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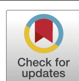

Identification of an intermediate for 1,8-dihydroxynaphthalene-melanin synthesis in a race-2 isolate of *Fulvia fulva* (syn. *Cladosporium fulvum*)

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Abstract

Fulvia fulva (syn. *Cladosporium fulvum*, Mycosphaerellaceae) is a dematiaceous fungus that causes tomato leaf mould. It is characterized by its biotrophic lifestyle and the synthesis of the bianthraquinone secondary metabolite, cladofulvin. The aim of the study was to characterize the dark pigment photochemically synthesized by *F. fulva* and to elucidate its biochemical pathway. We isolated a black pigment from *in vitro* cultures of the fungus. We determined the pigment to be 1,8-dihydroxynaphthalene (DHN)-melanin based on its chemical and photochemical characteristics, as well as the presence of flaviolin, when fungal reductases were inhibited by tricyclazole. Furthermore, the *pks1* gene involved in pigment synthesis has a KS domain already associated with DHN-melanin. Our findings support the relevance of studying melanization in *F. fulva*.

Keywords: Biochemistry, Genetics, Microbiology, Molecular biology

1. Introduction

Fulvia fulva (syn. *Cladosporium fulvum*, syn. *Passalora fulva*) is the causal agent of tomato leaf mould, a disease that affects mostly the leaves of tomato and occasionally stems, blossoms, petioles and fruit (Griffiths et al., 2018). This species is a member of the fungal family Mycosphaerellaceae, and is phylogenetically related to the genera *Stromatoseptoria*, *Hyalocercosporidium* and *Dothistroma* (Videira et al., 2017). When these fungi are cultured *in vitro*, the pigment produced within their mycelia varies according to growth substrate, and might be used as a diagnostic trait and/or may provide additional information regarding phylogenetically related fungi (Saparrat et al., 2009; Kowalski et al., 2016). Aguilera-Cogley et al. (2017) raised more experimental data about limitations of the use of colour of fungal colonies growing on agar cultures and their ability to synthesize pigments as criteria for species identification in Mycosphaerellaceae.

Several pigments and chromophores have been described in fungi; though their biological roles remain unclear (Toledo et al., 2017). Collemare et al. (2014) and Griffiths et al. (2016, 2018) reported that *F. fulva* synthesizes cladofulvin, the main secondary metabolite produced in agar cultures while experiencing stressful conditions. Several orthologous genes have also been identified that are likely involved in the synthesis of perylenequinone pigments such as elsinochrome and cercosporin toxins (Griffiths, 2015). However, information regarding the synthesis of other pigments synthesized by this fungus is lacking. Kowalski et al. (2016) suggested that more research is required to determine the chemical nature of several pigments synthesized by *Dothistroma septosporum* such as dothistromin. This toxic pigment, also produced by *D. pini* and other fungi in Family Mycosphaerellaceae

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(e.g., *Passalora arachidicola*, *Mycosphaerella* spp. and *Cercospora* spp.), alters its coloration in response to several interactions, such as those dependent on culture conditions (when the fungus grows *in vitro* versus *in planta*).

Dark pigments known as melanins are complex polymers composed of aliphatic and indole or phenol-type aromatic structures, synthesized by a wide range of organisms (Toledo et al., 2017). In fungi, they have been related with morphogenesis, pathogenesis and/or survival strategies (Bell and Wheeler, 1986; Elliott, 1995; Henson et al., 1999; Nosanchuk and Casadevall, 2003; Cordero and Casadevall, 2017). Since melanins are mostly insoluble in water and common organic solvents, and are amorphous solids not amenable to crystallographic studies, alternative approaches must be developed in order to characterize them. These limitations might be solved in different ways. While one method might be through pigment solubilization in hot, strong alkali, followed by a photometric estimation, another method might involve cultivating fungi in the presence of specific pigment inhibitors and inducers (Saparrat et al., 2009; Fernandes et al., 2016).

