

Response of the fungus *Pseudocercospora griseola* f. *mesoamericana* to Tricyclazole

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Abstract *Pseudocercospora griseola*, an anamorph of *Mycosphaerella*, causes Angular Leaf Spot (ALS). The mycelia and conidia from *P. griseola* are coloured due to the synthesis of 1,8 dihydroxynaphthalene (DHN)-melanin. The aim of this work was to identify in *P. griseola* f. *mesoamericana* isolate T4, a highly pigmented fungus, intermediary compounds as a result of the inhibition of melanin synthesis by tricyclazole, and to analyze at the structural level the localization of these dark pigments. The main metabolites were analyzed using ultraviolet matrix-assisted laser desorption-ionization mass spectrometry (UV-MALDI MS). Tricyclazole affected *P. griseola* f. *mesoamericana* in several different ways. The most evident effect was the reduction of melanin synthesis, and therefore diffusible shunt products were found and identified. Flaviolin was the main intermediate metabolite found in cultures supplemented with tricyclazole. This inhibitor, which affected pigmentation and the cell wall structure of mycelium, revealed macroscopically

by the reduction in growth, decreased the stratification and deposition of melanin in the hyphal wall. These results suggest a possible role of tricyclazole to control ALS.

Keywords *Pseudocercospora griseola* f. *mesoamericana* · *Phaseolus vulgaris* · Melanin · Tricyclazole · UV-MALDI-TOF MS · Microscopy · FTIR

Introduction

Pseudocercospora griseola (Sacc.) Crous & U. Braun, an anamorph of *Mycosphaerella* (Ascomycota), is the etiological agent of Angular Leaf Spot (ALS), a serious disease of common bean (*Phaseolus vulgaris* L.) around the world (Schwartz and Galvez 1980; Ddamulira et al. 2014) and the most important endemic disease in northwestern Argentina (20 to 50 % of yield losses, Vizgarra et al. 2010). *P. griseola* coevolved with

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its host, and much like the plant, the fungus has two major intraspecific groups, the formae *P. griseola* f. *griseola* (Andean group) and *P. griseola* f. *mesoamericana* (Mesoamerican group; Crous et al. 2006). Although both gene pools are pathogenic, *P. griseola* f. *mesoamericana* shows a broader range of virulence on bean varieties and a darker pigmentation and also tolerates higher temperatures, compared to those for *P. griseola* f. *griseola* (Crous et al. 2006).

Pseudocercospora griseola and other representatives of Mycosphaerellaceae synthesize dark pigments known as melanins. In contrast to other fungal species that need external precursors to synthesize this pigment (Franzen et al. 2008), *P. griseola* synthesizes melanin constitutively, which might be related to morphogenesis, pathogenesis and/or survival strategies of the fungus (Elliott 1995; Langfelder et al. 2003; Suryanarayanan et al. 2004). Recently, Saparrat et al. (2009) described that representatives of *P. griseola* from both gene pools synthesize 1,8-dihydroxynaphthalene (DHN) melanin. These same pigments have already been found in most Ascomycota, such as in *Alternaria alternata* (Carzaniga et al. 2002), *Bipolaris oryzae* (Moriwaki et al. 2004), *Bipolaris sorokiniana* (Kumar et al. 2015), *Cladosporium cladosporioides* (Llorente et al. 2012), *Colletotrichum lagenarium*, *Magnaporthe grisea* (Moriwaki et al. 2004), *Pseudocercospora fijiensis* (Beltrán-García et al. 2014) and *Sporothrix schenckii* (Romero-Martinez et al. 2000). However, information regarding the pathway of pigment synthesis as well as its localization at the ultrastructural level in *P. griseola* is lacking.

Strategies to manage ALS include cultural practices such as the use of resistance varieties of beans as well as chemical control (Stenglein et al. 2003; Paparu et al. 2014). The most effective fungicides to control *P. griseola* are dithiocarbamates and benzimidazols. While the former affect cytochrome P-450, the latter bind to tubulins, and this inhibits cellular division and therefore mycelial growth (Schwartz and Galvez 1980). Pigmentation of *P. griseola* hinders its control (Ddamulira et al. 2014). However, there is no information regarding the effect of these types of fungicides on melanin synthesis and/or their biological impact in this fungus. However, Llorente et al. (2012), while studying melanin synthesis on *Cladosporium cladosporioides*, found that Difenoconazole (Bogard 25 %, Syngenta Agro S.A., Argentina) and Clorotalonil (Daconil 72F, Syngenta Agro S.A., Argentina) generate a drastic reduction in fungal growth and conidiation. Concomitantly, cultures turned darker, most probably due to melanin deposition that seems to be a quencher of stress.

Tricyclazole (5-methyl-1,2,4-triazolo[3,4-b]benzothiazole; Fig. 1a) is a molecule that affects the DHN-melanin biosynthesis pathway through the inhibition of reductases that catalyze the transformation of 1, 3, 6, 8-tetrahydroxynaphthalene (1, 3, 6, 8-THN) to scytalone and of 1,3,8-trihydroxynaphthalene (1,

3, 8-THN) to vermelone (Bell and Wheeler 1986; Nobre and Ferreira 2004). Saparrat et al. (2009) and Llorente et al. (2012) demonstrated that this fungicide reduced growth and melanization both on *P. griseola* and *C. cladosporioides*. Furthermore, this compound is used to control plant pathogens such as *Magnaporthe grisea*, *Bipolaris oryzae*, *B. sorokiniana*, *Blumeria graminis*, *Colletotrichum lagenarium*, *C. lindemuthianum* and *Rhynchosporium secalis* (Mares et al. 2004; Kunova et al. 2012; Kumar and Mukherjee 2013; Kumar et al. 2015).

