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Effect of LED Blue Light on *Penicillium digitatum* and *Penicillium italicum* Strains

María T. Lafuente*, Fernando Alférez[1]

Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC). Consejo Superior de Investigaciones Científicas. Av. Agustín Escardino, 7. 46980, Paterna-Valencia, Spain

***Corresponding autor e-mail :** mtlafuente@iata.csic.es (María T. Lafuente)

[1]Current address: Citrus Research and Education Center. Institute of Agricultural and Food Sciences, University of Florida. 700 Experiment Station Rd, 3850 Lake Alfred, Fl, USA.

ABSTRACT

Studies on the antimicrobial properties of light have considerably increased due in part to the development of resistance to actual control methods. This study investigates the potential of Light Emitting Diodes (LED) blue light for controlling *Penicillium digitatum* and *Penicillium italicum*. These fungi are the most devastating postharvest pathogens of citrus fruit and cause important losses due to contaminations and to the development of resistant strains against fungicides. The effect of different periods and quantum fluxes and of delaying light application on the growth and morphology of *P.digitatum* strains resistant and sensitive to fungicides and of *P. italicum* cultured at 20 ºC was examined. Results showed that blue light controls the growth of all strains and that its efficacy increases with the quantum flux. Spore germination was always avoided by exposing the cultures to high quantum flux (700 $\mu molm^{-2}s^{-1}$) for 18 h. Continuous light had an important impact on the fungus morphology and a fungicidal effect when applied at a lower quantum flux (120 $\mu molm^{-2}s^{-1}$) to a growing fungus. Sensitivity to light increased with mycelium age. Results show that blue light may be a tool for *P. digitatum* and *P. italicum* infection prevention during handling of citrus fruits.

INTRODUCTION

Citrus fruits are subjected to various postharvest diseases that cause important economic losses. These losses are mostly due to pathogen contaminations in different strategic points in the packing-houses, such as drenchers, dip tanks, and cold storage rooms. Therefore, reducing inoculum of pathogens and maintaining good water quality in postharvest facilities is critical to reduce the incidence of decay. *Penicillium digitatum* (Pers.:Fr.) Sacc. (green mold) and *P. italicum* (blue mold) are the major pathogens of citrus fruits after harvesting. Green mold is most commonly responsible for postharvest decay of citrus, contributing up to 90 % of total losses (1). The use of fungicides constitutes the most common method to control postharvest diseases in citrus fruits. At present, *P. digitatum* and *P. italicum* are controlled by synthetic fungicides. However, due to the growing concern about human health and the environment, the use of chemicals is becoming increasingly restricted and there is a trend to develop alternative methods to control postharvest diseases (2-6). In the case of citrus fruits, the search of new strategies is also relevant because of the increase of fungicide resistant strains (1) and also because of low residue tolerance, disposal of used fungicide solutions in packinghouses, chemical costs and development of organic marketing programs (7). Induction of natural resistance in the fruit by using physical and chemical treatments (8-12) and biocontrol agents (3,13) is one of these alternatives. Nevertheless, the efficacy of these treatments is variable and is lower than that of synthetic fungicides. Therefore, appropriate infection prevention and measures for the reduction of pathogens contamination during postharvest handling and storage of citrus fruits is critical to control disease and reduce the use of fungicides.

The antimicrobial properties of light is a research area of increasing interest, not least due to the emergence of resistance to a number of other control methods including the use

of pesticides, disinfectants and antibiotics (14-18). Different reports have shown the potential of UV-treatments for reducing postharvest decay caused by different fungi (2,5,10,19,20). However, UV light has limitations because of its detrimental effects to workers, such as injury to the skin or eye on direct exposure. Lighting based on Light Emitting Diodes (LEDs) is one of the main emerging technologies in agriculture. In contrast with other illumination systems, LEDs possess features making them unique for industrial applications: higher irradiation with less heat dissipation, making easier control of humidity and temperature in closed environments such as storage rooms, easy installation and lower energy costs. Besides, a narrower bandwidth in the light spectrum achieved by this technology allows better control of the amount of light beam actually exciting photoreceptors in cells (21). Studies on the potential of LED blue light on different processes of agronomic interest has considerably increased during the last decade (21). Moreover, it has been shown that LED blue light may control food relevant fungi (22) and other pathogens that are harmful for consumers, such as *Listeria monocygenes* (23), that has caused important problems derived from fresh fruit contaminations during current postharvest practices (24). Such contaminations can occur in different strategic points in the packing-houses, such as drenchers, dip tanks or hydrocooling systems. Therefore, this technology might constitute an appropriate strategy to reduce not only postharvest decay caused by fungi but also the development of pathogens that can be harmful for consumers during postharvest handling of horticultural crops. Nevertheless, the potential of LED blue light to avoid losses of perishable crops during postharvest handling is almost unknown. In fact, to our knowledge, only two postharvest studies have been performed until now that suggest the industrial relevance of LED blue light reducing postharvest citrus fruit losses. On one hand, it has been shown that exposure of citrus fruit to this light emerging

technology, at a quantum flux of 40 µmolm^{-2}s^{-1} reduces infection by *P.digitatum* (25,26) although this quantum flux did not induce any change in mycelium growth and sporulation of the fungus *in vitro*. This finding indicated that blue light is able to induce resistance against *P.digitatum* in citrus fruit. On the other hand, Liao et al. (2013) showed that a quantum flux treatment of 120 µmolm^{-2}s^{-1} reduced *in vitro* fungal development. This result suggests, therefore, the potential of blue light for controlling mycelial growth and/or inhibiting conidia production of *P. digitatum* and that the efficacy of this physical treatment may increase with the quantum flux. Considering these results, that sanitation of packing- and store-houses is essential to reduce postharvest decay (11) and the high abundance of *P. digitatum* strains resistant to the two fungicides (thiabendazole (TBZ) and imazalil) most commonly used to control decay in citrus fruit (1), the aim of this study has been to investigate the potential of LED blue light for controlling sensitive and resistant strains of *P. digitatum* and by *P. italicum* during postharvest handling of citrus fruits. To that end, we have evaluated the effect of increasing blue light quantum flux and also time of exposure to such radiations on *in vitro P. digitatum* and *P. italicum* development and morphology. Conidial suspensions of three strains from *P.digitatum,* two of them resistant to fungicides, and one from *P. italicum* cultured on potato-dextrose agar (PDA) were used. Furthermore, as the effectiveness of chemical and physical treatments on controlling postharvest disease may vary if the treatment is applied after spore germination (27), the efficacy of the most effective blue light treatments was evaluated at different periods after inoculating the PDA plates with the conidial suspensions.

MATERIALS AND METHODS

Collection of *P. digitatum* and *P. italicum* isolates: *P. digitatum* and *P. italicum* strains used in this study were provided by Dr. González-Candelas and isolated from citrus fruit with typical green or blue mold collected from different orchards or packing-houses. Most strains are deposited in the Spanish Type Culture Collection (CECT). Petri plates containing 20 ml sterile PDA (39 g l^{-1}) were inoculated with *P. digitatum* (Pers.:Fr.) Sacc. isolates PHI-26 (CECT 20796), which is sensitive to TBZ and imazalil, and Pd1(CECT 20795) and PHI-41, resistant to both fungicides, and with *P. italicum* isolate PHI-I (CECT 20909) and incubated at 20 ºC for at least 7 days (8). Conidia from each isolate were rubbed from the agar surface by scrapping them with a sterile spatula and transferred to 10 ml of sterile water. The resulting conidial suspensions were filtered and the concentration titrated with a hemacytometer and adjusted to the desired final concentration (8).