Mycelia and conidia of fungi, such as *F. fulva*, *Pseudocercospora griseola* and *Stromatoseptoria castaneicola*, develop a dark mycelium when cultured *in vitro* due to the presence of melanin (Videira et al., 2017; Bárcena et al., 2018). However, *Hyalocercosporidium desmodii* and *Dothistroma* spp., which are phylogenetically related to *F. fulva*, have pale brown mycelia and hyaline conidia (Videira et al., 2017). The role of melanin within pathogens is still unclear, and the biochemical pathway synthesizing these dark pigments in *F. fulva* is unknown. Therefore, the aim of this study was to characterize the dark pigment photochemically synthesized by *F. fulva*, and to elucidate the biochemical pathway of melanin synthesis using several specific inhibitors and inducers.

2. Materials and methods

2.1. Fungal material

A monosporic culture of *F. fulva* race 2 was previously isolated from mouldy tomato leaf tissue (Medina et al., 2015). This fungal isolate (CIDEFI 300) was deposited in the Culture Collection of the Centro de Investigaciones de Fitopatología, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, La Plata, Argentina. The fungus was cultured on a potato dextrose agar (PDA) medium and cultures were stored at 4 °C.

2.2. Pigment extraction

Pigments were extracted from 21-day-old cultures grown on PDA at 25 °C (Gadd, 1982). Mycelia were collected with a clamp, washed extensively with hot water, and

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then were dried in an oven at 80 °C until constant weight was achieved. This biomass was frozen with liquid nitrogen and ground in a mortar. The powder material was stored at 25 °C. Pigment was extracted from 100 mg aliquots of dry fungal biomass, which was resuspended in 5 ml of 1 M NaOH and heated at 121 °C for 20 min (Saparrat et al., 2009). The sample was centrifuged at 5,000 x *g* for 10 min, the supernatant collected and the pH adjusted to 2 by adding 3 M HCl, as required. In order to precipitate melanin, the supernatant was incubated overnight at 4 °C. Pigments were recovered by centrifugation at 5,000 x *g* for 10 min, washed with distilled water until it had a neutral pH. Then, the pellet was dried in an oven at 40 °C overnight and was purified via acid hydrolysis using 6 M HCl at 100 °C for 2 h to remove carbohydrates and proteins. The non-hydrolysable residues were pelleted by centrifugation at 5,000 x *g* for 10 min, and then to remove lipids they were successively treated with ethanol and chloroform. The residue obtained was dried at 25 °C.

2.3. Spectroscopic analysis

Purified dark pigments, isolated from *F. fulva* CIDEFI 300, were dissolved in 1 M NaOH to analyse their photochemical behaviours, which were compared to the control made by an alkaline solution of a commercial melanin (Sigma Chemicals Co., St. Louis, USA). Both solutions were filtered through a 0.45 μm pore size membrane and then their absorption spectra and fluorescence excitation-emission matrices (FEEMs) were measured. The absorption spectra were measured on a Shimadzu UV-1800 at room temperature in quartz cells with 1.0 cm optical path length. The FEEMs were measured on air-equilibrated aqueous solutions using a Single-Photon Counting equipment FL3 TCSPC-SP (Horiba Jobin Yvon). The sample solution was irradiated in a quartz cell with a 450 W Xenon source through an excitation monochromator (FL-1004). After passing through an emission monochromator (iHR320), fluorescence was registered at 90° of the incident beam by using a room-temperature R928P detector. FEEMs were generated by collecting the data of successive emission spectra from 260 to 650 nm at excitation wavelengths that ranged from 240 to 580 nm, with 5 nm incremental steps. Excitation and emission slits were set to 5 nm (Medina et al., 2018).

2.4. Pigmentation experiments

Inhibition experiments relating to the melanin biosynthetic pathway involved the addition of one of three possible compounds to PDA medium, prior to culturing the CIDEFI 300 strain, that target specific synthesis genes in the pathway. Fifty ppm or 100 ppm of tricyclazole (5-methyl-1,2,4-triazolo[3,4-b] benzothiazole, Ultra Scientific Analytical Solutions, United States, dissolved in ethanol) was used to inhibit 1,8-dihydroxynaphthalene (DHN)-melanin synthesis. One-hundred ppm of sulcotrione (Riedel-de Haën™, Seelze, Germany, dissolved in water) was used to

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inhibit synthesis of piomelanin, and 100 ppm of kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone, Parafarm, Argentina, dissolved in water) served as inhibitor of DOPA-melanin synthesis. Alternatively, melanin inducer experiments were conducted involving compounds such as L-tyrosine and L-DOPA, which were added to PDA at 10 mM and 1 mM, respectively. Also, combinations of L-tyrosine (10 mM) with kojic acid (100 ppm), or L-tyrosine (10 mM) with sulcotrione (100 ppm) were added to the media. Plates were inoculated with a conidial suspension of CIDEFI 300 and were incubated in the dark at 25 ± 2 °C for 21 days, at which time colony growth (diameter) and melanin production were determined. Three replicate plates per treatment were made, including control plates free of inhibitors and/or inducers.

