Received: 24 January 2020 | Accepted: 29 April 2020

DOI: 10.1111/ppa.13207

ORIGINAL ARTICLE

Plant Pathology WILEY

Effect of light and dark on the growth and development of downy mildew pathogen *Hyaloperonospora arabidopsidis*

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Funding information
Turkish Ministry of Education; University of Worcester

Abstract

Disease development in plants requires a susceptible host, a virulent pathogen, and a favourable environment. Oomycete pathogens cause many important diseases and have evolved sophisticated molecular mechanisms to manipulate their hosts. Day length has been shown to impact plant–oomycete interactions but a need exists for a tractable reference system to understand the mechanistic interplay between light regulation, oomycete pathogen virulence, and plant host immunity. Here we present data demonstrating that light is a critical factor in the interaction between *Arabidopsis thaliana* and its naturally occurring downy mildew pathogen *Hyaloperonospora arabidopsidis* (Hpa). We investigated the role of light on spore germination, mycelium development, sporulation, and oospore formation of Hpa, along with defence responses in the host. We observed abundant Hpa sporulation on compatible *Arabidopsis* under day lengths ranging from 10 to 14 hr. In contrast, exposure to constant light or constant dark suppressed sporulation. Exposure to constant dark suppressed spore germination, mycelial development, and oospore formation, whereas exposure to constant light stimulated these three stages of development. A biomarker of plant immune system activation was induced under both constant light and constant dark. Altogether, these findings demonstrate that Hpa has the molecular mechanisms to perceive and respond to light and that both the host and pathogen responses are influenced by the light regime. Therefore, this pathosystem can be used for investigations to understand the molecular mechanisms through which oomycete pathogens like Hpa perceive and integrate light signals, and how light influences pathogen virulence and host immunity during their interactions.

KEYWORDS

Arabidopsis, circadian rhythm, downy mildew, light regime, oomycetes

1 | INTRODUCTION

Environmental factors such as light, temperature, and humidity play a significant role in the infection of plants by microbial pathogens and during disease development (Cheng *et al*., 2019). At the molecular level, adaptation to environmental fluctuations is influenced by circadian timing mechanisms that undergo daily adjustment and act as a seasonal timer for diverse organisms, including plants and plant-associated microbes (Johnson *et al*., 2003). Light is the one of most significant environmental signals for circadian regulation (Dunlap *et al*.,

2004). Many organisms have circadian regulation networks that operate through similar mechanisms. For plants, light is perceived by photoreceptors and acts as a signal to regulate circadian genes (Millar, 2004; Franklin *et al*., 2005). Discrete light at different times of the day has been reported to have defined and particular effects on phase changes (Johnson *et al*., 2003). The circadian clock has also been shown to have a major effect on regulation of plant immunity (Lu *et al*., 2017; Karapetyan and Dong, 2018).

Light is known to have an effect on sporulation of several fungal and oomycete species, and the circadian clock of one fungal phytopathogen has been linked to the pathogen's virulence programme (Hevia *et al*., 2015). In contrast, there are a limited number of publications on the relation of light with development or virulence in oomycetes (Rumbolz *et al*., 2002). Early studies reported positive phototaxis of *Phytophthora cambivora* zoospores (Rumbolz *et al*., 2002) and the effect of humidity and light on discharge of sporangia of different oomycete pathogens (Fried and Stuteville, 1977; Leach *et al*., 1982; Su *et al*., 2000). Similarly, in *Plasmopara viticola*, the downy mildew pathogen of grapevine, continuous light did not have any effect on the growth of the mycelium and formation of sporangiophores, but the shape of sporangia was observed to be immature (Rumbolz *et al*., 2002). In the lettuce downy mildew pathogen *Bremia lactucae*, exposure to dark induced sporulation while light inhibited sporulation in a temperature-dependent manner: at low temperature, light was suppressive; however, with increasing temperature, the effect of suppression was decreased (Nordskog *et al*., 2007). Light also suppressed sporulation in *Peronospora belbahrii*, downy mildew of sweet basil, but light-dependent suppression of sporulation was enhanced at higher temperature. Light is also known to regulate the balance between asexual and sexual spore formation in *Phytophthora infestans*, causative agent of potato blight (Xiang and Judelson, 2014), in which exposure to constant light suppressed sporulation on plants and artificial media (Harnish, 1965). The mechanistic basis of light effects on oomycete virulence are largely unknown and likely to comprise a combination of light-regulated programmes for the host as well as the pathogen. It is also conceivable that the interacting organisms could directly influence each other's circadian programmes.

Oomycetes cause many important diseases in crops and in natural ecosystems (Kamoun *et al*., 2015). Much recent progress has been made in understanding plant–oomycete interactions through the development of reference plant–oomycete pathosystems that are amenable to genomic, genetic, and molecular approaches (Herlihy *et al*., 2019). One such pathosystem is the downy mildew pathogen *Hyaloperonospora arabidopsidis* (Hpa) and its natural host *Arabidopsis thaliana* (Coates and Beynon, 2010). Like many oomycetes, Hpa establishes an intimate relationship with its host by forming structures called haustoria, which are used to obtain nutrients from the plant. The Hpa life cycle is completed by the formation of aerial sporangiophores, which produce asexual spores, and by sexual oospores that are formed in infected leaves (Koch and Slusarenko, 1990). Because Hpa is an obligate biotroph, it requires its host to remain alive in order to complete its life cycle (Coates and Beynon, 2010). Hpa also redirects the host's metabolism and suppresses the host defence mechanisms (Herlihy *et al*., 2019). In Hpa–*Arabidopsis* interactions, it has been established that 16 °C is the best temperature for Hpa sporulation under laboratory conditions (Dangl *et al*., 1992). However, the effect of different light/dark regimes on the sporulation of Hpa and the most productive light/dark time period for Hpa growth have not been reported. Elucidating the effect of light on the sporulation and growth of Hpa may also give some clue as to whether there is a circadian regulation of its life cycle. Here, we report the effect of different light/dark regimes on the germination, mycelial development, and sporulation of Hpa.