Infection and evaluation of fungal growth: Petri plates of 9 cm diameter were inoculated in triplicate for each light treatment and pathogen strain with 5µl of 10^6 conidia ml^{-1}. To determine the effectiveness of the LED blue light treatments reducing fungal growth, petri plates were treated with different light regimes: 1) immediately after being inoculated (0 days post-inoculation, 0dpi), 2) at 1 and/or 2dpi, after germination of spores begun, and 3) at 4 dpi, when the mycelium of each strain was abundant. Temperature was maintained constant at 20 ºC, both during the light treatments and during fungal growth in the plates under darkness, and monitored with an electronic datalogger. To evaluate how the light treatments may affect fungal growth, colonies diameters were measured from the reverse side in cm with a ruler in two directions along the time at 20 ºC for at least 7 days. For each experimental condition, three colonies were evaluated. Control samples consisted of inoculated plates maintained always under darkness at the same temperature.

126 The percentage of growth inhibition was also calculated using the following formula:

127 Percentage of growth inhibition = 100 x (GC-GSL)/GC,

128 where GC is growth of the control (continuous darkness) and GSL is the growth of the

129 fungal colony of the sample exposed to the light treatment (28).

130

131 **Degree of germination:** The degree of germination was determined in three replicate

132 samples using a Neubauer chamber. The number of germinated (already showing a germ

133 tube) and ungerminated spores were counted and the degree of germination was expressed

134 as the percentage of germinated spores. A total of 100 spores were counted in each replicate

135 sample.

136

137 **Blue light treatments:** To test whether the effectiveness of blue light in reducing the

138 pathogens' growth infective capacities may depend on both light quantum flux and time of

139 exposure to light, inoculated plates were exposed to light quantum fluxes ranging from 30

140 to 700 $\mu molm^{-2}s^{-1}$ during periods ranging between 5 min and 3 days. The first experiment

141 was performed with the Pd1 strain, since *P. digitatum* is the major pathogen of citrus fruits

142 and this strain is highly resistant to the fungicides TBZ and imazalil. The matrix blue light

143 quantum flux/exposure time to light first ranged between 30 and 120 $\mu molm^{-2}s^{-1}$ and

144 between 5 min and 6 h. On the basis of the results obtained, we further tested the effect of

145 applying 120 $\mu molm^{-2}s^{-1}$ for 6, 24 and 72 h using the Pd1 and the PHI-41 strains, both

146 resistant to imazalil and TBZ. Moreover, we investigated the effect of the most effective

147 treatment on the infective capacity of the strain Pd1 when the light was applied at different

148 dpi (0, 1, 2 and 4), and whether the effectiveness of the treatments was maintained after

149 removing the light stimulus. Finally, we studied whether light treatment duration may be

reduced by sharply increasing quantum flux to 700 μmolm^{-2}s^{-1}. Under such light regime, the effectiveness of time periods equal or lower than 24 h was tested and the effect of the selected treatment on fungal growth and morphology of the 4 strains was studied at 0 and 4 dpi.

Petri dishes were treated with the lid covers to avoid contaminations under the different light regimes described above in Mammoth Pro dark growth tents (60 x 60 x 160 cm) (Mammoth Pro 60, Eltac Hidrofarm, Spain) equipped with velcro-sealable ventilation panels (300mm x 200mm) and tough fabric lined with 95% reflective mylar to assure uniform light quantum flux. The light source was a LumiGrow Pro 650^{TM} LED array (LumiGrow, Novato, CA, USA) that emitted blue light at a center wavelength of 450 nm with a full width at half-maximum of 20 nm.. Light quantum flux was measured and adjusted using a spectroradiometer (GL Spectics, Sttutgart, Germany). The growth tents had sufficient capacity for air exchange and were placed in a temperature and humidity controlled room.

Statistical analysis: A mean comparison using the Tukey's test was performed to determine if means values were significantly different ($P \leq 0.05$) between blue light-treated and control samples (constant darkness) for each experimental light regime. The results were the means of three replicated samples ± S.E.M.

RESULTS AND DISCUSSION

Effect of LED blue light on growth of *P. digitatum* strains resistant to fungicides

Recently, it has been reported that LED blue light at a quantum flux of 40 $\mu molm^{-2}s^{-1}$ induces resistance against *P. digitatum* in citrus fruit (25) and also that blue light reduced fungal development when *P. digitatum* culture was exposed to a higher quantum flux (120 $\mu molm^{-2}s^{-1}$) (26). Therefore, we hypothesized that the effect of blue light inhibiting fungal growth may increase with light quantum flux and that the duration of light treatment may be shortened by increasing the quatum flux. Moreover, an interesting possibility would be that blue light would even have a fungicidal effect that could be used in the packing-houses as a mean of sanitation. This would be especially relevant since decay in citrus fruit is mainly controlled by two fungicides and strains resistant to them are abundant. To test such hypotheses, we first examined whether the growth of the *P. digitatum* Pd1 strain, resistant to TBZ and imazalil, was affected by exposing the cultured plates from 5 min to 6 h to light quantum fluxes ranging between 30 and 120 $\mu molm^{-2}s^{-1}$. Pd1 fungal growth was only slightly reduced when the plates were treated with a quantum flux of 120 $\mu molm^{-2}s^{-1}$ for 6 h (see Supplementary Materials Fig. S1), and such reduction was not statistically significant. Therefore, in a subsequent experiment, this light treatment was extended for 72 h.

The effectiveness of the 120 $\mu molm^{-2}s^{-1}$ treatment applied for 6, 24 and 72 h was tested for Pd1 and also for PHI-41, which is another *P. digitatum* strain resistant to both fungicides. Results confirmed that exposing the plates for 6 h to this quantum flux barely reduced fungal growth of both pathogens and showed that the efficacy of the light treatment may be increased by extending the time of exposure to blue light (Fig. 1). By day 7, the efficacy of the 72 h treatment inhibiting fungal growth of the PHI-41 strain was c.a. 3-fold higher than that of the 24 h treatment (Fig. 1). Results also showed that differences afforded by the 72 h treatment inhibiting fungal growth of the Pd1 and the PHI-41 strains were initially low, and such differences were lost as time progressed (Fig. 1). Therefore, results

197 indicate that susceptibility to blue light of strains from the same fungus genus may be
198 similar, while the effect of blue light varied when applying to different food relevant fungi
199 (22) or to various *Penicillium* fungal species that cause postharvest decay in citrus fruit
200 (26). Since the 72 h light treatment was only partially effective controlling fungal growth,
201 the question was whether increasing light exposure or quantum flux would be able to
202 control *Penicillium* growth and if such control might be even achieved if the light is applied
203 at different dpi.
204
205 **Effect of delaying LED blue light application on growth and morphology of *P.***
206 ***digitatum***
207 Considering the points highlighted above, we first focused on studying the effect of
208 exposing continuously, or for 3 days, the *P. digitatum* culture to the 120 $\mu molm^{-2}s^{-1}$ blue
209 light treatment at different dpi. The experiment was performed using the Pd1 strain. At 0
210 dpi, Pd1 fungal growth inhibition was about 45 % after 3 days, when the plates were
211 continuously incubated under light (Fig. 2, 0 dpi). The diameter of the Petri plates used was
212 of 9 cm. Therefore, this was the maximum fungal growth detected in the control plates
213 continuously maintained under darkness. This maximum was reached by day 11 in control
214 plates under dark conditions, but the experiment was prolonged to know whether this light
215 quantum flux may have a fungistatic effect although it was not possible to monitor growth
216 in control plates after that day. As shown in this graph (Fig.2, 0 dpi), by day 9, Pd1 fungal
217 growth was about 2-fold lower in the cultures held continuously under light than in those
218 transferred from light to darkness (non-continuous light). The above result reinforces the
219 idea that the efficacy of the treatment increases with its duration for the same light quantum
220 flux, and further suggests that *P. digitatum* fungal growth might be stalled by increasing