2.5. Isolation and identification of melanin pathway intermediates by UV−MALDI MS

Metabolites from CIDEFI 300 cultures grown on supplemented and non-supplemented (control) PDA were extracted with ethyl-acetate following the protocol of Wheeler and Klich (1995). After incubation in the dark at 25 ± 2 °C for 21 days the cultures were treated with ethyl-acetate. We made three independent UV-MALDI measurements on aliquots of the ethyl-acetate extracts of each treatment and three independent assays were conducted. A freshly prepared ethanolic solution of standard tricyclazole (50 ppm) was used as a reference. Samples were analyzed by ultraviolet matrix assisted laser desorption-ionization mass spectrometry (UV−MALDI MS), which was performed on the Bruker Ultraflex Daltonics TOF/TOF mass spectrometer as described by Bárcena et al. (2015). Two MALDI matrices were used: 2,5- dihydroxybenzoic acid (gentisic acid), GA and 9H-[3,4b] piridoindole (norharmane, nHo). Mass spectra were acquired in a linear positive ion mode and with the LIFT device in the MS/MS mode. External mass calibration was made using β-cyclodextrin (MW 1134) with nHo as matrices in positive 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 performed as described by Nonami et al. (1997), loading 0.5 μl of the matrix solution, analyte solution and matrix solution successively, once each layer dried at room temperature (1 atm atmosphere and 25 °C). 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 laser pulses per spot. Spectra were obtained and analyzed with the programs FlexControl and FlexAnalysis, respectively.

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2.6. Phylogenetic analysis

CIDEFI 300 DNA was extracted according to Medina et al. (2015). KS domain from CIDEFI 300 was amplified using the designing primers KS1-F (5′- GATTTC-TATGTCCATGCTCGGG-3′) and KS1-R (5′-AATTCTTGTTCCTGTGCCSTGC-3′). Reaction mixture contained 1× amplification buffer (Inbio Highway), 1.5 mM $MgCl_2$ (Inbio Highway), 15 ng of each primer, 200 mM each deoxynucleoside triphosphate (Inbio Highway), 20−40 ng of DNA template, and 1 U of Taq DNA polymerase (Inbio Highway) in a 15 μl volume. PCR was performed in a PTC-1152 Mini Cycler (MJ Research) programmed as follows: an initial step at 94 °C for 3 min; followed by 35 cycles of a denaturing step at 94 °C for 1 min, annealing at 59 °C for 45 s, and extension at 72 °C for 1 min; with a final extension at 72 °C for 5 min.

PCR product was resolved by electrophoresis in 1 % (w v^{-1}) agarose gel stained with 10 % ethidium bromide (v v^{-1}). A 100 to 1,000 bp DNA ladder marker (Inbio Highway) was used to estimate the size of the amplicons. Amplicon was purified as described by Sambrook et al. (1989), precipitated, and sequenced at MACROGEN Inc. (Seoul, South Korea). KS domain sequence was deposited at the National Center for Biotechnology Information (NCBI) GenBank (accession number: MK144330).

The phylogenetic analysis was conducted using MEGA7 (Kumar et al., 2016). The analysis included amino acid sequences for KS domains of PKS proteins from 24 ascomycetous fungi (Table 1), including the KS domain of PKS1 from *F. fulva* CIDEFI 300. The sequences were aligned using the default parameters of the ClustalW algorithm (gap opening penalty 15, gap extension penalty 6.66) (Thompson et al., 1994). The alignment was visually checked and manually optimized. Phylogenetic analysis was performed under Maximum-likelihood (ML) criteria (Jones et al., 1992). Clade stability was assessed based on 1000 bootstrap replicates.

