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Growth under Visible Light Increases Conidia and Mucilage Production and Tolerance to UV-B Radiation in the Plant Pathogenic Fungus *Colletotrichum acutatum*

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ABSTRACT

Light conditions can influence fungal development. Some spectral wavebands can induce conidial production, whereas others can kill the conidia, reducing the population size and limiting dispersal. The plant pathogenic fungus *Colletotrichum acutatum* causes anthracnose in several crops. During the asexual stage on the host plant, *Colletototrichum* produces acervuli with abundant mucilage-embedded conidia. These conidia are responsible for fungal dispersal and host infection. This study examined the effect of visible light during *C. acutatum* growth on the production of conidia and mucilage and also on the UV tolerance of these conidia. Conidial tolerance to an environmentally realistic UV irradiance was determined both in conidia surrounded by mucilage on sporulating colonies and in conidial suspension. Exposures to visible light during fungal growth increased production of conidia and mucilage as well as conidial tolerance to UV. Colonies exposed to light produced 1.7 times more conidia than colonies grown in continuous darkness. The UV tolerances of conidia produced under light were at least two times higher than conidia produced in the dark. Conidia embedded in the mucilage on sporulating colonies were more tolerant of UV than conidia in suspension that were washed free of mucilage. Conidial tolerance to UV radiation varied among five selected isolates.

INTRODUCTION

Survival, dispersal, growth and reproduction of fungi can be strongly influenced by exposure to solar radiation although its effects are diverse and often species-dependent (1–6). Among the main selective pressures that drive the perception of solar radiation by fungi is the protection against damage induced by the UV radiation (4,7,8). Due to the correlation between different wavebands in the solar spectrum, exposures to solar radiation are directly related to increases in temperature, dehydration and UV-induced damage in most organisms (6,9–11). Fungi react to solar radiation in different ways and the effects of the radiation depend on the duration of exposure, wavelength and irradiance of the incident photons that strike the cells (1). Exposure to moderate irradiances of visible light (400–700 nm) and near UV radiation (UV-A, 315–400 nm) stimulates conidial production, synthesis of photoprotective pigments and secondary metabolites in several fungal species (12–17). Light exposures can also pre-adapt fungal structures to forthcoming stresses. We reported that conidia of the entomopathogenic fungus *Metarhizium robertsii* produced under visible light are more tolerant to UV-B radiation and heat than those produced in darkness (18). There are also links between light-sensing and fungal pathogenicity and virulence in some animal and plant pathogenic fungi (4,19–21).

Ultraviolet radiation is a normal component of solar radiation and has a wide range of effects on the biology and chemistry of ecosystems (22). Only UV-A and UV-B (280–315 nm) reach the Earth's surface, as atmospheric ozone drastically reduces the penetration of radiation with wavelengths shorter than 320 nm and excludes those below 290 nm (23). Ultraviolet-B is the most harmful solar waveband for fungi, as demonstrated by the evaluation of action spectra for various species (1,5). Adverse effects of solar radiation on fungal conidia are well recognized. Direct exposure to solar radiation for a few hours can kill conidia of most species studied (5,9,10,24). Conidia are killed both by solar UV-A and UV-B radiation (5). In addition to killing conidia, exposures to sublethal doses of UV radiation can reduce conidial germination speed and virulence (1,5,25,26).

Colletotrichum is a large genus of ascomycete fungi containing several species that are common pathogens of a wide array of crops and non-cultivated plant species (27–29). *Colletotrichum*

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acutatum is one of the most pathogenic species of this genus and causes economically important losses of temperate, subtropical and tropical fruits worldwide (27,29). During the asexual stage of its life cycle on the plant host, *C. acutatum* produces acervuli with abundant unicellular conidia surrounded by a water-soluble mucilage (29,30). Conidia are dispersed by rain splash after the mucilage has been dissolved by water (29,31). Previous studies showed that *Colletotrichum* spp. mucilage acts as a conidial protectant enhancing their survival and environmental persistence. For example, mucilage prevents conidial germination before dispersion (32,33), acts as antidesiccant (34) and contains proteins that protect conidia from toxins produced in host tissues (35). Mucilage also protects *Colletotrichum* conidia against the detrimental effect of solar UV radiation (33,36).