exposure to blue light radiation for a given dose. Therefore, an interesting possibility is that blue light might exert a fungistatic effect on *P. digitatum* if appropriate light quantum flux is applied. This result is in concordance with data indicating that increasing radiation intensity may in some cases maximize the benefits of UV-C on reducing decay caused by *Botrytis* (20). However, mechanisms by which blue light may have a fungistatic effect on *Penicillium* or other postharvest pathogens should be at least in part different. In this regard it has to be considered that differences in sensitivity and responses of different fungi or plants species to light of different wavelengths might be related to the different sensitivity of light receptors (22, 29-31). The relevance of cryptochromes in the responses of plants and fungi to blue light has been described (31, 33). However, to date it has not been shown that cryptochromes can perceive UV-B or UV-C irradiations (34).

Delay in application of postharvest fungicides as well as of other alternative treatments controlling decay caused by pathogenic fungi may alter their efficacy reducing both decay incidence and *in vitro* growth (9,35,36). Therefore, we also investigated whether delaying LED blue light application may influence the growth of the fungus. Results revealed the potential of blue light for reducing *P. digitatum* fungal growth if the cultured plates are light-treated at different time points after pre-inoculation with the fungus since blue light (120 $\mu molm^{-2}s^{-1}$) was able to restrict growth of Pd1 *P.digitatum* strain regardless of time of light treatment delay (Fig 2). Fungal growth was almost stopped always after 8 days of continuous light application and was c.a. the 50 % that of the colonies held under darkness. These results indicate that the 120 $\mu molm^{-2}s^{-1}$ light quantum flux did not avoid spore germination although it may stall fungal growth. Moreover, it is interesting to note that the time required to achieve such effect when applying continuously this light quantum flux barely depended on the developmental stage of the fungus.

Results further showed that if the colonies treated at 0, 1, 2 or 4 dpi for 3 days with a 120 $\mu molm^{-2}s^{-1}$ blue light quantum flux were shifted to dark before reaching the stalling growth they still grew and that, after light removal, the fungal growth rate depended on the day in which the light was applied (Fig. 2). Thus, the slopes found for 0, 1, 2, and 4 dpi were 0.859, 0.802, 0.630 and 0.2418, respectively. Such slopes were calculated from the day it finished the light treatment for each dpi trial and during the period the growth was still lineal. These results indicate that the loss of light efficacy inhibiting fungal growth when the cultures were transferred to darkness was faster at 0 dpi and lower at 4 dpi, which suggests that the already grown mycelium losses the ability to cope with the damage inflicted by the light treatment.

We further examined whether the 120 $\mu molm^{-2}s^{-1}$ blue light quantum flux was able to stop the colonies' growth if they were shifted to the dark after reaching the stalling growth. This experiment was performed at 0 and at 4 dpi since these two post-inoculation periods showed the highest difference in growth rate when the plates were transferred to darkness after the 3 days light treatment. Moreover, the incubation period was considerably extended. Results confirmed the behavior found in the previous experiment since continuous light induced a delayed fungistatic effect after 8 days of fungal growthand this effect was maintained until the end of the experiment (22 days) (Fig. 3). Furthermore, the loss of light efficacy of the 3 days light treatment was faster at 0 dpi, (Fig. 3). Results also showed that no further growth occurred if the colonies treated with light at 0 or 4 dpi were transferred to darkness after the mycelium growth was stalled (Fig. 3). That is, the efficacy of the treatment was not reversed after removing the light stimulus.

Morphology of Pd1 *P. digitatum* strain grown under different light regimes is shown in Fig. S2 (see Supplementary Materials) and Fig. 4. In the 0 dpi trial, slight changes in the

morphology of the colonies occurred when this light quantum flux was applied for 3 days and then the plates were transferred to dark, but extending the light exposure period clearly affected the mycelium. Sporulation was already observed by day 4 in control plates held under darkness (Fig. S2a). In contrast, it was not observed in the cultures treated for 3 days with light and then transferred to darkness for 1 additional day (Fig. S2b) or in cultures held under continuous light (Fig. S2c). By day 7, the colonies held under darkness showed the characteristic green conidia and white mycelium of *P. digitatum* (Fig. 4a). The morphology of the cultures exposed to non-continuous light (Fig. 4b and c) was similar, although the 3 days light treatment delayed the fungal growth (Fig. 3). These results indicate that non-continuous light (3 days light) at a quantum flux of 120 $\mu molm^{-2}s^{-1}$ has only a slight effect retarding growth and sporulation when the light is applied at 0 dpi. However, extending light exposure (continuous light) had a noticeable effect (Fig 4d-f). By 7 days, the presence of brownished mycelium was already observed, although white mycelium still predominated (Fig. 4d). At day 11 under continuous light, the older part of the colony was mostly brown (Fig. 4e) but the remaining white mycelium was still able to sporulate as green conidia were very evident after shifting the plates to darkness for 4 days (Fig. 4f). Overall results indicate then that exposing *P.digitatum* to a 120 $\mu molm^{-2}s^{-1}$ blue light for a short period (3 days) at 0 dpi slightly affects growth and sporulation and, therefore, the effect of the treatment on reducing fungal growth may be reversed after removing the light. However, longer light exposure (11 days) may have a dramatic effect on fungal viability, which may explain, at least in part, the lack of reversibility of the effect of the 11 days light treatment on fungal growth after shifting the plates from light to darkness.

Examination of changes induced in morphology by continuous and non-continuous light at 4 dpi (Fig. 4g-l) in cultures treated with the same light quantum flux further

supported that older mycelium was more sensitive to this light quantum flux. Treating *P. digitatum* with light for 3 days at 4 dpi caused brownish on the center and older part of the colony (Fig. 4g), a fact not found at 0 dpi. Thus, by 7 days of fungal growth, colony appearance was very different depending on when the 3 days light treatment was applied, either at 0 or 4 dpi (Fig. 4b and g). This differential effect was maintained after further incubation in the dark (Fig. 4c and 4h). As shown in Fig. 4i, further incubation in the dark allowed the outer younger part of the colony treated at 4 dpi with light for 3 days to sporulate, however, colony growth was stalled (Fig. 3). Therefore, the 3 days light treatment did not completely avoided sporulation although it had an important inhibitory effect on fungal growth when it was applied at 4 dpi (Fig. 3). In contrast, it barely affected the fungus morphology and had only a transient inhibitory effect when it was applied at 0 dpi (Fig. 3). The multiple effects of UV treatments both in pathogens and fruits have been widely studied (10,37,38). Information related to blue light-emitting diodes induced responses against pathogens causing diseases in fruits is less abundant (39). As far as we know, there is only one report related to the study of the mechanisms of blue light effects on *P. digitatum.* This study showed that low blue light quantum flux (40 $\mu molm^{-2}s^{-1}$) reduces the activity of the pectolytic enzyme polygalacturonase produced by the fungus (26).