3. Results

3.1. Spectroscopic analysis

CIDEFI 300 cultures produced dark pigments that were solubilized in alkaline pH at high temperature (NaOH solution at 121 °C for 20 min). The isolated pigments were insoluble in 3 M HCl, as well as in water, and organic solvents such as acetone, chloroform and ethanol. These pigments could be decolorized using 6 M H_2O_2 (data no shown). The absorption spectra (inset) and emission spectra of alkaline solutions obtained from FEEM for excitation wavelengths between 300 nm and 450 nm of the dark pigment from *F. fulva* (a) and commercial melanin (b) are presented on Fig. 1. The main band of the emission spectra shifted from 400 nm to 480 nm as

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Table 1. Species names, accession numbers and references for protein sequences used in phylogenetic analysis.

Fungal species	Accession Number	Reference
Aspergillus flavus	AAS90093.1	Ehrlich et al. (2004)
Aspergillus fumigatus	AAC39471.1	Tsai et al. (1998)
Aspergillus fumigatus	XP_746435.1	Nierman et al. (2005)
Aspergillus nidulans	AAC49191.1	Brown et al. (1996)
Aspergillus nidulans	CAA46695.2	Mayorga and Timberlake (1992)
Aspergillus nidulans	XP_657754.1	Galagan et al. (2005)
Aspergillus terreus	XP_001217072.1	Unpublished. Annotation of the *A. terreus* NIH2624 genome
Cercospora nicotianae	AAT69682.1	Chung et al. (2003)
Cladosporium cladosporioides	JN205332.1	Llorente et al. (2012)
Colletotrichum lagenaria	BAA18956.1	Takano et al. (1995)
Elsinoe fawcettii	ABU63483.1	Liao and Chung (2008)
Fulvia fulva	**MK144330**	***This study***
Gibberella zeae	ABB90282.1	Kim et al. (2005)
Gibberella zeae	XP_390640.1	Unpublished. *F. graminearum* genome sequence
Glarea lozoyensis	AAN59953.1	Zhang et al. (2003)
Hypomyces subiculosus	ACD39762.1	Reeves et al. (2008)
Magnaporthe oryzae	XP_003715434.1	Dean et al. (2005)
Monascus purpureus	BAD44749.1	Shimizu et al. (2005)
Nectria haematococca	AAS48892.1	Graziani et al. (2004)
Nodulisporium sp.	AAD38786.1	Fulton et al. (1999)
Podospora anserina	XP_001910795.1	Espagne et al. (2008)
Pseudocercospora griseola	ALF44675.1	Unpublished
Sordaria macrospora	CAM35471.1	Engh et al. (2007)
Wangiella dermatitis	AAD31436.1	Ye et al. (1999)

the excitation wavelength increased from 300 nm to 450 nm in both samples containing the pigments of CIDEFI 300 and the commercial sample of melanin.

3.2. Pigmentation experiments

Only supplementation with 50 ppm of tricyclazole resulted in inhibition of melanin production, based on colonies that exhibit brown colouration and secretion of reddish-brown pigments (Fig. 2). Tricyclazole supplementation at 100 ppm prevented growth of the fungus, and none of the other compounds tested resulted in altered growth or pigmentation of the CIDEFI 300 strain (data not shown). UV-MALDI mass spectra of colonies from 21-day-old cultures of *F. fulva* CIDEFI

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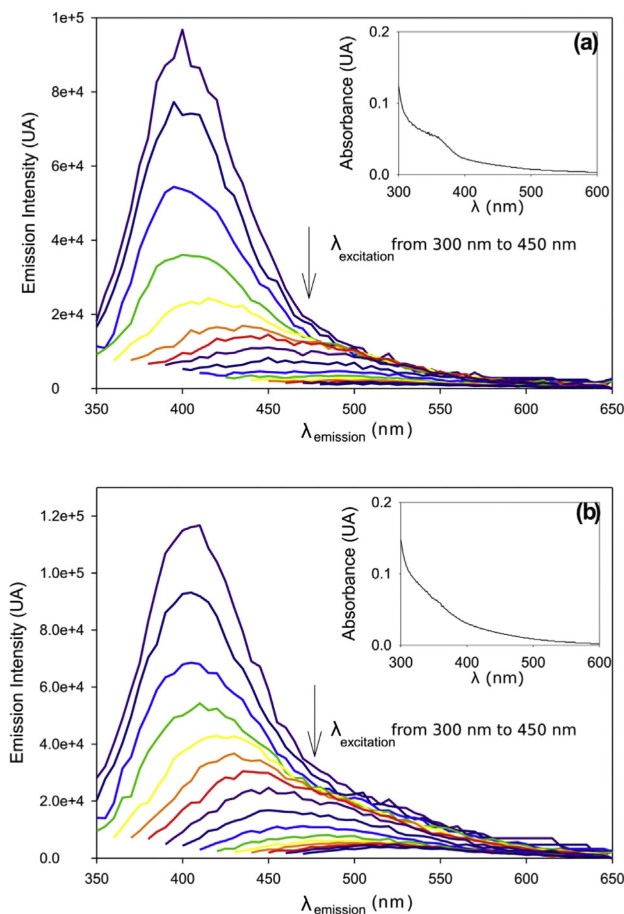