Colletotrichum species are exposed to solar radiation during at least part of their life cycle as the fungi grow in the aerial parts of the host plant and produce conidia on the plant surface (30). Although the effect of light on fungal development and physiology has been documented in a wide variety of species, data in this regard are scarce for *C. acutatum* and, as far as we know, there are no studies on light-mediated responses affecting conidial tolerance to environmental stresses such as UV radiation. Recently, we demonstrated that light-based approaches such as antimicrobial photodynamic treatment can be used to kill *Colletotrichum* spp. (37,38). Thus, the study of *C. acutatum* photobiology may help to better understand its population dynamics and epidemiology and perhaps to establish novel and more effective strategies to control this important plant pathogenic fungus.

The objectives of this study were to determine: (1) the effects on conidial and mucilage production from exposure to visible light during the mycelial growth and (2) the tolerance of the produced conidia to UV-B radiation, both on sporulating colonies (conidial masses imbedded in mucilage) and on individual conidia in suspension.

MATERIALS AND METHODS

Fungal species and strains. Colletotrichum acutatum strain CA 142, a causal agent of citrus postbloom fruit drop disease, was obtained from the Plant Pathogenic Fungi Collection of the Department of Plant Pathology and Nematology (Escola Superior de Agricultura "Luiz de Queiroz", University of São Paulo, Piracicaba, Brazil). *C. acutatum* strains FDC 03, FDC 52, FDC 82 and FDC 110 isolated from citrus were obtained from the Fungi Collection of the "Fundo de Defesa da Citricultura" (FUNDECITRUS, Araraquara, Brazil).

Fungal growth and conidia production. C. acutatum isolates were grown on 23 mL Difco™ Potato Dextrose Agar (Becton, Dickinson and Company, Sparks, MD) supplemented with 1 g L^{-1} Bacto™ Yeast Extract (Becton, Dickinson and Company) (PDAY) in Petri dishes (90 × 10 mm) at 28°C for 5 days in continuous darkness or with 12 h photoperiods (dark/light). Light was provided by two fluorescent lamps (Daylight Plus F30W/155-T8; Sylvania, Germany). Plates were covered with a 0.13 mm thick cellulose diacetate film to reduce medium dehydration. The measured irradiance in the visible waveband (700–400 nm) was 3.3 W m^{-2} at sample level (Fig. 1A). After growth, conidia were carefully scraped from the colonies and suspended in a 0.01% (v/v) Tween 80 (Sigma-Aldrich Chemie, St Louis, MO). Conidia concentration was determined with a hemocytometer and the suspension was diluted with the same Tween solution to 2×10^4 conidia mL^{-1}.

Irradiation chamber, lamps and filter. UV irradiation experiments were conducted in a temperature-controlled growth chamber (410 7NDR; Nova Ética, São Paulo Brazil) at 28 ± 1°C. The UV irradiance was provided by two UV-B fluorescent lamps (TL 20W/12 RS; Philips, Eindhoven, Holland). To achieve a stable level of radiation during the different trials, lamps were aged prior to the start of the experiments. The irradiated material (i.e. conidial suspensions or fungal colonies) was covered with a 0.13 mm thick cellulose diacetate film (JCS Industries), which blocked radiation below 290 nm. This allowed the passage of most UV-B and UV-A, but prevented sample exposure to UV-C (<280 nm) and short wavelength UV-B. All UV radiation measurements were made with an Ocean Optics USB4000 spectroradiometer (Dunedin, FL). The spectral irradiance of the filtered UV lamps is shown in Fig. 1B. The biologically effective weighted UV irradiance (UV_{BE}) at the samples level was 1900 mW m^{-2}. The DNA damage action spectrum developed by Quaite *et al.* (39) was used to calculate the UV_{BE}. We selected this spectral weighting function because Paul *et al.* (1) reviewed the spectral characteristics of nine fungal responses and concluded that this DNA damage spectrum closely approximated the fungal response. Musil (40) developed a formulation for this action spectrum which we normalized to unity at 300 nm and weighted the measured spectral irradiance from 290 to 365 nm.