The aim of this work was to evaluate the effect of tricyclazole on *Pseudocercospora griseola* f. *mesoamericana* growth, morphology and pigmentation when the fungus is grown in vitro. We studied melanin biosynthesis indirectly by identifying intermediary compounds as a result of the inhibitory activity of tricyclazole on reductases. Identifying biosynthetic intermediates provides an opportunity to investigate the role of the melanin in the causative agent of ALS and the potential use of tricyclazole to control it. Furthermore, we studied the localization of these dark pigments in fungal cells by Fourier Transform Infrared (FTIR) and microscopical analysis, methods that are diagnostic of melanin (Kayatz et al. 2001; Tavzes et al. 2009; Llorente et al. 2012).

Materials and methods

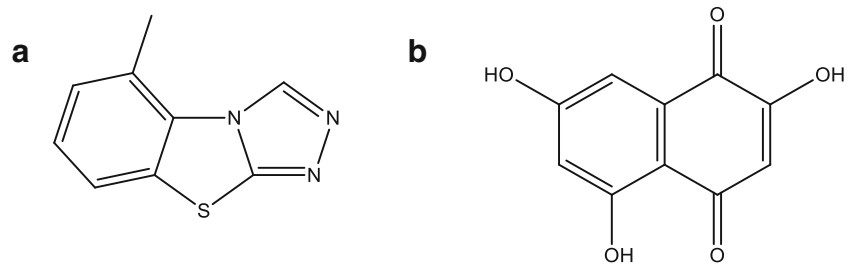
Fungal material

Isolate T4 of *P. griseola* representing the Mesoamerican (*P. griseola* f. *mesoamericana*) group was obtained from a typical leaf spot symptom (Stenglein and Balatti 2006). This fungal isolate was deposited in the Culture Collection of the Centro de Investigaciones de Fitopatología (CIDEFI), Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata as CIDEFI 232. It was kept at -20°C on filter paper impregnated with a conidia-mycelium suspension and as a culture grown for 7 days on a potato dextrose agar (PDA) medium at $24\pm 2^{\circ}\text{C}$ and maintained at 4°C . Inoculation was carried out by using a conidial suspension obtained according to Llorente et al. (2012), and unless specifically stated, cultures on PDA (control) and PDA supplemented with tricyclazole (Ultra Scientific Analytical Solutions, United States) were incubated in the dark at $24\pm 2^{\circ}\text{C}$ for 21 days.

Isolation and identification of melanin pathway intermediates by UV-MALDI MS

Intermediate metabolites of the fungus that was grown on PDA (control) and PDA supplemented with tricyclazole were

Fig. 1 Chemical structure of (a) tricyclazole, an inhibitor of 1,8-DHN melanin synthesis, and (b) flaviolin, the shunt product generated by the inhibition of the DHN-melanin biosynthesis pathway in *P. griseola* f. *mesoamericana* by tricyclazole



extracted with ethyl-acetate (Wheeler and Klich 1995). A representative culture growing on either PDA (control) or PDA supplemented with 100 ppm of tricyclazole was treated with ethyl-acetate for 2 and 24 h. On aliquots from the two ethyl-acetate extracts obtained from each culture type, three independent UV-MALDI analytic measurements were conducted. This procedure was repeated in a second experiment using the same replicate number. As a reference, we used a freshly prepared ethanolic solution of standard tricyclazole (2 mg ml⁻¹) in a straightforward manner. Samples were analyzed by ultraviolet matrix assisted laser desorption-ionization mass spectrometry (UV-MALDI MS) performed on the Bruker Ultraflex Daltonics TOF/TOF mass spectrometer. In preliminary assays, several compounds (α -cyano-4-hydroxycinnamic acid, CHCA; sinapinic acid, SA; 2,5-dihydroxybenzoic acid (gentisic acid), GA; 9H-[3,4b] piridoindole (norharmane), nHo) were evaluated as MALDI matrix showing similar broad signals for the molecular ions. Mass spectra were acquired in linear positive and negative ion modes and with the LIFT device in the MS/MS mode. External mass calibration was made using β -cyclodextrin (MW 1134) with nHo and GA as matrices in positive and negative ion mode. Sample solutions were spotted on a MTP 384 target plate polished steel from Bruker Daltonics. Matrix solutions were prepared by dissolving nHo or GA in water (1 mg ml⁻¹). Dry droplet sample preparation (sandwich method) was used according to Nonami et al. (1997), loading 0.5 μ l of matrix solution, analyte solution and matrix solution successively after drying each layer at normal atmosphere and room temperature. The matrix to analyte ratio was 3:1 (v v⁻¹) and the matrix and analyte solution loading sequence was: 1) matrix, 2) analyte, 3) matrix, 4) matrix. Desorption/Ionization was obtained by using the frequency-tripled Nd: YAG laser (355-nm). The laser power was adjusted to obtain high signal-to-noise ratio (S/N) while ensuring minimal fragmentation of the parent ions and each mass spectrum was generated by averaging 100 lasers pulses per spot. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively. The resolution of the signals detected was estimated according to their Full Width at Half-Maximum (FWHM), which in most cases was approx. 0.8 m/z unities.

Fourier transform infrared (FTIR)

Since FTIR has been used to identify several melanins in polymeric matrices by the presence of specific functional groups and chemical bonds in their structure (Tavzes et al. 2009; Tu et al. 2009; Llorente et al. 2012), the chemical composition of dry mycelium samples from three 21-day-old cultures grown on PDA (control) and PDA supplemented with tricyclazole at 100 ppm were analyzed by means of the potassium bromide (KBr) method on a Bruker Fourier-transform IR spectrophotometer. The fungal material from both types of cultures was washed with water at 100 °C for 5 min. Then, the resultant suspension was centrifuged at 5,000 g for 5 min and the pellet was dried in an oven at 40 °C overnight. Dry mycelium was embedded in infrared grade KBr disk (2 mg 20 mg⁻¹) and recorded in a Perkin-Elmer-Instruments spectrometer (scanning range: 400–4,000 cm⁻¹, resolution: 4 cm⁻¹, number of scans: 64), the data of which was processed by means of the software OMNIC (Thermo Nicolet, Madison, WI) according to Saparrat et al. (2010). This experiment was performed once with three replicates.