Changes in morphology found when continuous light was applied further support the higher susceptibility of the fungus to light at 4 dpi as compared to 0 dpi. As shown in Fig 4j-l, applying the light for 7 days at 4 dpi led to abundant brownish mycelium and completely inhibited sporulation. This effect was not reversed after shifting the plates to darkness. This might indicate severe hyphae damage or even cell death. In this regard, it should be noted the participation of oxidative stress in cell death and that the accumulation

317 of singlet oxygen is involved in the inhibitory effect of 450 nm blue light generated by LED
318 on growth of *B. cinerea* (40). Therefore, focusing on oxidative stress would be an
319 interesting approach to decipher the mode of action of blue light inhibiting *P. digitatum*
320 growth. Likewise, it should be considered that blue light-mediated damage to bacteria cells
321 has been related to the development of vacuoles within the cytoplasm, implying that
322 damage was related to intracellular chromophores excited by the blue light, to the release of
323 cytoplasmatic material to the surrounding environment and to cytoplasmic disruption (41)
324

325 **Fungicidal effect of blue light on *Penicillium digitatum* strains resistant and sensitive**
326 **to fungicides and on *Penicillium italicum***

327 The above results suggest that blue light might reach a fungicidal effect on *P.digitatum* if
328 the quantum flux is substantially increased and also that increasing the quantum flux might
329 favor a drastic shortening of the treatment. If these were the cases, then blue light might be
330 a useful tool for sanitation or for reducing contaminations even if it is applied after spore
331 germination. These ideas were tested by increasing c.a. 6-fold the light quantum flux (700
332 $\mu molm^{-2}s^{-1}$). The effect of this light dose was assayed in four different strains, three of them
333 from *P.digitatum* (Pd1, PHI-41 and PHI-26) and one from *P. Italicum* (PHI-1). Two of the
334 *P.digitatum* strains (Pd1 and PHI-41) are resistant to the fungicides imazalil and TBZ.

335 The effect of shortening the 700 $\mu molm^{-2}s^{-1}$ light treatment was first investigated in the
336 PHI-41 (*P. digitatum*) and PHI-1 (*P. italicum*) strains. Results show that the effectiveness
337 of this high light quantum flux also increases with duration of the treatment and that
338 applying the light for 18 h had already a fungicidal effect on both strains because the fungi
339 did not resume growing after the plates were returned to darkness (Fig. 5). After this light
340 treatment, the percentage of germination was 0 %, while c.a. 81 % of the spores germinated

in the control plates held under darkness. Reducing time of light application to 3 h had no inhibitory effect on PHI-41 growth and slightly inhibited the growth of PHI-1 (data not shown).

In a subsequent experiment, we extended the incubation period of the inoculated plates and further confirmed that applying 700 $\mu molm^{-2}s^{-1}$ blue light for 18 h has a fungicidal effect on all the studied strains if the light is applied at 0 dpi (Fig. 6). Growth of these strains was also reduced when this treatment was applied at 4 dpi (Fig. 6). Therefore, it appears that the fungal spores are more susceptible to the higher quantum flux doses but this different effect is not observed with 4 days old hyphae. Applying 700 $\mu molm^{-2}s^{-1}$ blue light for 18 h affected the mycelium of the 4 strains grown for 4 days. Changes in morphology of the PHI-26 strain grown for 4 days at 20 ºC and then exposed to this treatment are shown in Fig. 7. Patterns of changes in the rest of strains assayed were similar (not shown). Brownish

areas were already present after finishing the treatment (day 5) and also 2 days after transferring the cultures to darkness (day 7), although the white mycelia predominated and was able to further sporulate when the light was applied at 4 dpi for just 18 h. This would explain further fungal growth (Fig. 6 and 7) when this treatment was applied. As shown above, continuous light had a clear and not reversible detrimental effect on fungal growth even though it was applied at a lower quantum flux (Fig 4). Therefore, global results indicate that fungal spores cannot germinate under short high light quantum flux (0 dpi) and that a continuous lower light quantum flux is able to suppress both sporulation and mycelium growth when applied to already grown mycelium (4 dpi). Since blue light may avoid sporulation of *Penicillium* strains sensitive and resistant to the fungicides used to reduce disease incidence in citrus fruits, this new strategy might constitute a useful

alternative method for sanitation within integrated control programs for postharvest pest management of citrus fruits. Blue light would be of interest to reduce the use of fungicides by reducing contaminations of the fungicide solutions, which might allow reducing the concentrations applied or, at least, further re-utilization of such solutions. Although these solutions may be turbid, it is noticing the development of light processing methods for highly turbid fluids (42). Moreover, blue light could be used as a mean to minimize contaminations in cold storage rooms and other strategic points in the packing-houses. This would be especially relevant since decay in citrus fruit is mainly controlled by two fungicides (imazalil and TBZ) with different mode of action, whose use must be rotated in the packing- and store-houses for avoiding the development of strains resistant to them. Therefore, this study encourages further research simulating industrial conditions to implement blue light within integrated control programs for pest management of citrus fruits. Results indicate that the development of technologies able to induce high quantum fluxes of blue light would be helpful to shorten the light treatments. This study was limited by the light output of the LED array used but development of systems that use high-power LED arrays／light sources could enable the delivery of very high LED blue light doses for decontamination applications. This innovative emerging technology could be useful to the industry to reduce citrus fruit losses, and probably of other fruit and vegetable crops, caused by pathogens sensitive and resistant to fungicides. Moreover, it could help to open new markets demanding fruits non-treated with fungicides, while minimizing energy cost and toxic residues of fungicides

CONCLUSIONS

Results showed the potential of LED blue light for controlling the growth of *P. digitatum* and *P. italicum*, which are the major pathogens of citrus fruits after harvesting, and that efficacy increases with the duration of the treatment and with the light quantum flux. The older mycelium was more sensitive to blue light. Spore germination of strains sensitive and resistant to fungicides can be completely avoided by exposing them for a short period of time to high blue light quantum flux (700 $\mu molm^{-2}s^{-1}$). Lower quantum fluxes (120 $\mu molm^{-2}s^{-1}$) have a fungicidal effect when continuously applied to already grown mycelium (4 dpi). Therefore, the combination of high quantum flux followed by a continuous lower quantum flux may reduce both sporulation and mycelium viability. Global results indicate that blue light may be a tool to reduce contaminations and infections caused by *P. digitatum* and *P. italicum* strains in postharvest facilities by reducing pathogen inoculum.

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SUPPLEMENTARY MATERIALS

Additional Supplementary Information may be found in the online version of this article:

Figure S1. Growth of the Pd1 *P. digitatum* strain in potato-dextrose agar (PDA) after being treated from 5 min to 360 min with blue light at a quantum flux of 120 $\mu molm^{-2}s^{-1}$. Results represent the means of three replicates samples ± S.E.M. No significant differences ($P < 0.05$) were found between the control and any of the light-treated samples for the same storage period.

Figure S2. Morphology of *P. digitatum* in response to the 120 $\mu molm^{-2}s^{-1}$ LED blue light treatment. The fungus was grown in PDA at 20 ºC for 4 days and photographed: a) 4 days darkness (D, control), b) 3 days light (L) plus 1 day darkness (D), c = 4 days light (L).

421 **REFERENCES**

422 1. Sánchez-Torres, P. and J. J. Tuset (2011) Molecular insights into fungicide resistance
423 in sensitive and resistant *Penicillium digitatum* strains infecting citrus. Postharvest Biol.
424 Technol. 59,159-165.