UV tolerance of dark-cultured conidia. Colletotrichum acutatum isolates were grown for 5 days in continuous darkness and a conidial suspension (50 μL, 2×10^4 conidia mL^{-1}) was prepared as previously described. The suspension was spread over the surface of 5 mL of PDAY medium containing 0.08 g L^{-1} of deoxycholic acid sodium salt (Fluka, Italy) in Petri dishes (60 × 15 mm), using a sterile glass spreader. Deoxycholic salt at this concentration makes colonies grow more compactly, allowing the evaluation of fungal growth for several days (37,38). Conidia were immediately exposed to UV_{BE} irradiance of 1900 mW m^{-2} for 1, 2, 3, 4 or 6 h. Treatment doses at the end of the exposures were 6.8, 13.7, 20.5, 27.4 and 41 kJ m^{-2} UV_{BE}, respectively. Four replicate dishes per exposure time were irradiated. Control dishes were protected from radiation by aluminum foil inside the chamber. After exposure, dishes were incubated at 25°C in the dark. Colony forming units (CFU) were counted daily at 8× magnification for up to 7 days. For each trial, conidial relative percent survival after each exposure time was calculated by the following equation: *Relative survival* (%) = $(M_t/M_c) \times 100$, where M_t is the mean number of CFU of the four replicates at exposure time t and M_c is the mean number of CFU of the control dishes. Three independent experiments were performed.

Visible light effects on conidia and mucilage production and on UV tolerance of the produced conidia. Three independent experiments were

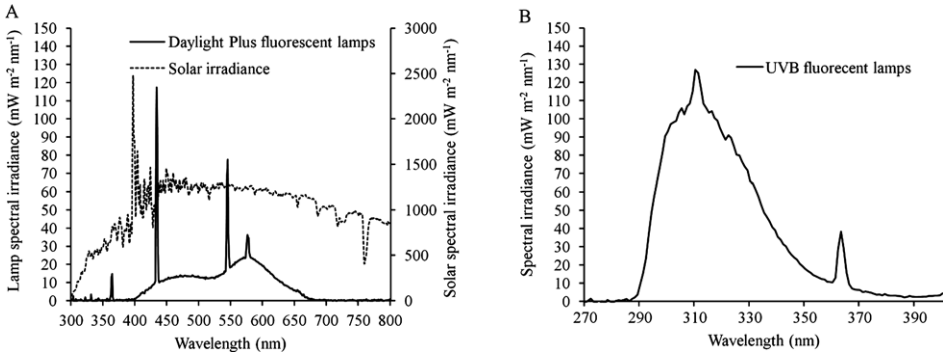

Figure 1. Spectral irradiance in the growth chamber at the sample level and midday solar spectral irradiance under clear sky recorded on August 25th 2014 in Ribeirão Preto, SP, Brazil (21°10′S latitude, 560 m elevation) (A) and spectral irradiance in the UV exposure chamber under cellulose diacetate at the sample level (B).

performed with two selected isolates (FDC 03 and FDC 52). These two isolates were selected because they have been recently isolated from commercial sweet orange orchards in São Paulo State, Brazil and produce conidia and mucilage abundantly. After growth, four 0.5 cm^2 disks of the culture medium were removed from each Petri dish and transferred to tubes containing 5 mL of Tween 80 solution (0.01% v/v). Tubes were vortexed for one minute and the mixture was filtered through cheesecloth to remove the culture medium disks and the hyphal debris. The filtrate was diluted (1:100) and conidial concentration was determined with a hemocytometer.

The production of mucilage by the colonies was determined indirectly by analyzing the absorption spectrum of the mucilage solutions. Disks of the colonies were removed and treated as previously described. After the filtration to remove the medium disks and hyphal debris, conidia were removed from the mixture by centrifugation. The supernatant was diluted (1:10) and its absorption spectrum was determined with an Ultraspec™2100 proUV-visible spectrophotometer (GE Healthcare, Germany).

The UV tolerance of the conidia produced by the colonies grown in the dark and with dark/light photoperiods was determined as described previously. Conidia were exposed to UV for 2 or 3 h.

UV tolerance of conidia on sporulating colonies. Petri dishes containing *C. acutatum* sporulating colonies (FDC 52) grown in the dark or with dark/light photoperiods were exposed to UV_{BE} irradiance of 1900 mW m^{-2} for 3, 5, 7 and 9 h (treatments of 20.5, 34.2, 47.9 and 61.6 kJ m^{-2} UV_{BE}, respectively). Plates not exposed to radiation were used as controls. After exposures, conidial samples from each treatment were carefully scrapped from the colonies and suspended in a 0.01% (v/v) Tween 80 solution. Conidial concentration was adjusted to 2×10^4 conidia mL^{-1} and 50 μL of the suspension were spread over the surface of 5 mL of PDAY medium containing 0.08 g L^{-1} of deoxycholic acid sodium salt in Petri dishes (60 × 15 mm) and incubated in the dark. Conidial relative percent survivals were calculated as previously described. Three independent experiments were performed.