2 | MATERIALS AND METHODS

2.1 | Plant lines, pathogen isolates, and propagation

Hyaloperonospora arabidopsidis isolate Emoy2 was maintained on *Arabidopsis* lines Ws-*eds1* (Parker *et al*., 1996) or Col-*rpp4* (Roux *et al*., 2011). Maintenance and preparation of inoculum for experiments was performed as described previously (Tör *et al*., 2002; Woods-Tör *et al*., 2018). Transgenic *PR1-GUS* lines were obtained from Xinnian Dong (Duke University, North Carolina, USA).

2.2 | Sporulation assay

Inoculated Col-*rpp4* seedlings were exposed to three different light (L)/dark (D) periods: 12 hr L/12 hr D, 14 hr L/10 hr D, and 10 hr L/14 hr D for 7 days at 16 °C, and the amount of sporulation was assessed.

Another experiment was designed to understand the effect of extreme light regimes on Hpa sporulation. The inoculated samples were exposed to four different light regimes: 7 days constant light; 7 days constant dark; constant light for 3 days post-inoculation (dpi) and constant dark after 3 dpi; and a light/dark regime of 12 hr L/12 hr D as control. As a light source, white fluorescent bulbs (300 $mmol \cdot m^{-2} \cdot s^{-1}$, 10 HQIL 400 W lamps plus four L40/60 W fluorescent bulbs; Osram) were used. To quantify sporulation, 10 infected seedlings from each replicate were taken and placed into an Eppendorf tube containing 250 µl water. Samples were vortexed and conidiospores were counted using a haemocytometer. All experiments had a minimum of three replicas and were repeated three times. All results were evaluated and compared statistically.

2.3 | Trypan blue staining

Cotyledons of 7-day-old Col-*rpp4* were spray inoculated with Hpa-Emoy2 and were exposed to a normal 12 hr L/12 hr D cycle, constant light, or constant dark. Cotyledons were examined at 3 dpi after staining with trypan blue to highlight the mycelial growth along with sexual spore (oospores) that are produced in the interior of the leaf,

and asexual fruiting bodies (sporangia) that form on the exterior of the leaf.

Seedlings were taken from infected samples at 0 and 12 hr post-inoculation and at 1, 2, 3, 4, 5, 6, and 7 dpi. Infected leaf segments were placed in an Eppendorf tube, covered with 1 ml or a sufficient amount of trypan blue solution (10 g phenol, 10 ml glycerol, 10 ml lactic acid, 10 ml water, 20 mg trypan blue (Merck) in ethanol [96%; 1:2 vol/vol]) and boiled at 100 °C for 1 min. The leaf segments were then destained for 1 hr in chloral hydrate (2 mg/ml) (Sigma). All steps were carried out in a fume hood. Pathogen structures were viewed under a Axioskop 4+ microscope (Zeiss).

2.4 | GUS assay

Transgenic *PR1-GUS* lines were used. Three-week-old seedlings were exposed to constant light or dark for 1–3 days. Then, seedlings were transferred to 24-well replica plates that contained 1 ml X-Gluc histochemical staining solution (50 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexylammonium salt [X-Gluc] in 50 mM $NaPO_4$, pH 7) and incubated overnight at 37 °C. After staining, leaves were treated with 70% methanol for up to 4 hr. The samples were washed with ethanol, immersed in glycerol, and tissues were examined for GUS staining under a dissecting microscope.

2.5 | Germination assay using cellophane

The germination assay using cellophane on MS agar (Murashige and Skoog, 1962) was carried out as described (Bilir *et al*., 2019). Sterile pieces of cellophane were placed on the surface of MS agar in the laminar flow cabinet. Hpa spore solution was prepared and centrifuged, all spores collected, and the pellet was then resuspended in sterile water. Approximately 10 µl spore solution were dropped on each piece of cellophane. Plates was grouped and put in the three different incubators: constant light, constant dark, and 12 hr L/12 hr D regime at 16 °C for 72 hr, and examined every 12 hr under the microscope. The number of germinated Hpa spores was counted using a haemocytometer.

2.6 | Determining biomass growth using quantitative PCR

The biomass of mycelium produced by Hpa-Emoy2 up to 3 dpi was measured from samples exposed to three different light regimes by real-time quantitative PCR (qPCR). The Hpa-*Actin* gene and *At-Actin* gene were used for quantification following the protocol of Anderson and McDowell (2015). After Col-*rpp4* seedlings were inoculated with Hpa-Emoy2, samples were separated and placed under normal (D/L), constant light, and constant dark regimes as three different groups. Every 24 hr, samples were taken, and their DNA extracted and measured with qPCR as described (Livak and Schmittgen, 2001). Sequences of primers used were: Hpa-Actin/F 5′-GTTTACTACCACGGCCGAGC-3′, Hpa-Actin/R 5′-CGTACGGAAACGTTCATTGC-3′, At-Actin/F 5′-AG CATCTGGTCTGCGAGTTC-3′, and At-Actin/R 5′-ACGGATTTAATG ACACAATGGC-3′.