425 2. Nigro, F., A. Ippolito and G. Lima (1998) Use of UV-C light to reduce *Botrystis*
426 storage rot of table grapes. Postharvest Biol. Technol. 13, 171-181.

427 3. Droby, S., M. Wisniewski, D. Macarisin and C. Wilson (2009). Twenty years of
428 postharvest biocontrol research: is it time for a new paradigm? Postharvest Biol. Technol.
429 52, 137–145.

430 4. Sharma, R. R., D. Singh, and R. Singh (2009) Biological control of postharvest
431 diseases of fruits and vegetables by microbial antagonists: a review. Biol. Control 50, 205–
432 221.

433 5. Romanazzi, G., A. Lichter, F. M. Gabler and J. L. Smilanick (2012) Recent advances
434 on the use of natural and safe alternatives to conventional methods to control postharvest
435 grey mold of table grapes. Postharvest Biol. Technol. 63, 141-147.

436 6. Ballester, A. R., M. T. Lafuente, C. H. R. De Vos, A. Bovy and L. González-Candelas
437 (2013) Citrus phenylpropanoids and defence against pathogens. Part I: Metabolic profiling in
438 elicited fruits. Food Chem. 136, 178-185.

439 7. Montesinos-Herrero, C., J. L. Smilanick, J. S. Tebbets, S. Walse and L. Palou (2011)
440 Control of citrus postharvest decay by ammonia gas fumigation and its influence on the
441 efficacy of the fungicide imazalil. Postharvest Biol. Technol. 59, 85-93.

442 8. Ballester, A. R., M. T. Lafuente and L. González-Candelas (2006) Spatial study of
443 antioxidant enzymes, peroxidase and phenylalanine ammonia-lyase in the citrus fruit-
444 *Penicillium digitatum* interaction. Postharvest Biol. Technol. 39**,** 115-124.

445 9. Eckert, J. W. and N. F. Sommer (1967) Control of diseases of fruits and vegetables by
446 postharvest treatment. Annu. Rev. Phytopathol. 5, 391–432.
447 10. Droby, S., E. Chalutz, B. Horev, L. Cohen, V. Gaba, C. L. Wilson and M. Wisniewski
448 (1993) Factors affecting UV-induced resistance in grapefruit against the green mould decay
449 caused by *Penicillium digitatum*. Plant Pathol. 42, 418–424.
450 11. Smilanick, J.L., M. F. Mansour and D. Sorenson (2006) Pre- and postharvest
451 treatments to control green mold of citrus fruit during ethylene degreening. Plant Dis. 90,
452 89–96.
453 12. Schirra, M., S. D'Aquino, A. Palma, A. Angioni and P. Cabras (2008) Factors
454 affecting the synergy of thiabendazole, sodium bicarbonate, and heat to control postharvest
455 green mold of citrus fruit. J. Agric. Food Chem. 56, 10793-10798.
456 13. Palou, L., J. L. Smilanick, and S. Droby (2008) Alternatives to conventional
457 fungicides for the control of citrus postharvest green and blue moulds. Stewart Postharvest.
458 Rev. 2, 1–16.
459 14. Malik, Z., J. Hanania and Y. Nitzan (1990) New trends in photobiology bactericidal
460 effects of photoactivated porphyrins-An alternative approach to antimicrobial drugs.
461 J. Photochem. Photobiol. B: Biol. 5, 281–293.
462 15. Papageorgiou, P., A. Katasambas and A. Chu (2000) Phototherapy with blue (415
463 nm) and red (660 nm) light in the treatment of acne vulgaris. Br. J. Dermatol. 142, 973–
464 978.
465 16. Ashkenazi, H., Z. Malik, Y. Harth and Y. Nitzan (2003) Eradication of
466 propionibacterium acnes by its endogenic porphyrins after illumination with high
467 intensity blue light. FEMS Immunol. Med. Microbiol. 35, 17–24.
468 17. Lipovsky, A., Y. Nitzan, H. Friedmann and R. Lubart (2009) Sensitivity of

469 *Staphylococcus aureus* strains to broadband visible light. J. Photochem. Photobiol. 85, 255–
470 260.
471 18. E. Endarko, M. Maclean, I. V. Timoshkin, S. J. MacGregor and J. G. Anderson
472 (2012) High-Intensity 405 nm Light Inactivation of *Listeria monocytogenes*. Photochem.
473 Photobiol. 88: 1280–1286.
474 19. D'hallewin, G., M. Schirra, E. Manueddu, A. Piga and S. Ben Yehoshua (1999)
475 Scoparone and scopoletin accumulation and ultraviolet-C induced resistance to postharvest
476 decay in oranges as influenced by harvest date. J. Am. Soc. Hortic. Sci. 124, 702–707.
477 20. Cote, S., L., Rodoni, E. Miceli, A. Concellón, P. M. Civello and A. R. Vicente (2013)
478 Effect of radiation intensity on the outcome of postharvest UV-C treatments. Postharvest
479 Biol.Technol. 83, 83-89.
480 21. Folta K. and K. S. Childers (2008). Light as a growth regulator: controlling plant biology
481 with narrow-bandwidth solid-state lighting systems. Hortsci. 43, 1957-1964
482 22. Schmidt-Heydt, M., C. Rüfer, F. Raupp, A. Bruchmann, G. Perrone and R. Geisen
483 (2011) Influence of light on food relevant fungi with emphasis on ochratoxin producing
484 species. Int. J. Food Microbiol. 145, 229-237.
485 23. Ondrusch, N. and J. Kreft (2011) Blue and red light modulates SigB-dependent gene
486 transcription, swimming motility and invasiveness in *Listeria monocytogene.* PLoS ONE
487 6(1): e16151. doi:10.1371/journal.pone.0016151
488 24. Nguyen, T. P., M. D. Danyluk and K. R. Schneider (2013) Growth, reduction, and
489 survival of bacteria on melon types. University of Florida IFAS Extension. FSHN12-07, 1-
490 45.
491 25. Alférez, F., H-L. Liao and J. K. Burns (2012) Blue light alters infection by
492 *Penicillium digitatum* in tangerines. Postharvest Biol. Technol. 63, 11-15.

493 26. Liao, H-L., F. Alférez and J. K. Burns (2013). Assessment of blue light treatments on
494 citrus postharvest diseases. Postharvest Biol. Technol. 81, 81-88.
495 27. Adaskaveg, J.E., H. Föster and N. F. Sommer (2002) Principles of postharvest
496 pathology and management of decay of edible horticultural crops. In Postharvest
497 Technology of Horticultural Crops: (Edited by A. A. Kader, Third edition), pp. 163-195.
498 Editorial University of California Agriculture and Natural Resources Publication 3311.
499 28. Fadda, A., A. Barberis, S. D'Aquino, A. Palma, A. Angioni, F. Lai, and M. Schirra
500 (2015). Residue levels and performance of potassium sorbate and thiabendazole and their
501 co-application against blue mould of apples when applied as water dip treatments at 20 or
502 53 ºC. Postharvest Biol. Technol. 106, 33-43.
503 29. Ensminger, P.A. and E. Schäfer (1992) Blue and ultraviolet-B light photoreceptors in
504 parsley cells. Photochem. Photobiol. 55, 437–447.
505 30. Losi, A. and W. Gärtner (2011) Old chromophores, new photoactivation paradigms,
506 trendy applications: Flavins in blue light-sensing photoreceptors. Photochem. Photobiol.
507 87, 491–510.
508 31. Heintzen, Ch. (2012) Plant and fungal photopigments. WIREs Membr. Transp.
509 Signal. 1, 411-432. Doi:10.1002/wmts.36.
510 32. Chico, J. M., G. Fernández-Barbero, A. Chini, P. Fernández-Calvo, M. Díez-Díaz and
511 R. Solano (2014) Repression of jasmonate-dependent defenses by shade involves
512 differential regulation of protein stability of MYC transcription factors and their JAZ
513 repressors in Arabidopsis. Plant Cell 26, 1967-1980.
514 33. Heijde, M. and R. Hulm (2012) UV-B photoreceptor-mediated signalling in plants.
515 Trends Plant Sci. 17, 230-237.