Microscopical analysis

Representative samples of mycelium taken from three 21-day-old cultures growing on PDA (control) and PDA supplemented with tricyclazole at 100 ppm were observed with light (LM), transmission electron (TEM) and confocal (CM) microscopes. Each experiment was performed twice.

For LM, the samples were directly observed with an Olympus BX51 light microscope equipped with an Olympus E-330 digital camera.

Samples for TEM were fixed with 2 % glutaraldehyde 2 h at 4 °C and then washed with phosphate buffer. They were post fixed 2 h at 4 °C with 1 % osmium tetroxide, washed with phosphate buffer, dehydrated with ascending grades of ethanol. The procedure ended with samples immersed in acetone. Then samples were embedded in Epoxy resin. Ultrathin sections of 60 nm were cut on a Reichart-J Super Nova Ultracut. Sections were placed on copper grids and stained with uranyl acetate 1 % and lead citrate (Kogej et al. 2006). Samples were viewed on a JEOL JEM 1200 EX II TEM and micrographs were taken with a digital camera Erlangshen ES 1000 W.

Samples for in situ detection of melanin in cell walls were oxidized by exposing to H_2O_2 6 M and light for 1 h according to Kayatz et al. (2001), and seen by CM in an Olympus FV300 CM (Olympus Latin America) equipped with a 60 X 1.2 N.A. water immersion objective. Melanin fluorescence was detected using a 488 nm laser (excitation) and emission filters at 505–525 nm. Since autofluorescence of vesicles was found at this range of wavelength when samples were not exposed to hydrogen peroxide and light, the amount of fluorescence signal on both cell wall and intracellular particles was processed and quantified according to Ricardi et al. (2012). Means of the treatments were contrasted by the Student's *t* Test (at $p \leq 0.05$) using the Statistix 8.0 software for Windows.

Colony growth and pigmentation under a range of tricyclazole concentrations

The PDA medium was amended with tricyclazole at a concentration of 12.5, 25, 100 and 200 ppm (van de Sande et al. 2007). Colony growth was estimated by determining colony diameter and color was measured by estimating darkness (*k*) of the colonies. To do this, scanned images of colonies were analysed with a grey scale using Adobe® Photoshop® 8.0.1 (Griffith et al. 2007). The relationship between the percentage of growth inhibition and the concentration of the fungicide

was estimated; this allowed us to calculate the concentration of fungicide that caused 50 % of growth inhibition (IC_{50} ; Llorente et al. 2012). This experiment was performed twice with three replicates at each concentration. Data were analyzed by means of a one-way analysis of variance (ANOVA) and means of the treatments were contrasted by the Tukey Test (at $p \leq 0.05$) using the Statistix 8.0 software for Windows.

Results

UV-MALDI-mass spectrometry analysis of metabolites from ethyl-acetate extracts of *P. griseola f. mesoamericana* determined the presence of DHN-melanin precursors or related metabolites. Figure 2 shows UV-MALDI mass spectra of the samples obtained from 21-day-old cultures of isolate T4 grown on PDA without (control) and with addition of tricyclazole 100 ppm (Fig. 2a and b, respectively). In the presence of tricyclazole, a signal at $m/z=206.52$ was detected, compatible with the compound flaviolin (2,5,7-trihydroxy-1,4-naphthoquinone; Fig. 1b, exact mass 206.02), which is an oxidation product of 1,3,6,8-THN. This was confirmed by MS/MS spectrum of the ion detected [M^+ , $\text{C}_{10}\text{H}_6\text{O}_5$] and its fragmentation pattern (Fig. S1 and Scheme S1). This signal at m/z 206 was absent in the control (Fig. 2a) and is not a matrix