2.7 | Statistical analysis

For statistical analysis, paired Student's *t* tests were performed on data obtained from plant infection and germination assays.

3 | RESULTS

3.1 | Optimal light regime for Hpa sporulation

We began by testing how Hpa sporulation is affected by three different light/dark periods, representing day lengths commonly encountered by the plant and pathogen in natural environments. We used a compatible interaction between the Hpa isolate Emoy2 and a mutant in the *Arabidopsis* accession Columbia (Col) that inactivates the disease resistance gene *RPP4* (*Recognition of Peronospora parasitica gene 4*, Roux *et al*., 2011). Sporulation was quantified at 4 and 7 dpi under the following light regimes: 14 hr L/10 hr D, 12 hr L/12 hr D, and 10 hr L/14 hr D. Plants grown under all three regimes supported abundant sporulation, which increased between 4 and 7 dpi (Figure 1). We observed only small, statistically insignificant

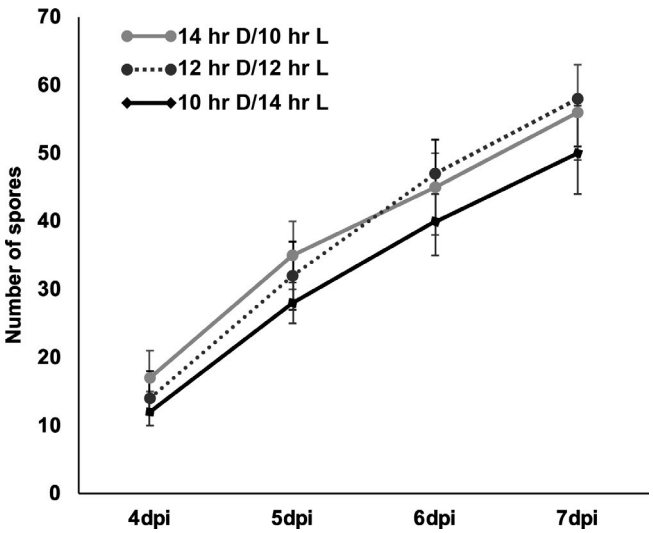

FIGURE 1 Optimization of the light (L)/dark (D) period for sporulation of *Hyaloperonospora arabidopsidis* (Hpa). Three different light/dark periods were tested to compare the amount of Hpa sporulation sporulation. These periods were 14 hr L/10 hr D, 12 hr L/12 hr D, and 10 hr L/14 hr D. Spores were harvested 4–7 days post-inoculation (dpi) and counted using a haemocytometer. Average and standard error of three replicates are shown. This experiment was repeated three times with similar results

differences in sporulation between the three regimes. We selected 12 hr L/12 hr D as the reference time period for subsequent experiments.

3.2 | Effect of constant light or dark on sporulation

The next set of experiments were designed to test how Hpa sporulation was affected by constant light or constant dark. Different light/dark conditions were compared to the 12 hr L/12 hr D reference. As expected, abundant sporulation was observed between 4 and 7 dpi on plants grown under the 12 hr L/12 hr D light regime (Figure 2a). In contrast, sporulation was dramatically reduced on seedlings exposed to constant light or dark after 3 dpi. Moreover, sporulation was almost totally suppressed on plants grown under 7 days of constant light or 7 days of constant dark regime that commenced immediately after inoculation (Figure 2b). When infected seedlings were exposed to constant light or dark after 3 dpi, there were hardly any new conidiophores and the amount of sporulation after 7 dpi was the same as at 3 dpi (Figure 2a). These experiments demonstrate that disruption of a normal light/dark regime can significantly affect the pathogen's capacity to complete the asexual phase of its life cycle.

3.3 | Recovery from suppression of sporulation by constant light

We tested whether asexual sporulation could be restored by returning plants to 12 hr L/12 hr D after treatment with constant light or dark as described above. Interestingly, seedlings that were returned to a normal 12 hr L/12 hr D regime after exposure to 7 days of constant light supported light sporulation 2 days after the shift and moderate sporulation after 4 days (Figure 2c). A similar recovery was observed in seedlings returned to the reference regime after treatment with constant light from 4 to 7 dpi (Figure 2b). In contrast, seedlings exposed to constant dark immediately after inoculation began to show a chlorotic phenotype after 4 days and the seedlings did not recover after shifting to a normal light regime, and no sporulation could be recorded (Figure 2c). Similarly, seedlings that were exposed to constant dark between 4 and 7 dpi did not survive after 7 dpi, and thus no sporulation could be recorded (Figure 2b). When seedlings were exposed to constant light or constant dark beginning immediately after inoculation for 3 days, then shifted to a normal light regime, light sporulation was recovered 7 dpi in samples exposed to constant dark. Abundant sporulation was observed 7 dpi in samples exposed to constant light, similar to plants grown under a normal light regime (Figure 2d). These experiments demonstrate that the suppression of sporulation by constant light treatment of varying durations is not a permanent effect, and that sporulation can be recovered by returning the plants to a normal regime.