516 34. Wade, H. K., T. N. Bibikova, W. J. Valentine and G. I. Jenkins (2001) Interactions
517 within a network of phytochrome, crytochrome and UV-B phototransduction pathways
518 regulate chalcone synthase gene expression in *Arabidopsis* leaf tissue. Plant J. 25, 675-685.
519 35. Sugar, D. and S. R. Basile (2011) Orchard calcium and fungicide treatments mitigate
520 effects of delayed postharvest fungicide applications for control of postharvest decay of
521 pear fruit. Postharvest Biol. Technol.60, 52–56.
522 36. Gatto, M. A., A. Ippolito, V. Linsalata, N. A. Cascarano, F. Nigro, S. Vanadia and D.
523 Di Venere (2011) Activity of extracts from wild edible herbs against postharvest fungal
524 diseases of fruit and vegetables. Postharvest Biol. Technol. 61, 72-82.
525 37. Asad, L. M. B. O., A. A. de Carvalho, I. Felzenszwalb, A. C. Leitao and N. R. Asad
526 (2000) H_2O_2-induced cross-protection against UV-C killing in *Escherichia coli* is blocked
527 in a *lexA (Def)* background. J. Phytochem. Photobiol. B Biol. 54, 67–71.
528 38. Zlatev, Z., F. J. C Slidon and M. Kaimakanova (2012) Plant physiological responses
529 to UV-B radiation. Emir. J. Food Agric. 24, 481-501.
530 39. Kim, K., H-S. Kook, Y-J. Jang, W-H. Lee, S. Kama-Kannan, J-J. Chae and K-J. Lee
531 (2013) The effect of blue-light-emitting diodes on antioxidant properties and resistance to
532 *Botrytis cinerea* in tomato. J. Plant Pathol. Microb. 4:203.doi:10.4172/2157-7471.1000203.
533 40. Imada, K., S. Tanaka, Y. Ibaraki, K. Yoshimura and S. Ito (2014) Antifungal effect of
534 405-nm light on *Botrytis cinera*. Letters Appl. Microbiol. 59, 670-676.
535 41. Dai, T., A. Gupta, Y-Y. Huang, R. Yin, C. K. Murray, M. S. Vrahas, M. E. Sherwood,
536 G. P. Tegos, M. R. Hamblin (2013) Blue light rescues mice from potentially fatal
537 *Pseudomonas aeruginosa* burn infection: efficacy, safety and mechanism of action.
538 Antimicrob. Agents Chemother. 57, 1238-1245.

539 42. Huan, Y., R. Sido, R. Huang and H. Chen (2015) Application of water-assisted pulsed
540 light treatment to decontaminate raspberries and blueberries from *Salmonella.* Int. J. Food
541 Microb. 208, 43-50.

542

543

544

Figure legends

Figure 1. Effect of treating PHI-41 and Pd1 *P. digitatum* strains with 120 µmolm^{-2}s^{-1} LED blue light for 6 (○), 24 (□) and 72 h (▽) on fungal growth and on fungal growth inhibition. Control samples were always maintained under darkness (●). The treatments were applied immediately after inoculating (0 dpi) the plates with 5µl of 10^6 conidia ml^{-1}. Results represent the means of three replicates samples ± S.E.M. Significant differences ($P < 0.05$) between the light-treated PHI-41 and Pd1 strains and their respective controls continuously held under darkness for the same storage period were found from day 3 when the samples were treated with light for 72 h. Significant differences were only maintained till day 5 when the light was applied for 24h, and no statistic difference was found when it was applied for 6 h.

Figure 2. Effect of treating the Pd1 *P. digitatum* strain with 120 µmolm^{-2}s^{-1} LED blue light at different dpi on fungal growth. The cultures were treated continuously with this light quantum flux (○), or for 3 days and then shifted to darkness (non-continuous light, □). Control samples were always held under darkness (●). The arrows indicate when the light treatments started (0, 1, 2 and 4 dpi). All the samples were inoculated with 5µl of 10^6 conidia ml^{-1}. Results in the upper panels represent the means of three replicate samples ± S.E.M and the asterisks indicate significant differences ($P < 0.05$) between the treated and the control samples for the same storage period. The statistical analysis was not performed for the 14 days samples since the diameter of the control colony held under darkness was close or higher than 9 cm. Data missing in each plot corresponding to the day 14 were close or higher than 9 cm. Data in the lower panels show the rate of growth when the cultures were treated with non-continuous light at different dpi.

569 **Figure 3**. Effect of treating the Pd1 *P. digitatum* strain with 120 μmolm^{-2}s^{-1} LED blue light
570 at 0 and 4 dpi on fungal growth. The cultures were treated continuously with this light (L)
571 quantum flux (○), or for 3 days (□) and then shifted to darkness (D), in each dpi trial.
572 Other sets of samples for each dpi were treated with light for 11 days, to reach the
573 mycelium growth stalling, and then shifted to darkness (△). Control samples were always
574 maintained under darkness (●). The arrows indicate when the light treatments started or
575 finished (0 and 4 dpi). The L in the arrows indicates treatment with continuous light, while
576 LD3 and LD11 indicate the days the plates were shifted to darkness after being treated for 3
577 and 11 days with light, respectively. All the samples were inoculated with 5μl of 10^6
578 conidia ml^{-1}. Results represent the means of three replicates samples ± S.E.M. Significant
579 differences ($P < 0.05$) between the treated and the control samples for the same storage
580 period were found from day 3 at 0 dpi and from day 8 at 4 dpi. The diameter of the control
581 plates (darkness, D) was close or higher than 9 cm after day 11 and, therefore, these values
582 were not included in the plots.
583
584 **Figure 4**. Changes in the morphology of *P. digitatum* during its growth under different
585 light regimes. The quantum flux of the LED blue light was 120 μmolm^{-2}s^{-1} light, the light
586 was applied both at 0 (a-f) and at 4 dpi (g-l), and the fungus was grown in PDA at 20 ºC
587 and photographed at different time points. In the photographs, d indicates the number of
588 days, D darkness and L light. The symbol + indicates the shifting from light to darkness or
589 from darkness to light.
590
591 **Figure 5**. Effect of treating the PHI-41 (*P. digitatum*) and PHI-1 (*P. italicum*) strains with
592 700 μmolm^{-2}s^{-1} LED blue light for 6 (◇), 9 (○), 18 (▽) or 24 h (□) on fungal growth and