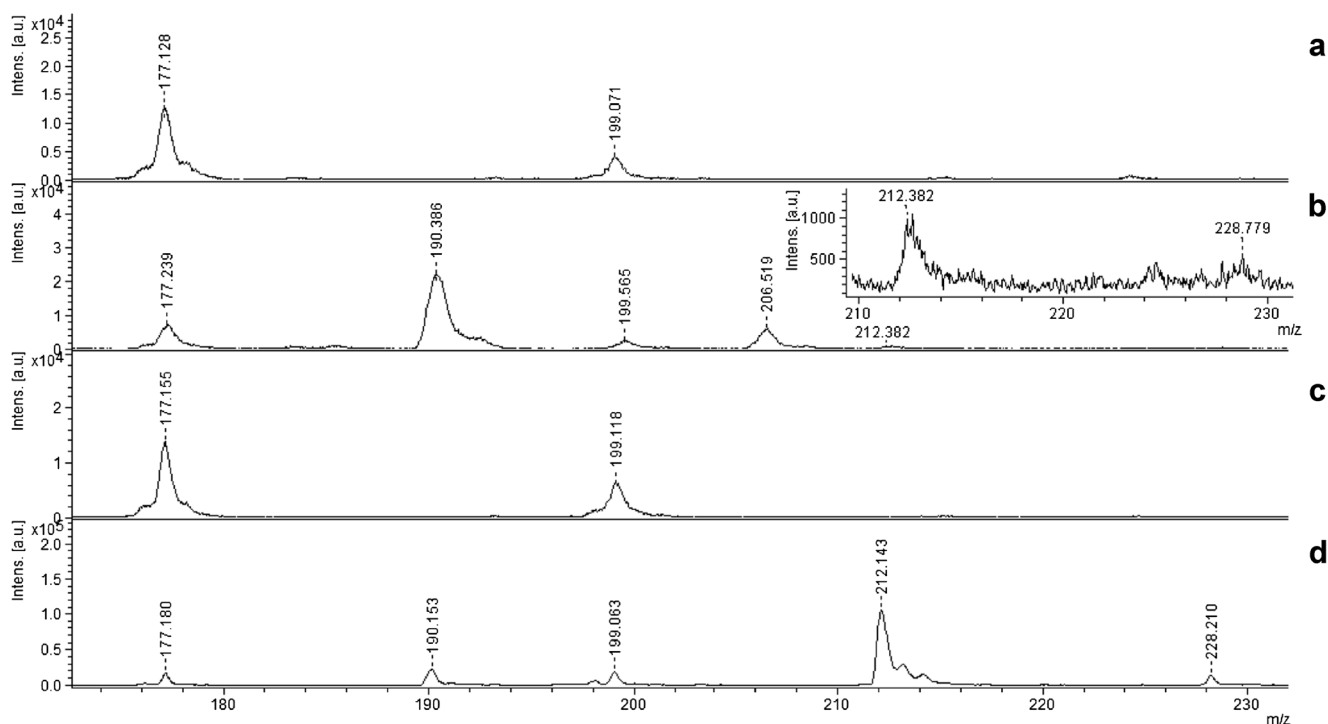


Fig. 2 Positive ion MALDI mass spectra of ethylacetate extract corresponding to 21-day-old cultures of T4 grown on PDA: **a**) control 24 h; **b**) supplemented with 100 ppm tricyclazole 24 h, including an inset shows the zoom between m/z region 210 and 230; **c**) gentisic acid

(matrix) and **d**) tricyclazole. Matrix: gentisic acid. Peaks assignment: $m/z=190$ [$\text{Tricyclazole}+\text{H}$] $^+$; $m/z=206$ [flaviolin] $^+$; $m/z=212$ [$\text{Tricyclazole}+\text{Na}$] $^+$; $m/z=228$ [$\text{Tricyclazole}+\text{K}$] $^+$

cluster (Fig. 2c). Concomitantly with this, the mycelium and extracellular medium of tricyclazole-grown cultures developed a reddish coloration (Fig. 5b). Under such conditions, a signal at $m/z=190.39$ was detected (Fig. 2b). In order to determine if this signal corresponds to the intermediate 2-hydroxy juglone (2-HJ; exact mass=190.02) or to protonated tricyclazole (exact mass=190.04), the mass spectrum of tricyclazole was measured (Fig. 2d). As expected, the protonated ($m/z=190.15$), sodiated ($m/z=212.14$) and potassiated tricyclazole ($m/z=228.21$) ions were detected. Additionally, UV-MALDI MS/MS mass spectra of the ion with $m/z=190$ observed in the tricyclazole spectrum and that observed at similar m/z value in the spectrum of ethyl-acetate extract in the presence of inhibitor were obtained (Fig. 3). The fragmentation patterns of both MS/MS spectra obtained were similar, indicating that the later signal (Fig. 2b) corresponded to the tricyclazole moiety. Furthermore, symmetric Gaussian patterns in the signals detected on the UV-MALDI MS spectra for flaviolin (Fig. 2b) and tricyclazole (Fig. 2b and d) showed relevant levels of intensity (approx. 6000 a.u.), which according to m/z values, is accurate and therefore reveals the presence of a single molecule species.

The FTIR spectra of dry mycelia from control as well as 100 ppm tricyclazole-treated cultures presented similar bands of absorption. They were characterized by peaks between $1670\text{--}1600\text{ cm}^{-1}$ that correspond to conjugated double bonds $C=C$ and $C=O$ in the aromatic ring as well as $C=O$ in

secondary amines, diagnostic features of typical conjugated quinoid structures such as those of melanins (Fig. 4).

Hyphae and conidia of cultures supplemented or not supplemented with 100 ppm tricyclazole were analyzed microscopically (Fig. 5a and b). When control cultures were examined by LM, it was observed that fungal walls were melanized (Fig. 5c). In fungi grown with tricyclazole, cell walls were clearer (Fig. 5d). Further analysis with TEM showed that while the hyphal walls from mycelia developed on control PDA presented an organized inner electron-transparent layer and an outer electron-dense one, those from fungi grown with tricyclazole did not (Fig. 6). Furthermore, when mycelia were treated with H_2O_2 and light, and were then examined with CM, a fluorescent melanin-type material was found in the cell wall of all cultures (Fig. 7a.2 and b.2). Additionally, when the fungal material was not treated with H_2O_2 -light, no fluorescence in the hyphal wall was detected (Fig. 7a.1 and b.1), but lower signal was detected in cultures grown with tricyclazole (Fig. 7b.2), and an autofluorescent signal for intracellular vesicles was found both in the control cultures and in those treated with tricyclazole. Therefore, a higher cell wall/vesicles fluorescence relationship was found in control cultures treated with H_2O_2 -light than in those from cultures supplemented with tricyclazole (Fig. 8).

Isolate T4 was grown on PDA medium and PDA supplemented with 12.5, 25, 100 and 200 ppm tricyclazole, which affected growth and pigmentation (Fig. 9a and b respectively). This increase in concentration led to the development of