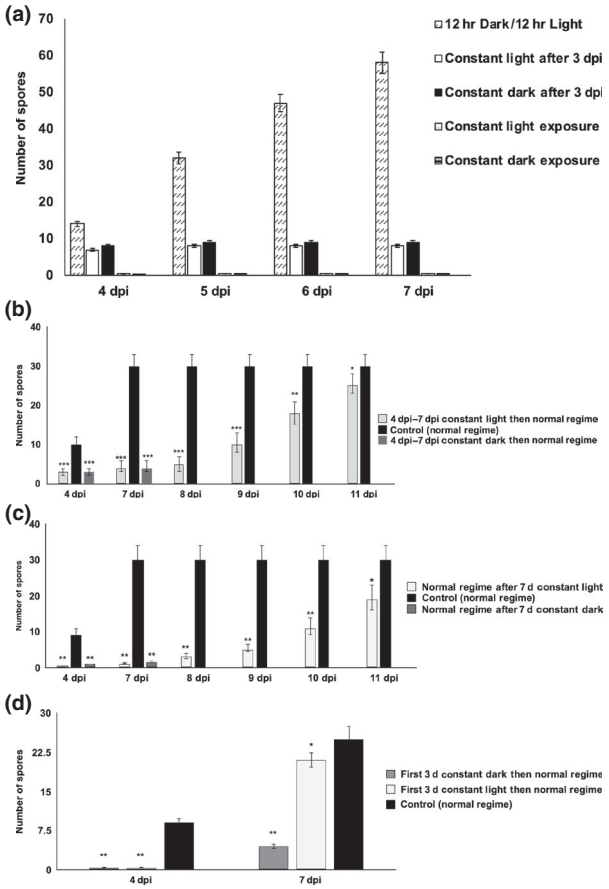

FIGURE 2 Amount of sporulation under different light regimes. (a) Five different light/dark conditions were tested. These were 12 hr light (L)/12 hr dark (D), constant light after 3 days post-inoculation (dpi), constant dark after 3 dpi, constant light exposure for 7 days, and constant dark exposure for 7 days. Spores were harvested 4–7 dpi and counted using a haemocytometer. (b) Samples were exposed to the reference light regime during the first 3 days (control regime), then were exposed to either constant light or constant dark over the subsequent 4 days (4–7 dpi). At 7 dpi, the samples were transferred to the reference light regime again and sporulation was recorded until 11 dpi. (c) Samples were exposed to constant light or constant dark for 7 days immediately after inoculation. At 7 dpi samples were transferred to the normal light regime again and sporulation was recorded until 11 dpi. (d) Samples were exposed to constant light or constant dark beginning immediately after inoculation for 3 days, then shifted to a normal light regime, with sporulation recorded at 4 and 7 dpi. All experiments were repeated three times. All results were evaluated and compared statistically. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, paired Student's *t* test

3.4 | Effect of different light conditions on mycelial growth of Hpa in leaves

Considering that plants grown under constant light for 7 days supported abundant Hpa sporulation after they were returned to a normal 12 hr L/12 hr D regime (Figure 2), it seemed likely that mycelium may have grown inside the leaf during exposure to constant light, but did not produce sporangia until a normal light regime was restored.

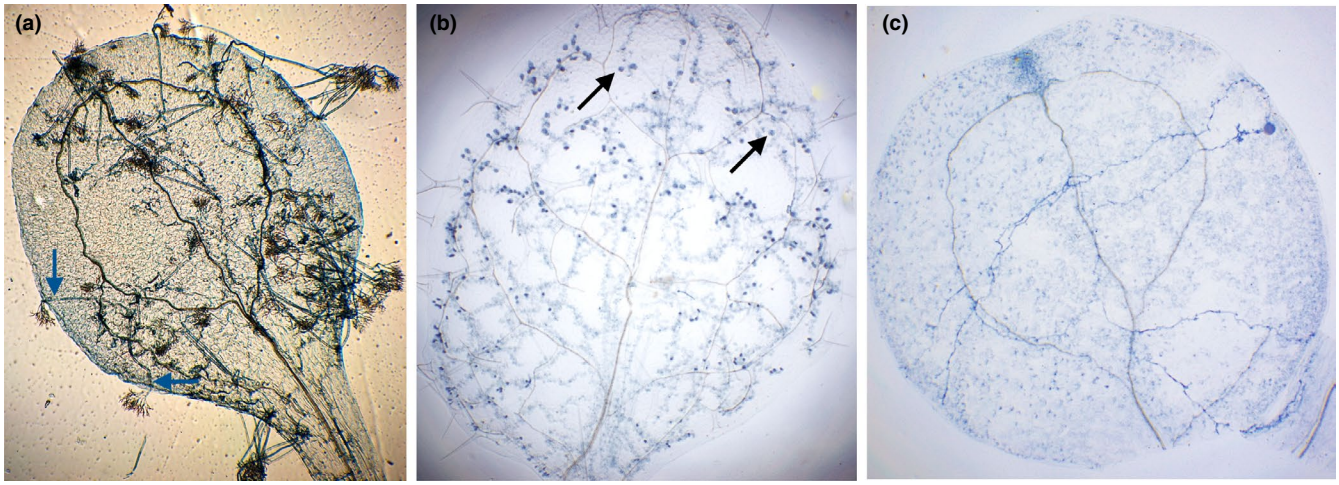

FIGURE 3 *Hyaloperonospora arabidopsidis* (Hpa) development in *Arabidopsis thaliana* leaves under different light regimes. (a) Infected plants grown under the reference 12 hr light/12 hr dark cycle; (b) infected plants grown under a constant light regime; and (c) infected plants grown under constant dark regime. Infected seedlings were stained with trypan blue 3 days post-inoculation. Blue arrows indicate conidiophores and black arrows indicates oospores [Colour figure can be viewed at wileyonlinelibrary.com]