Fig. 1. Emission spectra extracted from FEEM for excitation wavelengths between 300 nm and 450 nm for (a) dark pigment from *F. fulva* and (b) commercial melanin sample. Inset: Absorption spectra.

300, grown on non-supplemented PDA (control) and on media supplemented with 50 ppm tricyclazole are shown in Fig. 3a and b, respectively. In the presence of 50 ppm tricyclazole, we found a signal at m/z = 206.27, which is approximately the size of flaviolin (2,5,7-trihydroxy-1,4-naphthoquinone; exact mass 206.02), an

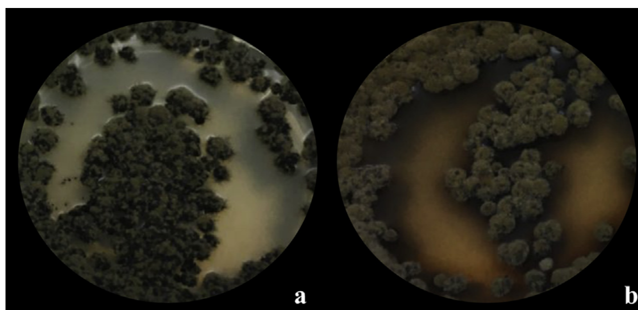

Fig. 2. Cultures of *F. fulva* strain CIDEFI 300 grown on PDA in non-supplemented medium (a), and grown on medium supplemented with 50 ppm of tricyclazole (b) for a 21-day period.

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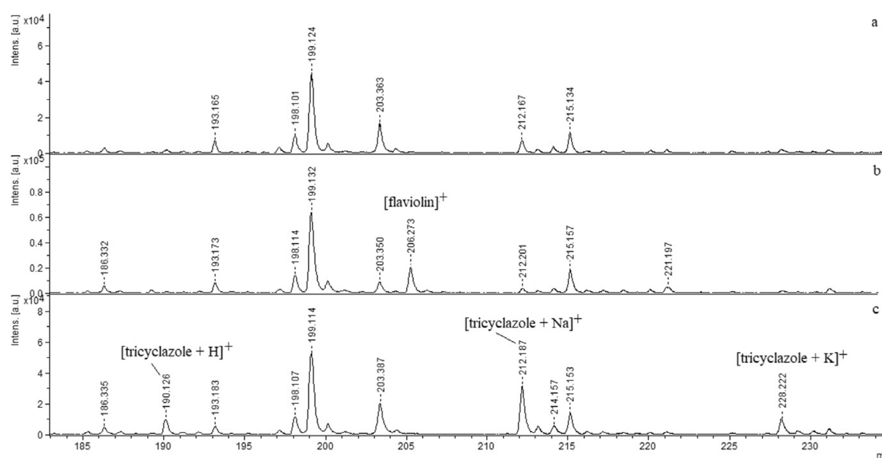

Fig. 3. Positive ion MALDI mass spectra of ethyl-acetate extract corresponding to 21-day-old cultures of *F. fulva* CIDEFI 300 grown on PDA: non-supplemented control (a); supplemented with 50 ppm tricyclazole with peak assignment for $[\text{flaviolin}]^+$ (m/z = 206; b); and of an ethanolic solution of standard 50 ppm tricyclazole with peak assigments for $[\text{Tricyclazole} + \text{H}]^+$ (m/z = 190), $[\text{Tricyclazole} + \text{Na}]^+$ (m/z = 212), and $[\text{Tricyclazole} + \text{K}]^+$ (m/z = 228; c). Matrix: gentisic acid.