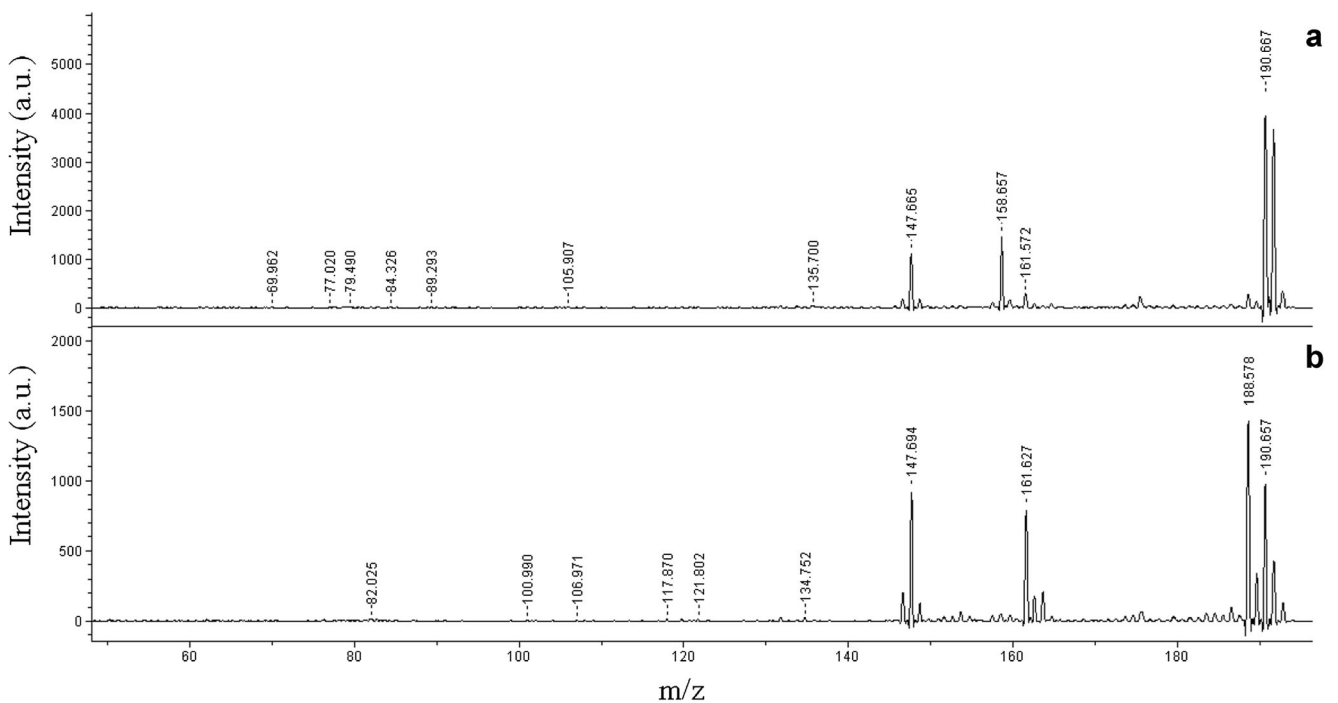


Fig. 3 Positive ion UV-MALDI MS/MS mass spectra of $[M+H]^+$ ion with m/z 190 from **a**) ethylacetate extract corresponding to 21-day-old cultures of T4 grown on PDA supplemented with 100 ppm tricyclazole 24 h and **b**) tricyclazole standard

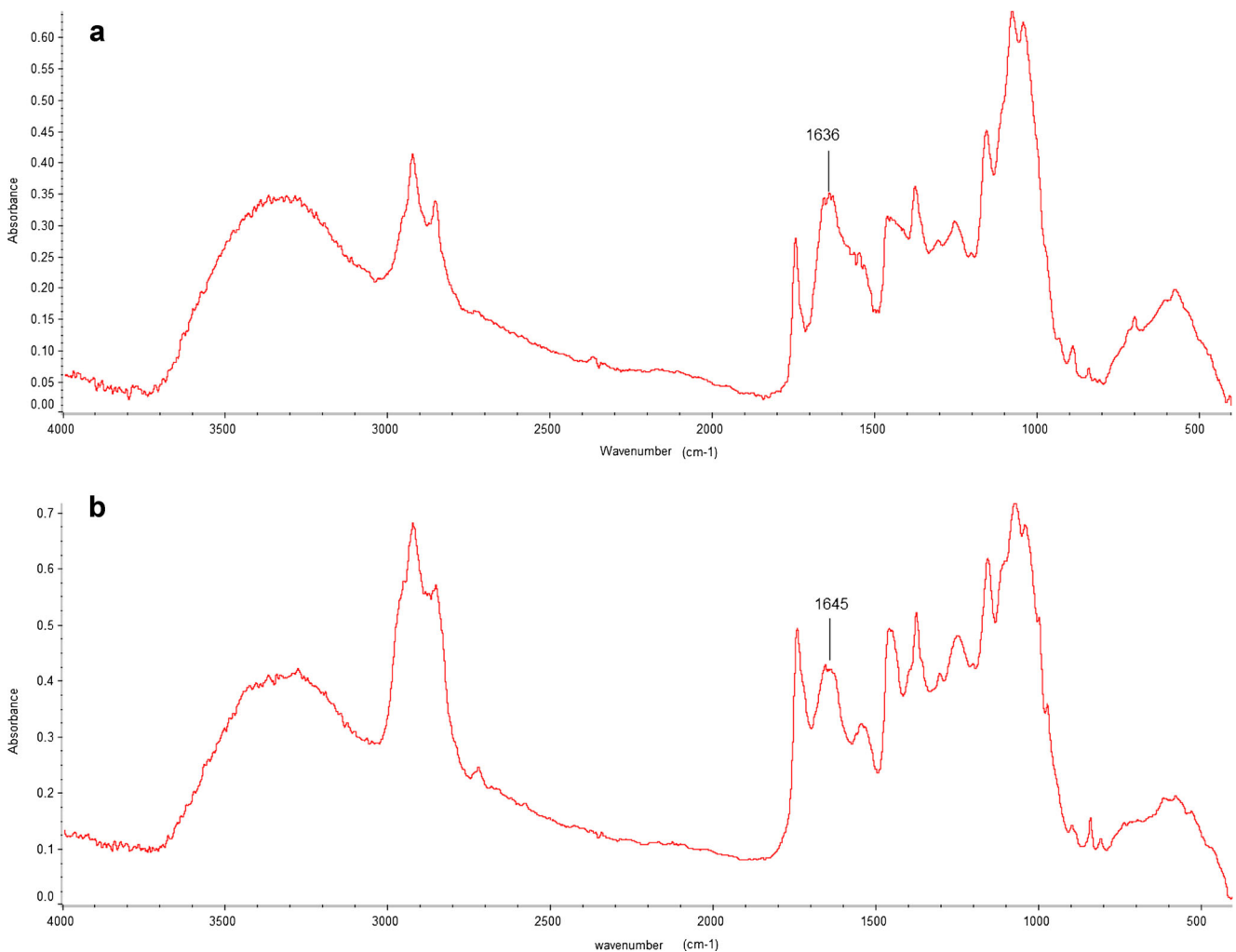


Fig. 4 FTIR spectrum of mycelium of *Pgriseola f. mesoamericana* grown on PDA (basal medium) (a) and on PDA+100 ppm of tricyclazole (b). This experiment was performed once with three repetitions

lighter colonies, which resulted in a reduction of the darkness “k” from 12.5 ppm (Fig. 9b). Though colony size was substantially reduced at 100 ppm (Fig. 9a), the concentration of fungicide that caused 50 % reduction of growth was 118.75 ppm.