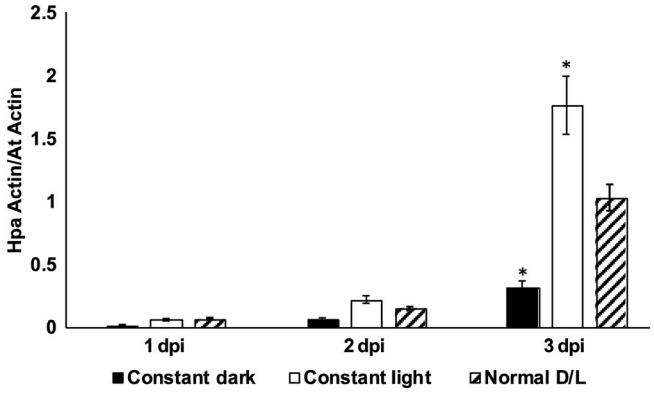

FIGURE 4 *Hyaloperonospora arabidopsidis* (Hpa) mycelial biomass production under different light regimes. Normal light, constant light, and constant dark regimes were applied. After Hpa inoculation, samples were taken every day from infected *Arabidopsis thaliana* leaves until 3 days post-inoculation (dpi) as mycelial growing phase is usually completed within the first 3 days. In all samples, mycelial growth is calculated by quantitative PCR and compared with each other. Student's *t* test, $^*p < .05$

To check this possibility, infected seedlings were stained with trypan blue 3 dpi.

In plants grown under the normal light cycle, mycelia had grown throughout cotyledons, sporangia had formed, and sporulation was observed over the whole surface of the cotyledon (Figure 3a). In contrast to the normal light cycle, in cotyledons exposed to constant light, there were extensive mycelia 3 dpi and abundant oospores, but no conidiophores (Figure 3b). These results indicate that vegetative growth and sexual sporulation can proceed under constant light, but asexual sporulation is suppressed.

In cotyledons exposed to constant dark, less mycelial development was observed in comparison to those that were exposed to either a normal light cycle or constant light (Figure 3c). A small number of oospores were observed, similar to that observed under the constant light experiment (Figure 3b).

To precisely assess Hpa growth in planta, we used a qPCR assay in which Hpa DNA is quantified as a proxy for pathogen biomass. During evaluation over 3 days, mycelial biomass showed an increase in all groups (Figure 4). However, the lowest biomass was observed with constant dark exposure, whilst the constant light gave the highest biomass production on every day. Constant light conditions produced a significant increase in biomass compared to that observed with normal light conditions, especially at 3 dpi. On the other hand, under constant dark conditions, biomass was significantly decreased compared to that obtained with the normal light conditions (Figure 4). Altogether, these results confirm that light is an important factor for vegetative growth and reproduction for Hpa.

3.5 | Effect of different light conditions on spore germination

Because the light and dark affect Hpa vegetative growth and sporulation, we questioned whether the light or dark affect germination of spores and whether it is necessary to have a regular light/dark regime for germination. It is challenging to accurately quantify germination on plant leaves, because trypan blue staining and clearing during the early stages of infection eliminate spores on the leaf surface. Thus, cellophane strips were used for germination assays instead of seedlings.

The germination assay was first carried out with the reference light regime (12 hr L/12 hr D). Under this regime, spores germinate after 6–8 hr and a germ tube emerges (Figure 5a). After 12 and 24 hr, germ tubes have extended on the surface of the cellophane (Figure 5b,c). After 48 hr, formation of mycelial branches was obvious and most branches were laterally oriented as they covered the surface (Figure 5d).