Statistical analysis. Survival data were subjected to one-way analysis of variance (ANOVA) to compare the effect of the different treatments. No data transformations were required in any experiment. Comparisons between treatments were performed using orthogonal contrasts (41). *P* values < 0.05 were considered significant. All analyses were carried out using PROC MIXED in SAS version 9.0 (SAS Institute, Inc., Carey, NC).

RESULTS

UV tolerance of dark-cultured conidia

A strong dose-dependent negative effect of UV radiation on conidial survival was observed for all *C. acutatum* isolates but the isolates differed in UV tolerance (Fig. 2). After 1 h of exposure, isolates CA 142 and FDC 03 showed relative survival lower than the other isolates. Exposures of 2 h were enough to kill between 50% (FDC 82 and FDC 52 isolates) and 80% (FDC 03) of the conidia. Exposures of 4 h killed approximately 95% of the conidia of the most tolerant (FDC 52) and exposure of 6 h killed 100% of the conidia of all the five isolates (Fig. 2).

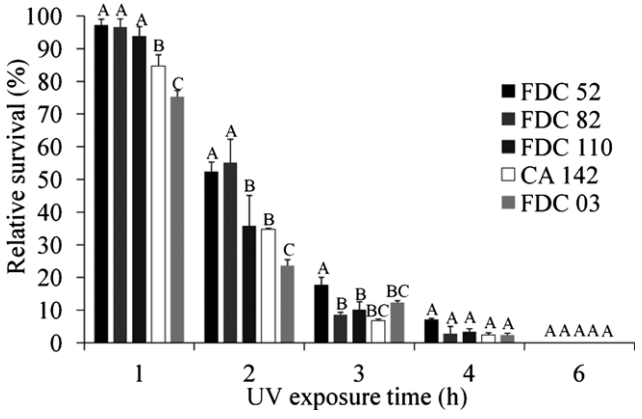

Figure 2. Relative percent survival after UV treatment of *Colletotrichum acutatum* conidia from five different isolates cultured in the dark. The UV_{BE} irradiance was 1900 mW m^{-2}. Letters indicate the significance of the comparison among strains for each exposure time ($P < 0.05$). Survival was calculated in relation to non-irradiated controls. Error bars are standard deviations of the three independent experiments.

Visible light effects on production of conidia and mucilage and on UV tolerance of the produced conidia

Colonies grown under a 12 h photoperiod produced approximately 1.7 times more conidia than colonies grown in continuous darkness (Fig. 3A). Exposure to visible light also stimulated the production of mucilage by the fungus (Fig. 3B and Supplementary Fig. S1). The mucilage produced by the colonies grown under light presented absorption peaks in the UV-C and UV-B at 310 nm (Fig. 3B).

Conidia of the isolates FDC 52 and FDC 03 produced under light were much more tolerant to UV radiation than those from colonies grown in the dark (Fig. 4). After 2 h of UV exposure, survival of the conidia of the isolate FDC 52 produced under light was 96.8% whereas survival of the conidia produced in the dark was only 50%. Similar results were observed for isolate FDC 03. Differences in survival between conidia produced under light or in darkness were significant for all comparisons ($P < 0.0001$).

UV tolerance of conidia on sporulating colonies

Survival of the conidia embedded in mucilage on the sporulating colonies grown under light was higher than those on sporulating colonies grown in continuous darkness ($P < 0.0001$ for all comparisons). Also, tolerance to UV radiation of conidia embedded in mucilage was higher than those free of the mucilage (compare Fig. 5 with Figs. 2 and 4).

DISCUSSION

Solar UV radiation is one of the main environmental factors that can kill conidia of several fungal pathogens (42–44). This study shows that conidia produced in dark-grown cultures and washed free of the surrounding mucilage are all inactivated by a few hours of exposure to UV irradiance equivalent to that found in nature. While this was true for all five isolates, we observed some variability in UV tolerance among them. Variation in conidial tolerance to UV-B radiation was previously observed in other fungal species and at least part of it has been explained by the adaptation of the isolates to different environmental UV levels associated with the sites of origin (25,45–47). Our results indicate that in *C. acutatum* variation in conidial tolerance to UV occurs even among strains isolated in the same habitat (commercial orange orchards in São Paulo State) and in sites close to each other.