oxidation product of 1,3,6,8- tetrahydroxynaphthalene (1,3,6,8-THN). Interestingly, extracts of control samples lack such signal (Fig. 3a). As expected, the protonated (m/z = 190.15), sodiated (m/z = 212.14) and potassiated tricyclazole (m/z = 228.21) ions were detected in an ethanolic solution of standard 50 ppm tricyclazole (Fig. 3c).

3.3. Phylogenetic analysis

We amplified a 415 bp fragment corresponding to a partial gene sequence of *pks1*, which contains the complete KS domain sequence. We found that the KS domain obtained from CIDEFI 300 shared 100 % identity with that published by de Wit et al. (2012). Our analysis inferred a close phylogenetic association for the KS domain of *pks1* in CIDEFI 300 with representative amino acid sequences of fungi belonging to other Dothideomycetes, particularly those included in the Orders Capnodiales (Davidiellaceae and Mycosphaerellaceae) and Myriangiales (Elsinoaceae), which incidentally have been previously associated with both DHN-melanin and elsinochrome synthesis (Fig. 4). In addition to this, all KS domains that clustered with the DHN-melanin synthesis corresponded to representatives of Dothideomyceta and Sordariomyceta.

4. Discussion

Melanins are secondary metabolites that are made up of a complex array of heterogeneous polymers, whose properties are, as expected, a function of their chemical structure and source of origin (Toledo et al., 2017). Melanins are characterized by

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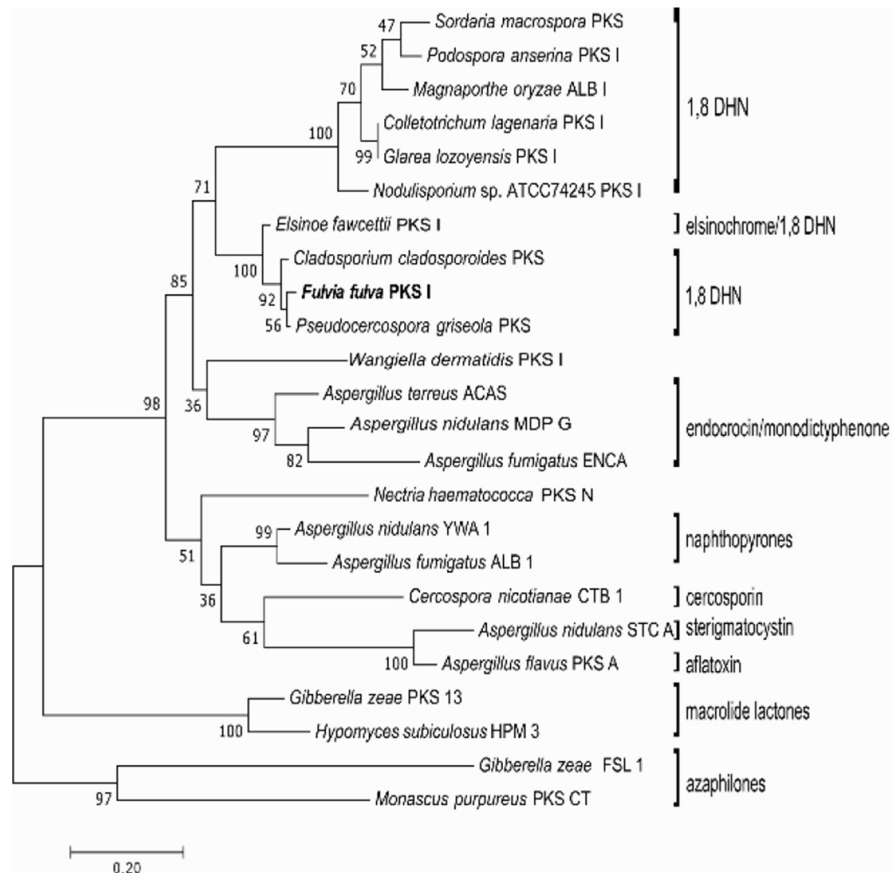

Fig. 4. Molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 24 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 178 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

means of several features like solubility in alkaline pH as well as susceptibility to oxidizing agents. In addition to this, another helpful tool is the spectral analysis of melanins, since they have an unusually broad absorption spectrum, which is typical of dark pigments since they absorb almost all visible wavelengths. This spectrum increases monotonically, which is inversely related to the band wavelength.