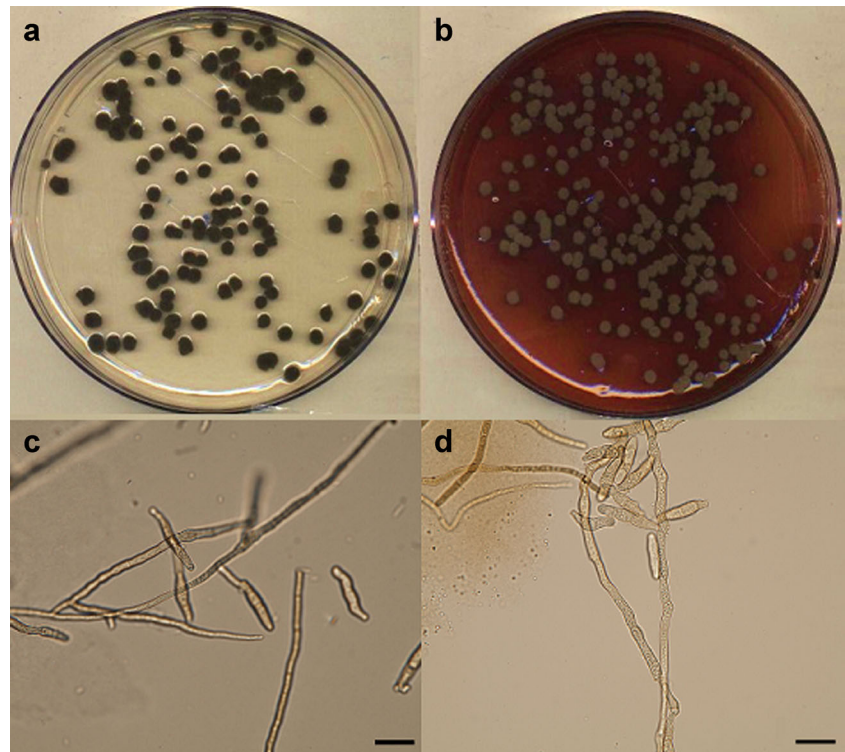
Discussion

Melanins are widely distributed among dematiaceous fungi such as *Mycosphaerellaceae* (Hoss et al. 2000). These polymeric substances might have a biological role such as virulence and/or survival against environmental stress factors (Bell and Wheeler 1986; Cooke and Whipps 1993; Chaskes et al. 2008; Onofri et al. 2008; Singaravelan et al. 2008; Tabora et al. 2008). Saparrat et al. (2009) showed that *Pseudocercopora griseola* synthesizes DHN-melanins, a process that was inhibited by tricyclazole. Here, we confirmed

that this inhibition leads to a reduction in fungal growth and pigmentation. While 12.5 ppm of tricyclazole affected colony pigmentation, it did not affect fungal growth, at least at such concentration. Fernandez et al. (2013) also reported that melanization of *Cenococcum geophilum* was sensitive to tricyclazole, but it was mostly unrelated to fungal growth. A similar reduction in pigmentation was observed in several phytopathogenic fungi that produce DHN-melanin, like *Alternaria solani*, *Botrytis cinerea*, *Magnaporthe grisea*, *Sclerotinia minor* and *Thielaviopsis basicola*, as well as in the human pathogens *Aspergillus terreus*, *Aspergillus tubingensis*, *Nannizzia cajetani* and *Trichophyton violaceum* (Mares et al. 2004).

Regarding synthesis of other pigments, when isolate T4 was grown in the presence of tricyclazole, a reddish pigment diffused out of the mycelium. Our results suggest that tricyclazole reduced rather than blocked melanin synthesis since the FTIR analysis, such as reported by Tavzes et al.

Fig. 5 Colony, mycelia and conidia of *P.griseola f. mesoamericana* grown on PDA in the absence (a+c) and presence of 100 ppm of tricyclazole (b+d). Scale 20 μ m



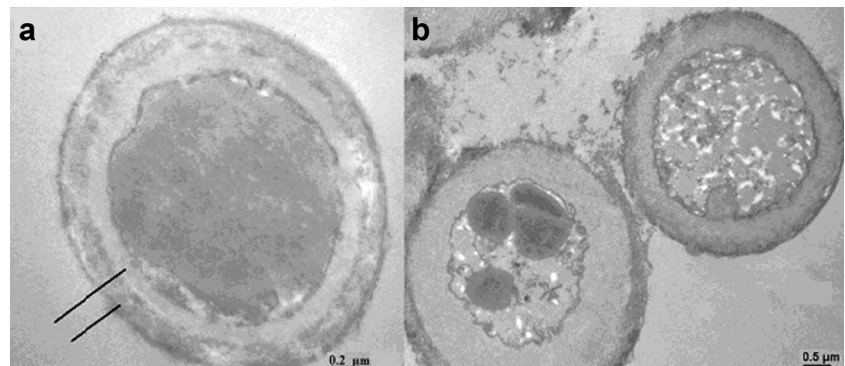
(2009), revealed the presence of melanin in hyphal walls whether the fungus was or was not grown in the presence of the inhibitor. Concomitantly with this, the reddish pigment was released. Based on all these evidences, Saparrat et al. (2009) hypothesized that the reddish pigments might be the soluble compounds released from fungal cultures grown in the presence of tricyclazole, which might be intermediate metabolites of melanin. Such a reduction in melanin synthesis, at least at certain concentrations of the inhibitor, affected mycelial growth; therefore, the melanin level in fungal wall may also be a determining factor in the organization of cell structure.