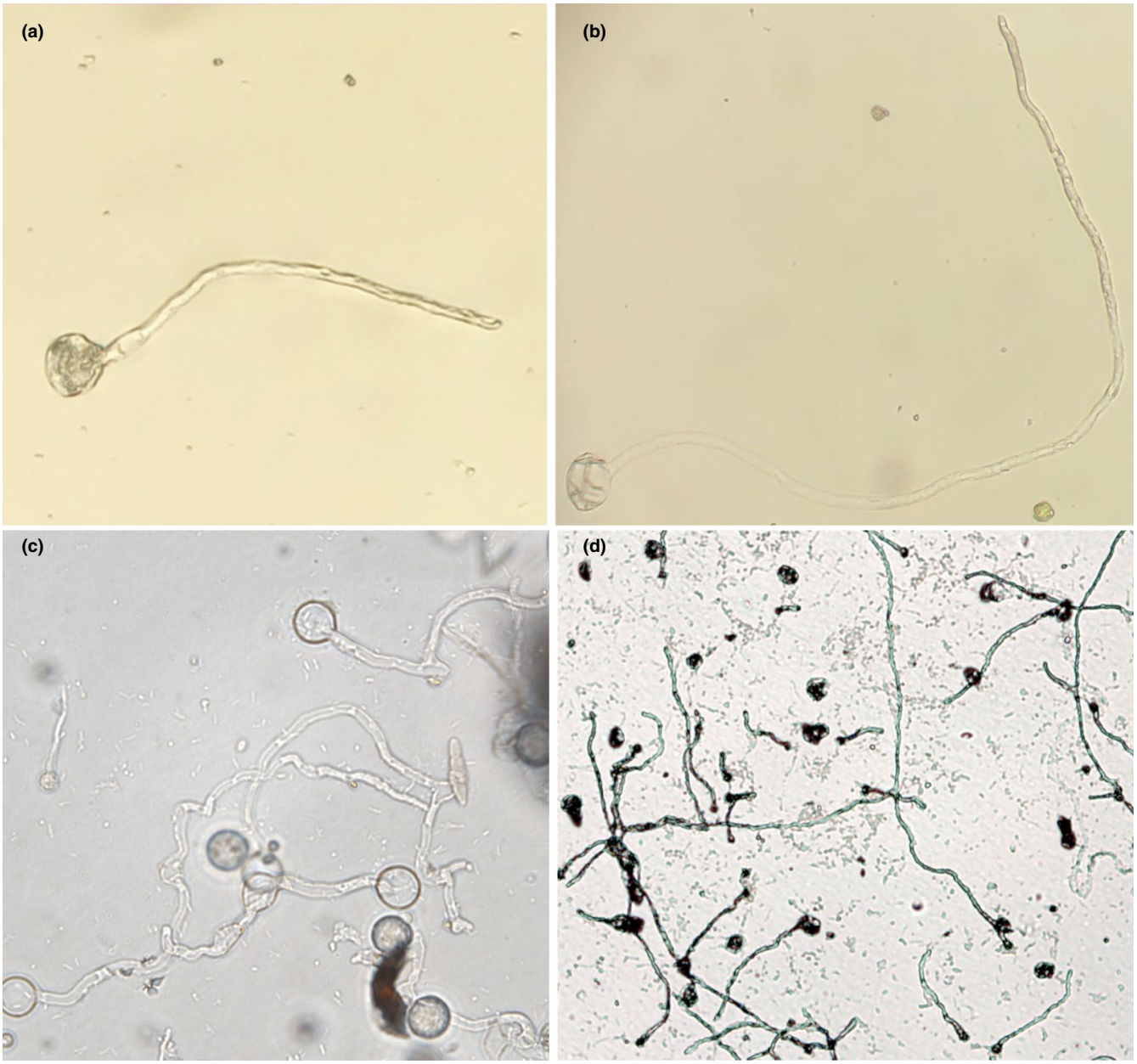

FIGURE 5 Germination of *Hyaloperonospora arabidopsidis* spores on cellophane under 12 hr light/12 hr dark regime. Spores were placed on cellophane strips and examined at regular intervals. (a) After 6 hr, spore had germinated and germ tube was produced; (b, c) after 12 and 24 hr, respectively, the germ tube became longer; (d) after 48 hr, lateral mycelial branches were obvious and hyphae began to cover the surface of the cellophane [Colour figure can be viewed at wileyonlinelibrary.com]

Germination using cellophane strips under constant light and constant dark was assessed in comparison to the reference light regime. The germination rate under the reference regime was 33% after 24 hr. The spores that were exposed to 24 hr constant dark showed a 22% germination rate, which was the lowest percentage observed within this time period. Under constant light at 1 dpi, 37% of Hpa spores were germinated on cellophane (Figure 6). At 2 dpi, the germination percentage increased for all treatments. The germination rate under constant dark was the lowest with 31%, the reference regime was 57%, and constant light was 49%. At 3 dpi, the percentage of germination under constant dark and constant light was the same as at 2 dpi. However, in the reference light regime, germination increased and reached the highest percentage. At the end of 3 days, germination seemed to be completed and spores appeared to have lost their viability. These results indicate that light is an important factor for spore germination independently of the host, and that optimal germination of spores occurs under a normal light/dark regime.

3.6 | Effect of inoculation time on Hpa mycelial biomass growth

If there is a synchronized circadian regulation of Hpa development and host defence, the inoculation time should be important for

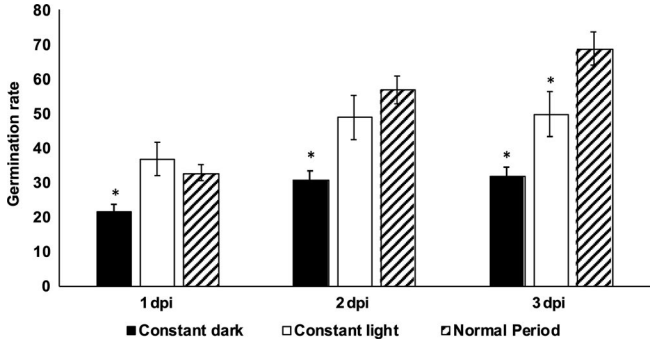

FIGURE 6 Germination rate of *Hyaloperonospora arabidopsidis* spores under different light conditions on cellophane. The spore germination on cellophane, which was exposed to constant light, constant dark, or 12 hr light/12 hr dark regimes (Normal), was determined after 1, 2, and 3 days post-inoculation (dpi). Values represent means of three experiments, and error bars correspond to the standard error of the means. Asterisks indicate statistically significant differences to the reference regime in two-tailed Student's *t* test ($p < .05$)

optimal colonization. Accordingly, previous reports have demonstrated that the time of day for inoculation can affect the degree to which Hpa can successfully colonize *Arabidopsis*, due at least in part to circadian up-regulation of host immune responses during a time period that encompasses subjective dawn. Due to these observations, the optimal infection time for Hpa development was not obvious. Therefore, biomass production between two inoculation times was compared using qPCR. Two zeitgeber time points were determined to observe the effect of day and night (or light and dark) on the development of pathogenicity. ZT0 refers to the beginning of daylight in an entrained cycle and ZT12 is the beginning of night, under experimental conditions of 12 hr L/12 hr D. One sample was inoculated at dawn (ZT0); this was followed by the beginning of the light period, and then the dark period, therefore this sample was called L/D. The other sample was set up as the opposite, with inoculation at dusk (ZT12), called D/L. To determine the dynamic range of qPCR assays, we used an infection time course of virulent Hpa-Emoy2 on Col-*rpp4* (Figure 7). All samples showed a greater biomass