593 fungal growth inhibition. Control samples were always held under darkness (●). The
594 treatments were applied immediately after inoculating (0 dpi) the plates with 5µl of 10^6
595 conidia ml^{-1}. Results represent the means of three replicates samples ± S.E.M. Significant
596 differences ($P < 0.05$) between the control and the samples subjected to any treatment for
597 the same storage period were found from day 2.
598
599 **Figure 6**. Effect of treating the Pd1, PHI-26, PHI-41 *P. digitatum* strains and the PHI-1 *P.*
600 *italicum* strain with 700 $\mu molm^{-2}s^{-1}$ LED blue light for 18 h at 0 (○) and 4 dpi (□) on
601 fungal growth. Control samples were always held under darkness (●). All the plates were
602 inoculated with 5µl of 10^6 conidia ml^{-1}. Results represent the means of three replicated
603 samples ± S.E.M. Significant differences ($P < 0.05$) between the control and the samples
604 subjected to the light treatment at 0 and 4 dpi were found along the whole experiment for
605 the same storage period. Data missing in each darkness plot or in the plot corresponding to
606 the PHI-26 culture treated with light at 4 dpi were equal or higher than 9 cm**.**
607
608 **Figure 7**. Changes in the morphology of the PHI-26 *P. digitatum* strain during growth at 20
609 ºC. Cultures were held continuously under darkness (D) (pictures in upper panels), or were
610 treated for 18 h with 700 $\mu molm^{-2}s^{-1}$ LED blue light (L) at day 4 (4 dpi) and then shifted for
611 different number of days (d) to darkness. This light regime avoided sporulation at 0 dpi and
612 therefore, no photograph corresponding to 0 dpi is shown. Pattern of changes in the rest of
613 strains assayed was very similar and, therefore, only the photographs corresponding to the
614 PHI-26 strain are shown to better visualize the effect of blue light.

Fig. 1

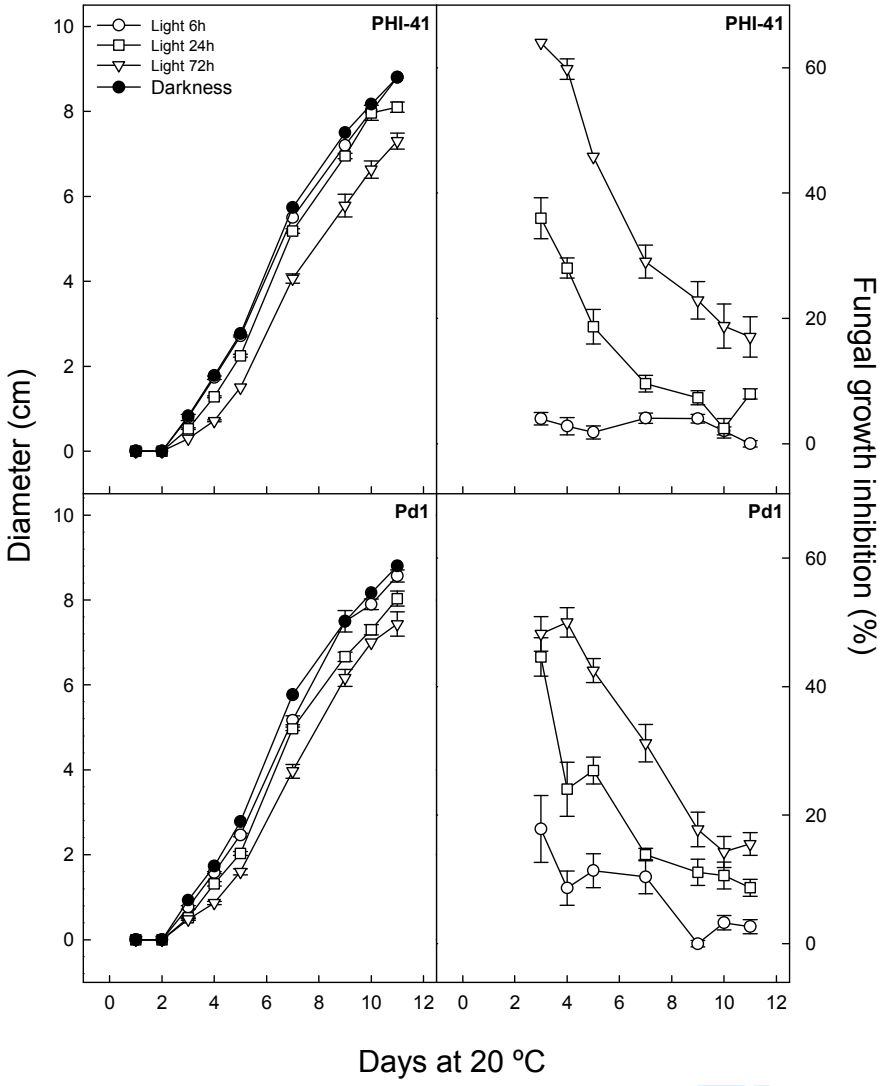

Light 6h
Light 24h
Light 72h
Darkness
PHI-41
PHI-41
Pd1
Pd1
Diameter (cm)
Fungal growth inhibition (%)
Days at 20 ºC
0
2
4
6
8
10
12
0
20
40
60