UV-MALDI MS is a key analytical tool to identify the chemical nature of compounds of low molecular mass, such as intermediate metabolites involved in melanin synthesis. In

this work, we found that such an intermediate metabolite had a MS/MS spectrum and a retro Diels-Alder like fragmentation pattern typical of flaviolin (McLafferty and Turecek 1993), which was the main metabolite associated with mycelium of *P. griseola f. mesoamericana* grown on medium with tricyclazole 100 ppm, probably as a result of the autoxidation of 1, 3, 6, 8-THN. Flaviolin might be a shunt product from the inhibition of reductases by tricyclazole (Zeng et al. 2012), since isolate T4 synthesizes melanin through the DHN-pathway (Saparrat et al. 2009).

Furthermore, no trace of 2-HJ, another diagnostic tool to identify DHN-melanin (Butler and Day 1998), was found. In cultures of *Alternaria solani* grown with high concentrations of tricyclazole, DHN-melanin was inhibited, 2-HJ was found and traces of flaviolin were detected (Lazarovits and Stoessel

Fig. 6 Hyphae of *P.griseola f. mesoamericana* isolate T4 grown on PDA (basal medium) (a) and on PDA+100 ppm of tricyclazole (b). Lines indicate layers with different electron-density



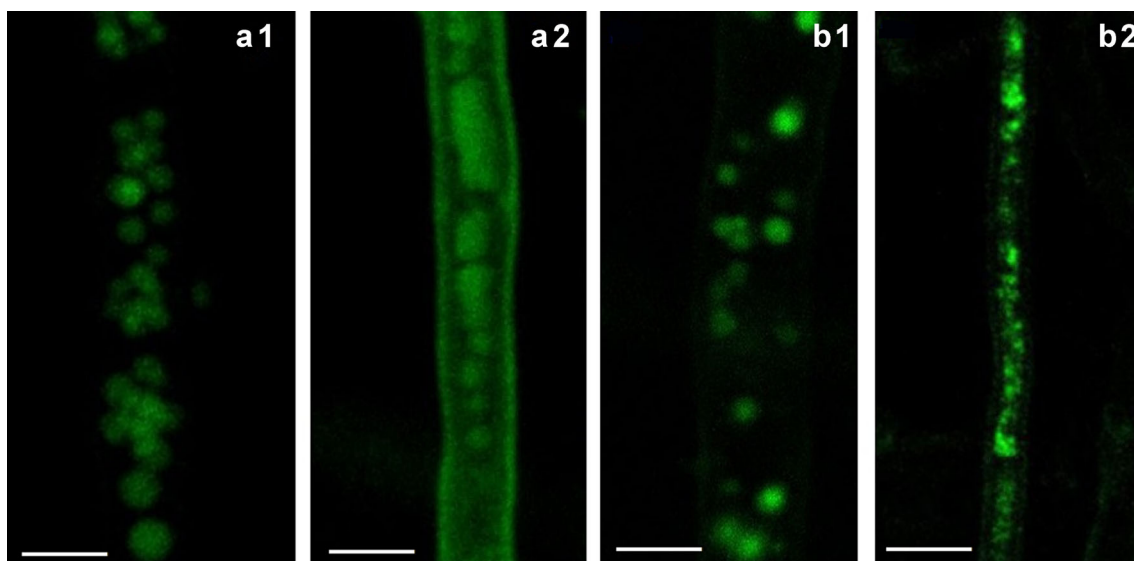


Fig. 7 Hyphae of *P.griseola f. mesoamericana* isolate T4 from cultures grown on PDA alone (a) and on PDA+100 ppm of tricyclazole (b), either nontreated (1) or treated (2) with H₂O₂ 6 M and light. Scale 5 μm

1988). In cultures of *Verticillium dahliae*, 1 to 20 ppm of tricyclazole resulted in an increase of 2-HJ and a decrease in flavin level (Lazarovits and Stoessl 1988). According to Lazarovits and Stoessl (1988), the type of melanin shunt products in the presence of tricyclazole and their accumulation are dependent on the fungicide concentration and the fungus tested. Since we only extracted the metabolites from cultures grown at one concentration of tricyclazole, the absence of traces of 2-HJ might at the least be due to a differential inhibition of the fungicide on specific reductase steps. Therefore, our results suggest that reductases of *P. griseola f. mesoamericana* that catalyze the conversion of 1,3,6,8 - THN to scytalone are more sensible to tricyclazole than those generating vermeline. Kogej et al. (2004) also reported that reductases involved in the different steps of melanin synthesis from both *Colletotrichum lagenarium* and *Magnaporthe grisea* showed different sensitivities to tricyclazole.

The effect of tricyclazole on fungal growth and pigmentation also was accompanied with alterations in morphology of black fungi, more precisely the hyphal wall, at the

ultrastructural level (Franzen et al. 2006; Kogej et al. 2006). Chattopadhyay et al. (2013) and Kumar et al. (2015) found that the tricyclazole in the range between 25 and 100 ppm provoked several changes in *Bipolaris sorokiniana*, such as colony morphology, spore size and number of septa in conidia. Also a decrease in the deposition of electron-dense granules in hypha walls was found in cells of *Trimmatostroma salinum* and *T. abietis*, chlamydo spores of *Thielaviopsis basicola*, and sclerotia of *Botrytis cinerea* and *Sclerotinia sclerotiorum*, when they were cultured in a medium with 1–30 ppm of tricyclazole (Wheeler and Stipanovic 1979; van de Sande et al. 2007; Butler et al. 2009). Our observations show that the hyphal walls of the isolate T4 had an inner electron-transparent layer and an outer electron-dense one, which also has been described for walls of spores from *Cladosporium cladosporioides* (Latzg  et al. 1988) and *Humicola grisea* (Ellis and Griffiths 1974). Tricyclazole at 100 ppm reduced stratification and deposition of melanin in the hyphal wall of isolate T4, as seen by TEM and CM respectively. Kumar et al. (2015) also associated the presence