Growth of *C. acutatum* colonies under low irradiance of visible light increased conidia and mucilage production and also the tolerance to UV radiation of the produced conidia. The increase in conidia production induced by visible light was previously observed in other fungal genera including *Colletotrichum* (15,48). Exposure to light also increased mucilage production and changed its absorption spectrum. Mucilage produced by the colonies exposed to light present absorption peaks in UV-C and

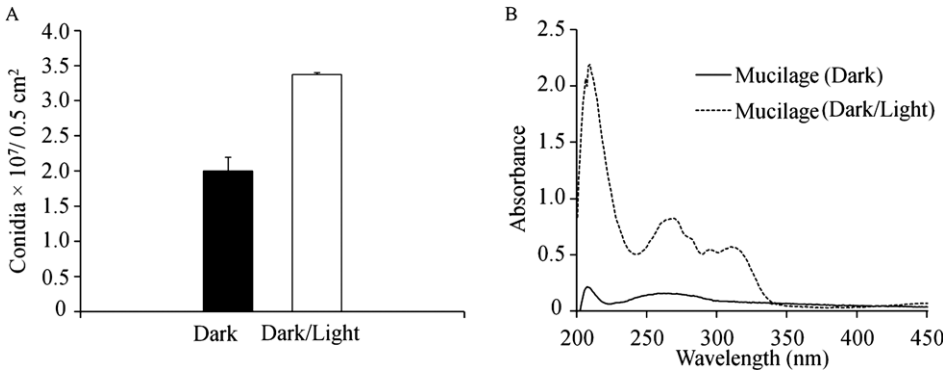

Figure 3. Conidial production (A) and absorption spectra of mucilage (B) produced by *Colletotrichum acutatum* isotate FDC 52. Colonies were grown under a 12 h photoperiod (dark/light) or continuous darkness. Error bars are standard deviations of the three independent experiments.

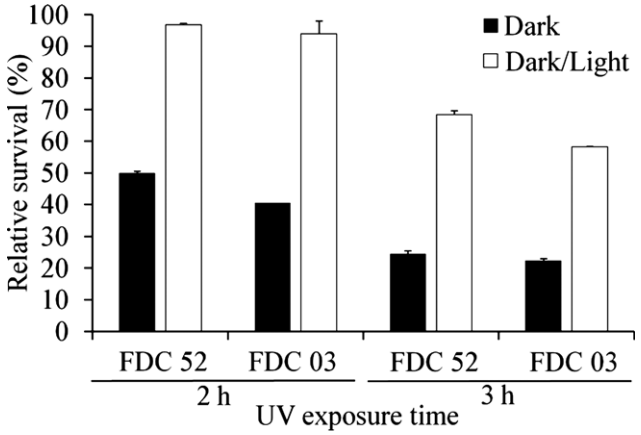

Figure 4. Relative percent survival of *Colletotrichum acutatum* conidia (isolates FDC 03 and FDC 52) exposed for 2 and 3 h to UV_{BE} irradiance of 1900 mW m^{-2}. Colonies were grown under a 12 h photoperiod (dark/light) or in continuous darkness. Survival was calculated in relation to non-irradiated controls. Error bars are standard deviations of the three independent experiments. Differences in survival of conidia produced under light or in darkness were significant for all comparisons ($P < 0.0001$).

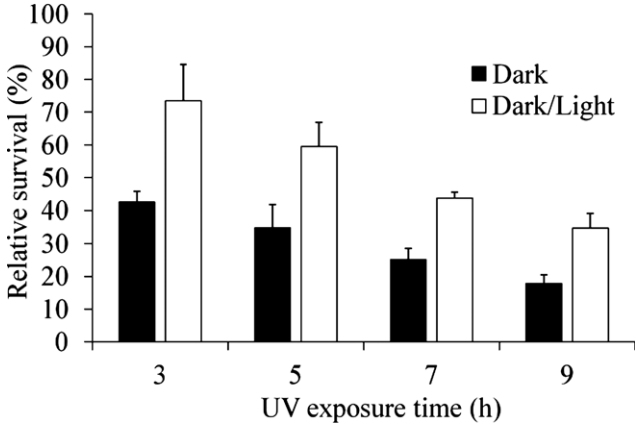

Figure 5. Relative percent survival of conidia of *Colletotrichum acutatum* (isolate FDC 52) embedded in the mucilage treated with various UV exposures. The UV_{BE} irradiance was 1900 mW m^{-2}. Prior to UV treatment, colonies were grown under a 12 h photoperiod (dark/light) or in continuous darkness. Survival was calculated in relation to nonexposed controls. Error bars are standard deviations of the three independent experiments. Differences in survival between conidia produced under light or in darkness were significant for all comparisons ($P < 0.0001$).