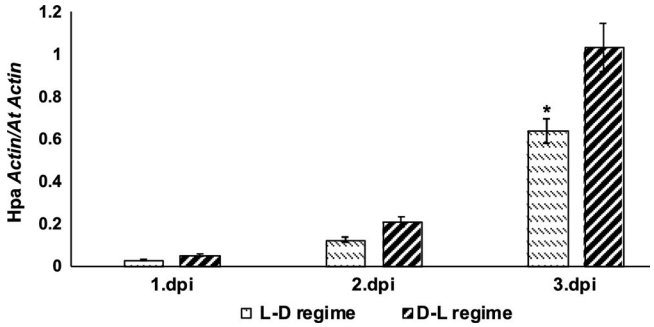

FIGURE 7 Effect of inoculation time on *Hyaloperonospora arabidopsidis* (Hpa) Emoy2 biomass on Col-*rpp4*. Col-*rpp4* seedlings were infected with Hpa at dawn (ZT = 0), labelled L-D, or dusk (ZT = 12), labelled D-L. At 1, 2, and 3 days post-inoculation (dpi), samples were taken and biomass was calculated using quantitative PCR and compared with each other. Student's *t* test, $^*p < .05$

of mycelium on the D/L cycle than L/D cycle and showed a regular increase in mycelium growth over the 3 days (Figure 7). The highest biomass was observed in the D/L cycle, where by 3 dpi, pathogen biomass in samples under the D/L cycle was approximately 56% higher than in samples inoculated under L/D cycle. The results suggest that initiating infection at dusk promotes a higher degree of virulence than initiating infection at dawn.

3.7 | Effect of continuous light/dark regimes on an immune response biomarker

Plant immune responses are characterized by the activation of a set of pathogenesis-related (*PR*) genes (Ward *et al*., 1991; Uknes *et al*., 1992). A *PR1promoter-GUS* reporter gene is considered to be a valid marker gene for activation of immunity in *A. thaliana* (Uknes *et al*., 1992), enabling transgenic *Arabidopsis PR1-GUS* plants to be employed to detect activation of immune responses. We set up an experiment to test whether the reporter gene activity can be induced by constant light and constant dark treatment with the transgenic plants containing *PR1-GUS*. In this assay, when the *GUS* reporter gene is activated by any stress factor, the plant tissues are observed to be stained blue.

In *Arabidopsis* seedlings grown under normal light regime with no pathogen infection, there were no blue-stained cells, indicating that the *GUS* gene was not induced under this condition, as expected (Figure 8a). In contrast, seedlings exposed to constant light (Figure 8b) and constant dark (Figure 8c) for 72 hr showed *GUS* activity, indicating constant light and dark regimes trigger immunity. These results indicate that induction of immunity under constant light or dark exposure could contribute to the suppression of the sporulation of Hpa.

4 | DISCUSSION

Using the Hpa–*Arabidopsis* reference system, we showed that light regimes significantly affect several stages of the Hpa disease cycle, including spore germination, mycelial development, oospore formation, and sporulation. We also obtained preliminary results suggesting that light regimes can influence the immune status of the host. These observations complement recent studies showing that the plant circadian clock system regulates the immune system in the interactions between *Arabidopsis* and Hpa (Wang *et al*., 2011; Zhang *et al*., 2013). However, the previous studies focused mainly on incompatible interactions with resistant plant hosts and did not address how light might affect Hpa in a disease-susceptible host. Therefore, this work was undertaken to investigate the effect of light on a virulent Hpa isolate.

Our initial investigations led to the selection of 12 hr L/12 hr D as a reference regime for the pathogen for ongoing experiments. Subsequent experiments showed that exposure of plants to constant light or dark regimes had a suppressive effect on sporulation (Figure 2).

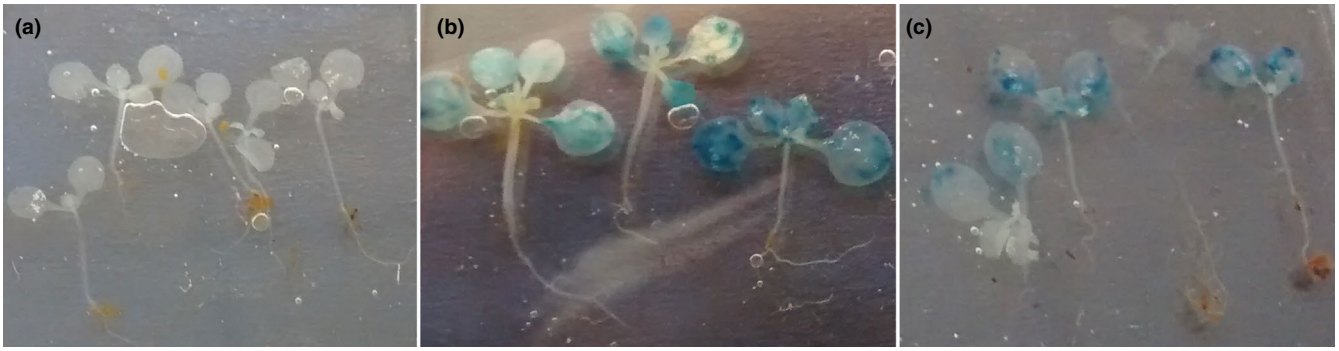

FIGURE 8 *GUS* expression in *Arabidopsis thaliana* seedlings exposed to different light regimes. (a) Seedlings grown under normal 12 hr light/12 hr dark regime; (b) seedlings exposed to constant light; and (c) seedlings exposed to constant dark. After 48 hr of exposure to these regimes, histochemical GUS assays were carried out. These experiments were repeated three times with similar results [Colour figure can be viewed at wileyonlinelibrary.com]