Our results prove that the dark pigments being produced by the *F. fulva* CIDEFI 300 strain are melanins due to several observations: 1) solubility in high pH (1 M NaOH) and insolubility in low pH (4 M HCl), water, or organic solvents (Montes-Avila et al., 2018); 2) similar absorption spectra to that of commercial as well as synthetic melanins (Bell and Wheeler, 1986; Nighswander-Rempel et al., 2005a,b; Kayatz et al., 2001; Meredith and Riesz, 2004; Selvakumar et al., 2008; Ozturk et al., 2017); and 3) similar emission spectra to those previously reported for synthetic melanin (Gallas and Eisner, 1987; Perna et al., 2009; Meredith and Riesz, 2004;

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Nighswander-Rempel et al., 2005a). Additional studies should be performed to confirm, with greater accuracy, the chemical identities of all these dark fungal pigments.

Fungi may synthesize melanin using more than one pathway (Toledo et al., 2017). Inhibitors and inducers of putative pathways of melanin synthesis might help to elucidate these routes, and this can be used to infer the chemical nature of the pigment (Saparrat et al., 2009; Toledo et al., 2017). Although the synthesis of DHN-melanin is typical among representatives of Ascomycota, other types of melanins have been found in several other fungal families such as DOPA-melanin in Aspergillaceae (Pal et al., 2013), Davidiellaceae (Llorente et al., 2012) and Mycosphaerellaceae (pyomelanin; Keon and Hargreaves, 1998). The synthesis of dark pigments in cultures of *F. fulva* was inhibited only by tricyclazole. Concomitantly, the fungus secreted a reddish pigment into the culture medium. We hypothesized that such compounds are probably the result of the inhibitory activity of tricyclazole on fungal reductases (Zeng et al., 2012), which has already been described in cultures of *Cladosporium cladosporioides* and *Pseudocercospora griseola* (Bárcena et al., 2015; Llorente et al., 2012). Our findings suggest that the main dark chromophore of colonies of *F. fulva* might be melanin that is synthesized through the DHN-pathway, as described for other typical fungi from Ascomycota.

The UV-MALDI-mass spectrometry analysis of an ethyl-acetate extract from cultures grown on tricyclazole amended PDA media suggests that the fungus synthesizes DHN-melanin, and that tricyclazole (50 ppm) led to the accumulation of shunt by-products like flaviolin. This is likely due to the autoxidation of 1,3,6,8-THN, an intermediate metabolite in DHN-melanin pathway, which might be due to the inhibitory effect of tricyclazole on reductases (Zeng et al., 2012). Interestingly, no trace of 2-hydroxy juglone (2-HJ) was detected, which turned out to be another diagnostic tool that proved that the pigment is DHN-melanin (Butler and Day, 1998). The type and amount of melanin shunt products are dependent on tricyclazole concentration and the fungus tested (Lazarovits and Stoessl, 1988). Tricyclazole at 100 ppm appeared to be toxic to *F. fulva*, since no growth occurred under such conditions. The absence of traces of 2-HJ might be due to a differential inhibition of the fungicide on specific reductases. Our results suggest that reductases of *F. fulva* catalyze the conversion of 1,3,6,8-THN to scytalone, and it appears that these reductases are more sensitive to tricyclazole than those that generate vermelone. Furthermore, Kogej et al. (2004) and Bárcena et al. (2015) found that the reductases involved in the different steps of melanin synthesis in *Colletotrichum lagenarium*, *Magnaporthe grisea* and *Pseudocercospora griseola* f. *mesoamericana* differed in their sensitivity to tricyclazole. All this together suggests that *F. fulva* synthesizes mostly DHN-melanin, though it also might synthesize other pigments such as cladofulvin, which is the main secondary metabolite that this fungus produces when it is cultured under stress conditions (Collemare et al., 2014; Griffiths et al., 2016, 2018).