Figure 2

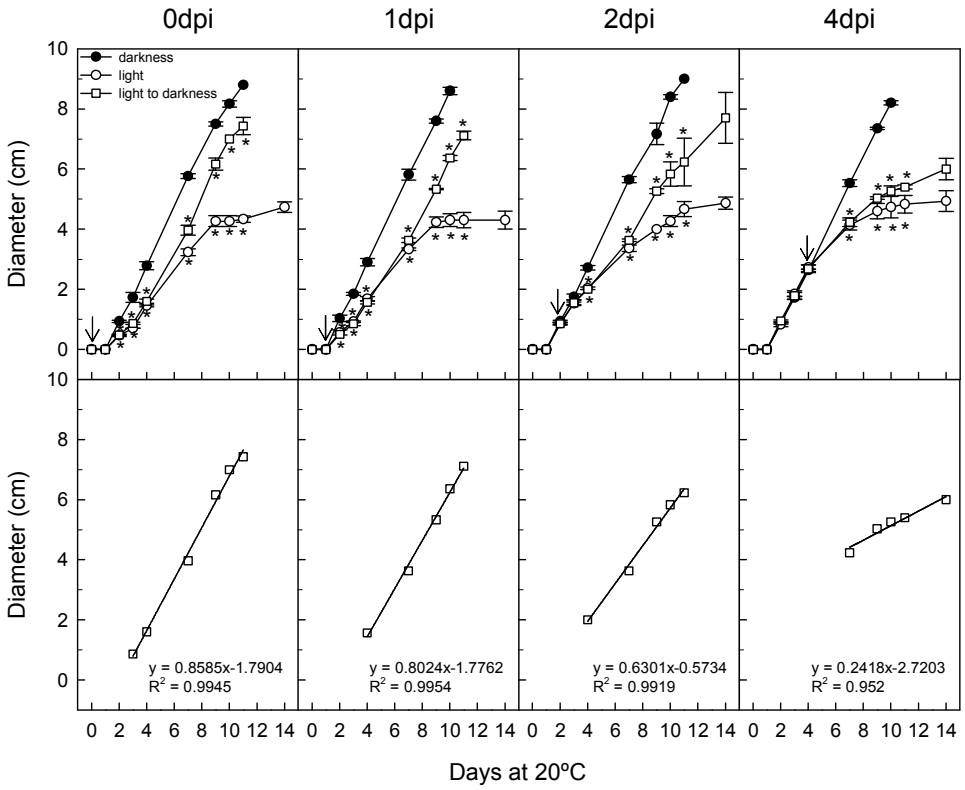


Figure 3

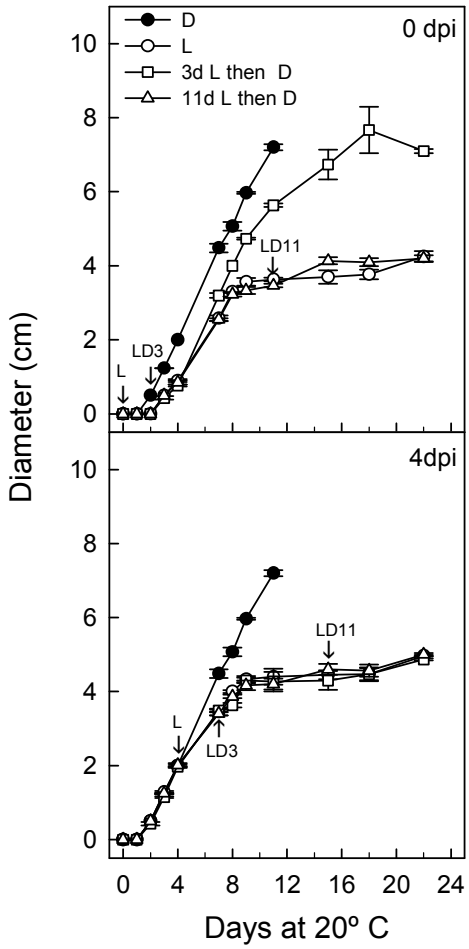
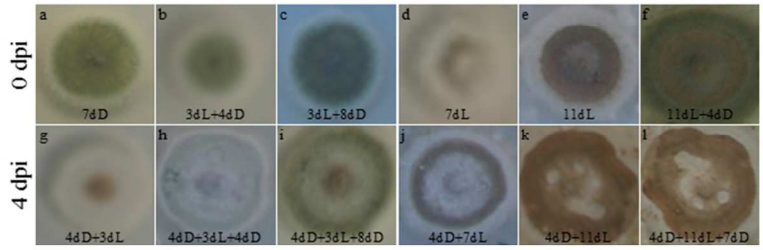

D
L
3d L then D
11d L then D
0 dpi
4dpi
LD3
LD11
L
Diameter (cm)
Days at 20º C
0
2
4
6
8
10
0
4
8
12
16
20
24

FIG 4



190x254mm (96 x 96 DPI)

Fig. 5

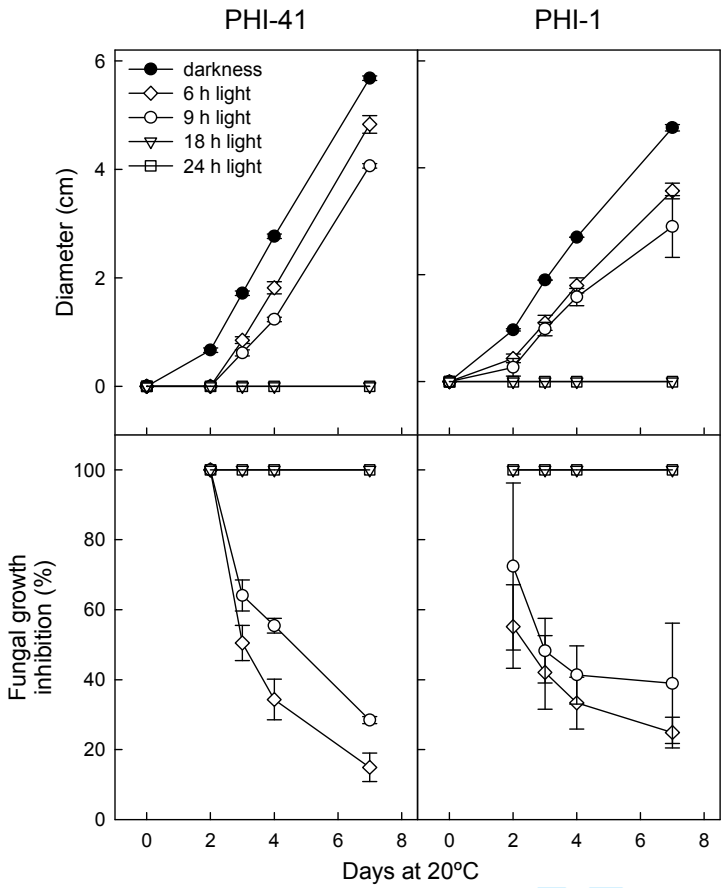

PHI-41
PHI-1
darkness
6 h light
9 h light
18 h light
24 h light
Diameter (cm)
Fungal growth inhibition (%)
Days at 20ºC

Figure 6

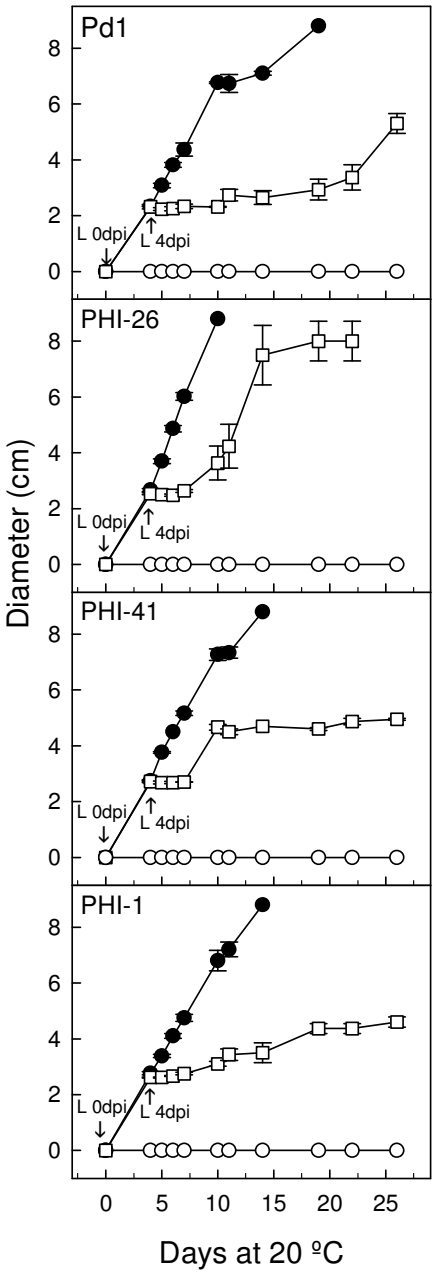
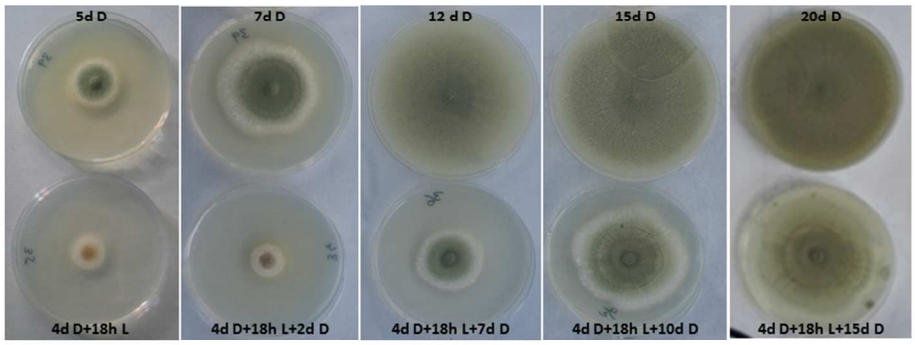

Pd1
PHI-26
PHI-41
PHI-1
L 0dpi
L 4dpi
Diameter (cm)
Days at 20 ºC
0
2
4
6
8
0
5
10
15
20
25

Fig. 7



254x190mm (96 x 96 DPI)