UV-B regions. Previous studies with *C. graminicola* and *C. musae* showed that mucilage is chemically complex and contains UV-absorbing compounds such as mycosporines that absorb specifically at 240 and 310 nm. These compounds act as photoprotectants in other fungal species (49–51). Suspended conidia produced by colonies exposed to light were much more tolerant to UV radiation than conidia produced by colonies grown in the dark. Because in these experiments mucilage was washed out before conidia had been exposed to UV radiation, the difference in tolerance was most likely due to the induction of endogenous photoprotectants. Conidial tolerance to solar UV radiation is a quantitative trait determined by protective mechanisms (i.e. UV-absorbing pigments) that prevent or reduce the occurrence of damage to intracellular compounds, and by several systems that repair the damage caused by radiation (i.e. photoreactivation and nucleotide excision repair) (6,52–54).

Conidia on sporulating colonies that grew exposed to light were also more tolerant than those produced in the darkness. As expected, mucilage-surrounded conidia on the sporulating colonies were more tolerant to UV radiation than conidia in suspension. After 9 h exposure to UV-B treatment, approximately 30% of the mucilage-surrounded conidia were still able to germinate and form colonies. The protective effect of mucilage against UV radiation was previously reported in *C. acutatum* and *C. musae* (36,55) but our study is the first to document this with environmentally realistic UV radiation fluences.

In natural environmental conditions, *Colletotrichum* conidia are exposed to solar radiation either in conidial masses surrounded by mucilage or in a less-protected state after rain disperses conidia from the mucilage. In conidial masses, solar UV is partially shaded by the surrounding conidia and mycelia and also by the mucilage which absorbs UV and acts as a sunscreen. After dispersal, conidial protection against the detrimental effects of solar radiation will depend mainly on the presence of endogenous photoprotectants.

In mid-latitude locations, total daily UV_{BE} irradiances in early summer would approximate our 5-hour treatment (56). At the latitudes where our isolates originated (21°S to 23°S), late spring through early summer daily UV_{BE} irradiances would be higher and would be represented by the range of the 6–7 h treatments. Thus in nature, where conidia develop under sunlight, we would expect only a portion of the conidia in acervuli to be inactivated by a day of unobstructed sunlight. Inactivation would be higher in dispersed conidia washed free of mucilage. On the basis of

our results, we can speculate that solar UV radiation plays a major role in the control of natural populations of this species. Studies with other fungal pathogens demonstrated that in addition to killing conidia, exposure to sublethal levels of UV radiation can cause other adverse effects that reduce the ability of fungi to infect the host (5,6).

The effects of solar UV irradiance and fluence on the severity of fungal diseases vary greatly and would need to be assessed on a case-by-case basis for each pathogen-host association. For example, increasing ambient UV-B significantly reduced the number of lesions caused by *Septoria tritici* in wheat (*Triticum aestivum*) as a result of conidia germination and germ tube growth being strongly inhibited by UV-B (1,26). Similarly, maize (*Zea mays*) leaves exposed to relatively low levels of solar radiation exhibited a significantly greater percentage of lesions caused by *C. graminicola* than did those exposed to higher levels (57). The opposite was also reported. Ningen *et al.* (11) observed that increased shade decreased anthracnose severity caused by *C. gloeosporioides* on fortune's spindle (*Euonymus fortune*) cultivars. Different light wavelengths have a major effect on *C. acutatum* virulence by influencing melanin production. Higher pigmentation was visually observed under white and blue light, while the least pigmentation was observed in mycelia incubated in the dark (20).

In conclusion, light conditions during *C. acutatum* growth influence both the quantity and the characteristics of the produced conidia. Growth under visible light increases conidia and mucilage production and tolerance to UV-B radiation of the conidia. Exposure to UV-B radiation is highly detrimental to *Colletotrichum* conidia. A few hours of exposure to an UV-B irradiance easily found in nature are sufficient to inactivate washed conidia. Conidia embedded in the mucilage on sporulating colonies were much more tolerant to UV-B radiation than washed conidia. This study shows the importance of using realistic culture conditions when evaluating the UV tolerance of fungal plant pathogens.

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SUPPORTING INFORMATION

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

Figures S1 and **S2** can be found online on DOI: 10.1111/php.12410

Figure S1. Macroscopic appearance of *Colletotrichum acutatum* isolate FDC 52 cultured for 5 days (A) under 12 h photoperiod (dark/light) or (B) continuous darkness.

Figure S2. Microscopic appearance *Colletotrichum acutatum* isolate FDC 52 cultured for 5 days (A) under 12 h photoperiod (dark/light) or (B) continuous darkness.

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