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However, we failed to detect cladofulvin, most likely because its synthesis is repressed by glucose, and we cultured *F. fulva* on PDA.

Polyketide synthases are key multi-enzymes that contribute to the biosynthesis of metabolites such as pigments, including DHN-melanin, and toxins (Llorente et al., 2012). A total of 23 functional core secondary metabolite genes have been described within the genome of *F. fulva* (de Wit et al., 2012), 10 of which are PKSs (Collemare et al., 2014). This genetic information suggests that the fungus can synthesize pigments like those with structures similar to perylenequinone-type, elsinochrome and cercosporin toxins (Griffiths, 2015). However, only cladofulvin and melanin have been found in cultures of *F. fulva* (Agosti et al., 1962; Davies and Hodge, 1974; Collemare et al., 2014). Our results suggest that *F. fulva* synthesizes, by means of the pentaketide pathway, DHN-melanin-like compounds like other ascomycetous fungi. The first step of this synthesis involves catalysis by a non-reducing type I PKS, an enzyme that plays a key role in the biosynthesis of DHN-melanin as well as other secondary metabolites such as aflatoxins (Thywißen et al., 2011). Within genome sequence of *F. fulva* published by de Wit et al. (2012), Collemare et al. (2014) found the *pks1*, a putative enzyme involved in the synthesis of melanin-like pigment and elsinochrome.

Ökmen et al. (2014) described that black mutants of *F. fulva* obtained by a deletion of the conserved transcriptional regulator *CfWor1* failed to synthesize cladofulvin, although the *pks1* gene was the only core secondary metabolite gene that was induced. In this work, this phenotype was associated with the synthesis of an unknown black pigment (Ökmen et al., 2014). Although fungal type I PKSs have up to eight types of functional domains (acyl transferase, acyl carrier protein, ketosynthase (KS), ketoreductase, enoyl reductase, dehydratase, thioesterase, and methyltransferase), the KS domain has been reported as a highly conserved motif within multifunctional enzymes, that might be used for the identification of novel *pks* genes and/or genes homologous (Bingle et al., 1999; Llorente et al., 2012). Our phylogenetic analysis suggests that *pks1* in the genome of *F. fulva* might play a crucial role in synthesis of melanin and elsinochrome, though there are no reports regarding the synthesis of the second pigment in cultures of *F. fulva*. In a recent study, Ebert et al. (2018) compared and characterized perylenequinone and DHN-melanin biosynthetic gene clusters in Ascomycota. They found that gene cluster for elsinochrome synthesis is related to the DHN-melanin production and that the *pks1* gene of *Elsinoë fawcettii* clustered closely to PKSs involved in the synthesis of DHN-type-melanin. Also, in *Aspergillus niger*, a single PKS has been associated with the synthesis of two different classes of polyketides, DHN-melanin and the naphtho- γ −pyrones (Chiang et al., 2011). Therefore, our results suggest that *F. fulva* has a pentaketide-pathway that plays a key role in the synthesis of DHN-melanin-like compounds, the dark pigment of this fungus. Incidentally, Tricyclazole, a specific inhibitor of DHN melanin synthesis, has been used to manage diseases of

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plants caused by pigmented fungi including those of the Mycosphaerellaceae, so evidently melanization in *F. fulva* might play a key role in plant pathogen interactions and deserves to be studied further.

5. Conclusion

The chemical and photochemical characteristics of the dark pigment synthesized by *F. fulva*, as well as the presence of flaviolin when fungal reductases were inhibited by tricyclazole, proved that the observed pigment is DHN-melanin. The *pks1* gene involved in pigment synthesis has a KS domain already associated with DHN melanin.

Declarations

Author contribution statement

Mario Saparrat: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Rocio Medina: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; materials, analysis tools or data; Wrote the paper.

Gabriela Petroselli: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Janina Alejandra Rosso, Rosa Erra-Balsells: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

César Lucentini: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ernesto Franco: Performed the experiments; Analyzed and interpreted the data.

Pedro Balatti: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Argentina; CONICET (PUE INFIVE), Argentina; CICPBA, Argentina; UNLP, Argentina.

Competing interest statement

The authors declare no conflict of interest.

Additional information

Data associated with this study has been deposited at GenBank under the accession number MK144330.1.

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