Niyogi, 2009); 2) at very high intensities, blue light can photochemically destroy photopigments and some other molecules, which then act as free radicals and can cause oxidative damage (Jourdan et al., 2015); 3) scoparone has a suppressive effect on reactive oxygen species and protects the mitochondrial electron transport chain system (Lee & Jang, 2015). Hence these results suggest that although LBL is able to induce scoparone in citrus fruit, this coumarin does not play a critical role

in LBL-induced resistance against *P. digitatum* in citrus fruits. They also indicate that flavonoids and other phenolics are not relevant in this process.

In line with this idea, our findings show that ethylene production rapidly increases in citrus fruit peel in response to the strongest LBL intensity (Fig. 3), but might not increase while eliciting resistance when applying a lower quantum flux (Fig. 6B). Therefore, the rise in ethylene could be a stress response, at least in part. Along these lines, previous work by our group have indicated that ethylene production increases in response to abiotic stresses in non climacteric citrus fruit, and revealed the link between the rise in ethylene production and oxidative stress in this fruit crop (Establés-Ortiz, Romero, Ballester, González-Candelas, & Lafuente, 2016; Lafuente et al., 2001).

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

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

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Polymethoxylated flavones (PMFs) hexamethyl-O-gossypetin (3',4',3,5,7,8-hexamethoxyflavone), hexamethyl-O-quercetagenin (3',4',3,5,6,7-hexamethoxyflavone), tetramethyl-O-scutellarein (4',5,6,7-tetramethoxyflavone) and heptamethoxyflavone (3',4',3,5,6,7,8-heptmethoxyflavone) were kindly supplied by Dr. J.M. Sendra (IATA-CSIC, Valencia, Spain).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.09.089>.

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