Similar inhibitory effects of light on sporulation of fungal and oomycete pathogens, including downy mildews, have been reported for decades (referenced in the Introduction and reviewed by Rotem *et al*., 1978). However, these studies generally have not directly addressed whether constant light inhibited vegetative (mycelial) growth in planta and/or sporulation. Our investigations showed that constant dark inhibited Hpa growth while constant light supported higher levels of Hpa biomass production than that in normal light/dark or constant dark regimes. Interestingly, constant light exposure induced abundant oospore formation. This apparent inhibition of asexual sporulation by constant light or dark was reversible; plants that were returned to the reference light regime after 4 days of constant light or dark could support abundant sporangiophore production. Similar observations have been reported for other downy mildew pathogens, for which a "recovery" period of 4 hr in the dark was sufficient to enable sporulation (reviewed by Rotem *et al*., 1978). The mechanism behind this recovery is unknown but was postulated at the time to involve enzymatic degradation of a light-induced "antisporulant". Such hypotheses can now be tested with the experimental tools of the Hpa–*Arabidopsis* pathosystem.

In this context, we tested whether constant light- or dark-treatment was sufficient to activate the plant immune system in the absence of pathogen infection. Using transgenic plants containing a fusion of *PR1* promoter to a *GUS* reporter gene, it was clear that after 24 hr, the *PR1* promoter was activated by 24 hr constant light and 48 hr constant dark (Figure 4). These results are similar to those reported in previous publications (Evrard *et al*., 2009). It has been reported that plant defence responses and hypersensitive response-associated programmed cell death triggered by pathogens is activated by light in tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*), and *Arabidopsis*, and the activation of inducible resistance is dependent on phytochrome functions (Guo *et al*., 1993; Chandra-Shekara *et al*., 2006). The blue light receptor cryptochromes (*CRY*) and red/far-red light photoreceptor phytochromes (*PHY*) work together in *Arabidopsis* and they regulate many light-controlled defence responses and entrainment of the circadian clock. The photoreceptor gene *CRY1* regulates systemic acquired resistance (SAR) positively and in the *cry1* mutant, salicylic acid (SA)-induced pathogenesis-related gene *PR-1* expression is reduced, but enhanced in *CRY1-ovx* (*CRY1-overexpressor*) plants under light conditions (Wu and Yang, 2010).

We also tested whether the timing of inoculation affected Hpa's capacity to colonize the plant. A previous report demonstrated that effector-triggered immunity and basal immunity against Hpa is more efficient early in the day (Wang *et al*., 2011), and we confirmed this observation by using a different virulent isolate of Hpa. Our results demonstrate that plants inoculated at dusk supported significantly more mycelial growth than plants inoculated at dawn, even at 3 dpi. Our experiments do not point directly to an underlying mechanism, but we hypothesize that this might reflect a difference in timing of basal defence mechanisms that limit growth of virulent Hpa. Wang *et al*. (2011) noted that SA-dependent gene expression was stronger in the day than at night; accordingly, it was reported that morning and midday inoculations lead to higher SA accumulation, quicker and more intense *PR* (pathogenesis-related) gene activation and expression, and hypersensitive response, than inoculations at dusk or at night (Griebel and Zeier, 2008). These previous reports on different systems support our data and help to explain why night time inoculation is more efficient than day time inoculation.

It is important to emphasize that all experiments involving Hpa grown in planta could reflect influence of light on both the pathogen and the host. Fungal and oomycete pathogens have been shown previously to incorporate light perception into their development and virulence programmes. For example, 48 hr constant white light exposure inhibits sporulation of *P. infestans* on potato or agar plates (Xiang and Judelson, 2014). Because Hpa is an obligate pathogen that can only complete its life cycle on a compatible *Arabidopsis* host, we cannot directly assess how light influences sporulation away from the host. However, our in vitro spore germination assay indicates that light does affect the Hpa life cycle and suggests that Hpa can perceive light.

In conclusion, we have reported several lines of evidence that light is a critical factor during development of downy mildew disease on *Arabidopsis* and can influence responses in the pathogen and the host. We can now exploit this system to understand the mechanistic basis of these effects, using the well-developed tools for *Arabidopsis* in combination with a new protocol for reverse genetics in Hpa. Our future studies will focus on circadian regulation on both the host

and pathogen side. While it is well established that circadian regulation of host immunity is an important factor in immunity against Hpa and other pathogens in *Arabidopsis*, the role of circadian regulation in oomycete virulence is unexplored and therefore could be an enlightening area for future inquiries.

ACKNOWLEDGEMENTS

Financial support for O.T. from the Turkish Ministry of Education and for C.J.-Q. from the University of Worcester are gratefully acknowledged.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author on reasonable request.

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How to cite this article: Telli O, Jimenez-Quiros C, McDowell JM, Tör M. Effect of light and dark on the growth and development of downy mildew pathogen *Hyaloperonospora arabidopsidis*. *Plant Pathol*. 2020;69:1291–1300. https://doi.org/10.1111/ppa.13207