Fig. 8 Relation of fluorescence signal for averaging area in cell wall and intracellular vesicles in control cultures (0) and cultures supplemented with tricyclazole (100) treated with H₂O₂-light (black bars) and non-treated with H₂O₂-light (white bars). Bars with the same capital letter are not significantly different (Student's *t* Test, *p*<0.05)

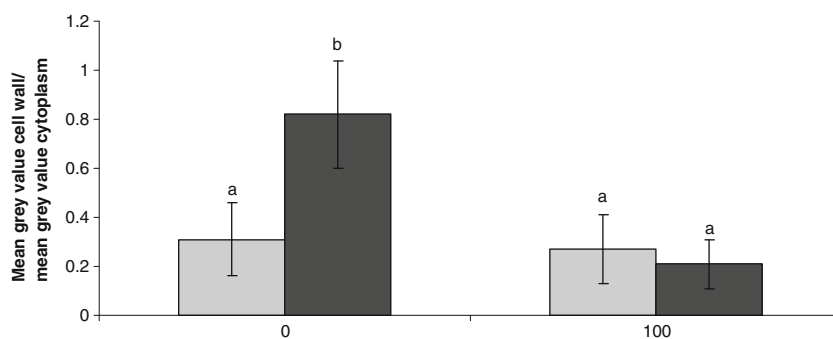
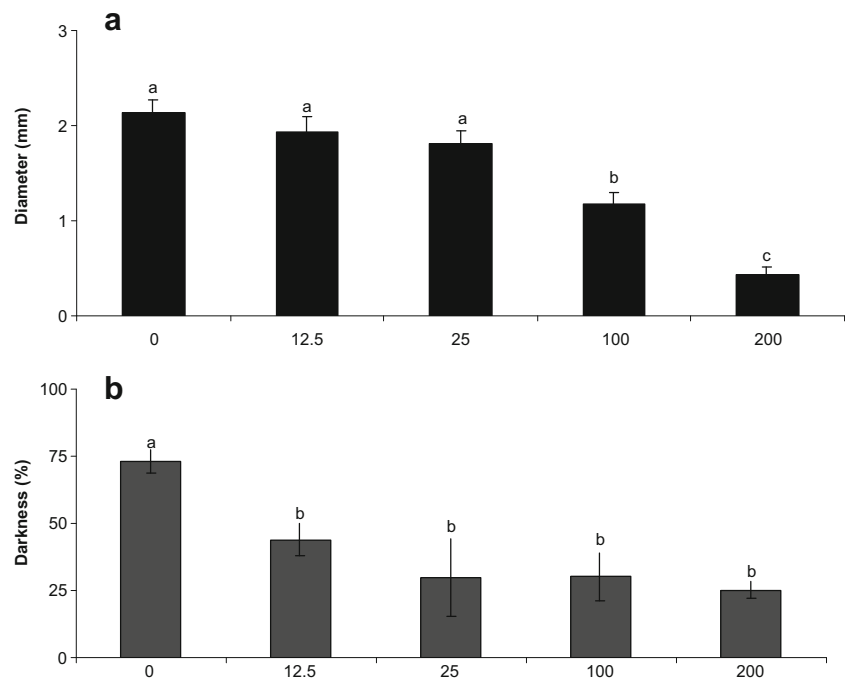


Fig. 9 Growth (a) and pigmentation (b) of colonies of *P. griseola mesoamericana* isolate T4 on PDA in the absence (0) and presence (12.5, 25, 100 and 200 ppm) of tricyclazole for a 21-day-period. Values are means of three replicates. Bars with the same capital letter are not significantly different ($p < 0.05$). This experiment was performed twice with similar results



of this inhibitor with morphological changes in the cell walls of hyphae and conidia of the black *B. sorokiniana* isolates, which also was associated with a lower level of melanization. The organization of the hyphal wall in phytopathogens and the localization and amount of their dark pigments has been reported to be an important factor in protecting of several dark fungi from various environmental insults (Mandal et al. 2007). Therefore, tricyclazole might provide an efficient method of control of such phytopathogenic fungi.

The bases of activity of tricyclazole in the pathogen–plant interaction reside on its ability to alter several mechanisms, such as those related to fungal biology as well as the hormonal and antioxidant systems of plants (Habibzade et al. 2012; Shanmugapriya et al. 2013). Tricyclazole has been used to control of *M. grisea* on rice. Recently, it has been demonstrated that this molecule also has fungicide activity against other pathogens of cereals, such as *Bipolaris oryzae* and *B. sorokiniana*, *Blumeria graminis* and *Rhynchosporium secalis*, as well as *Colletotrichum lagenarium* and *C. lindemuthianum*, pathogens of cucumber and beans, respectively (Mares et al. 2004; Gupta et al. 2013; Kumar and Mukherjee 2013; Kumar et al. 2015). Although Saparrat et al. (2009) reported the effect of tricyclazole in melanization of *Pseudocercospora griseola*, there is no additional data on the role this compound might play in the control of ALS. However, all the evidences presented in this work suggest that tricyclazole might be an effective fungicide for ALS control.

Tricyclazole affected *Pseudocercospora griseola* f. *mesoamericana* isolate T4, in several different ways. The most evident effect was on pigment synthesis. It reduced melanin synthesis, and therefore diffusible shunt product from an

intermediary of melanin synthesis, identified by UV-Maldi MS, was found. In addition to this, we also found that tricyclazole affected the cell wall structure of mycelia, revealed macroscopically by the reduction in growth. As suggested by Kunova et al. (2012), the knowledge about tricyclazole's mode of action that controls pathogenic fungi is crucial information to support its registration. However, information regarding the efficiency of tricyclazole in field experiments is not available. Future studies should be aimed to evaluate if this molecule can be used to control fungal diseases provoked by DHN-melanized fungi in the field. So far, *Pseudocercospora griseola* has not been controlled by inhibiting secondary metabolites like melanin that play a biological role. Therefore, the question arises about the effect that an inhibitor of pigment synthesis might have on the pathogenic activity and/or virulence of the fungus. To address this question, future work should be aimed at generating fungal mutants impaired in pigment synthesis.

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