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Cladosporium cladosporioides LPSC 1088 Produces the 1,8-Dihydroxynaphthalene-Melanin-Like Compound and Carries a Putative *pks* Gene

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Abstract *Cladosporium cladosporioides* is a dematiaceous fungus with coloured mycelia and conidia due to the presence of dark pigments. The purpose of this study was to characterize the dark pigments synthetized by *Cladosporium* sp. LPSC no. 1088 and also to identify the putative polyketide synthase (*pks)* gene that might be involved in the pigment biosynthesis. Morphological as well as molecular features like the ITS sequence confirmed that LPSC 1088 is *Cladosporium cladosporioides*. UV-visible, Fourier Transform Infrared (FTIR) and Electron Spin Resonance (ESR) spectroscopy analysis as well as melanin inhibitors suggest that the main dark pigment of the isolate was 1,8 dihydroxynaphthalene (DHN)-melanin-type compound. Two commercial fungicides, Difenoconazole and Chlorothalonil, inhibited fungal growth as well as increased pigmentation of the colonies suggesting that melanin might protect the fungus against chemical stress. The pigment is most probably synthetized by means of a pentaketide pathway since the sequence of a 651 bp fragment, coding for a putative polyketide synthase, is highly homologous to *pks* sequences from other fungi.

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Introduction

Cladosporium Link: Fr. (teleomorph *Davidiella*, *Davidiellaceae*, *Capnodiales*) is one of the largest genera of dematiaceous hyphomycetes with more than 772 species [1–3]. It includes fungi with morphologically plastic conidial system and also endophytic, fungicolous, human pathogens, phytopathogenic and saprotrophic forms [4]. Within the genus, *C. cladosporioides* is a cosmopolitan saprotrophic fungus that may be an opportunistic invader of necrotic tissue of many host plants. It also has been isolated from diverse environments such as air, soil and several other substrates [5], and/or might also be a common endophytic or quiescent fungus [3]. *C. cladosporioides* is responsible of several pulmonary and cutaneous infections as well as other human problems such as allergy [6]. However, *C. cladosporioides* is a complex species that includes isolates that though phylogenetically different, are morphologically indistinguible from *C. cladosporioides sensu-strictus* [3].

The mycelia and conidia of *C. cladosporioides* and other representatives of *Davidiellaceae* are coloured due to the synthesis of dark pigments known as melanins [3, 6, 7]. These are secondary metabolites that frequently protect fungi against environmental stresses such as UV-light, predation, desiccation as well as heavy metals [8–11]. Latgé et al. [12] characterized the isolate of *C. cladosporioides* LCP 404 based on its ultrastructure and the composition of the conidial wall, which contained 1,8-dihydroxynaphthalene (DHN)-type melanin. Chodurek et al. [13] described the chemical characteristics of melanin of *C. cladosporioides*, such as the presence of indole derivatives/substructures, which are mostly related to 3,4-dihydroxyphenylalanine (DOPA)-melanin [14]. Buszman et al. [15] and Pilawa et al. [7] provided evidence of the existence of both eu- and pheomelanin. Dadachova et al. [16] found several substructures related to both types of melanins such as DHN- and DOPA-one in an ATCC-VA strain of *C. sphaerospermum*, a species phylogenetical-related to *C. cladosporioides*. The nature of melanins in these dematiaceous fungi seems to be complex, therefore we analyzed the types and role of dark pigments of *Cladosporium* sp. LPSC no. 1088, an organism associated to typical leaf mold lesions on *Solanum lycopersicum* L. bv "Zorzal". The purpose of this work was to characterize the dark pigment synthetized by *C. cladosporioides* LPSC no. 1088 and identify the presence in the organism's genome of the putative genes that might be involved in pigment synthesis.

Materials and Methods

Fungal Material

A monosporic isolate of *Cladosporium* sp. strain LPSC no. 1088 (Culture collection of the La Plata Spegazzini Institute) was obtained from a typical leaf mold symptom on *Solanum lycopersicum* L. bv "Zorzal". Diseased leaves were collected in 2007 from a farmer of the horticultural area close to the city of La Plata (Argentina). The fungus was cultured and maintained on a potato dextrose agar (PDA) medium at 4 °C. It was deposited in the culture collection of the Instituto Spegazzini, UNLP, La Plata, Argentina as LPSC no. 1088.

Extraction and Characterization of Pigments

Dark pigments of *Cladosporium* sp. were extracted from 10 days old cultures that were grown on PDA at 25 °C [11, 17–19]. Briefly, fungal biomass containing mycelia and spores (150 mg, dry mass) was collected from the surface of cultures grown on PDA, washed 3 times each with 5 ml of water and then was boiled in distilled water for 5 min. Then, it was centrifuged at 5,000*g* for 5 min and the pellet was washed with 5 ml of distilled water. To extract the pigment, the fungal pellet was resuspended in 5 ml of 1 M NaOH, then the suspension was heated at 121 °C for 20 min. The pigmented NaOH sample was centrifuged at 5,000*g* for 5 min and the supernatant was collected. The alkaline extract of the pigment was adjusted to pH 2 with concentrated HCl to precipitate melanin. The precipitate was washed with 3 ml of distilled water and dried overnight in an oven at 40 °C. Qualitative diagnostic tests for fungal melanins were conducted as described by Selvakumar et al. [20], including peroxide, polyphenol test and the UV-visible, Fourier Transform Infrared (FTIR) and Electron Spin Resonance (ESR)

spectroscopy analysis. For FTIR spectroscopy, both the *Cladosporium* pigment and the melanin standard (Sigma Chemicals Co., St. Louis, USA) were dried at 40 °C overnight and embedded in infrared grade KBr disk (2 mg 20 mg^{-1}). The FTIR spectrum was recorded in a Perkin-Elmer-Instruments spectrometer (scanning range: 400–4,000 cm^{-1}, resolution: 4 cm^{-1}, number of scans: 64), which was calculated by means of the software OMNIC (Thermo Nicolet, Madison, WI) and was processed according to Saparrat et al. [21]. Bands selected as index peaks reflecting functional groups associated with the fungal melanins were assigned according to Babitskaya et al. [22], Saparrat et al. [11] and Tavzes et al. [23] (Table 1). The ESR spectrum of the *Cladosporium* pigment in dry state was taken in a Bruker EMX-Plus spectrophotometer (room temperature; instrumental parameters: microwave frequency, 9.87 GHz; microwave power, 3 mW; field modulation, 0.5 G) [20].

Inhibition of Melanin Synthesis

Melanin synthesis was analyzed by adding to PDA medium aliquots of stock solutions (1x10^4 ppm) to make up final concentrations of 1, 10 and 100 ppm of tricyclazole (5-methyl-1,2,4-triazolo[3,4-b] benzothiazole, Ultra Scientific Analytical Solutions, United States, dissolved in ethanol) and kojic acid (5-hydroxy-2-(hydroxymethyl)-4-pyrone, Parafarm, Argentina, dissolved in water), a specific inhibitor of DHN-melanin synthesis and an inhibitor of DOPA-melanin synthesis respectively. Plates were inoculated by spreading a conidial suspension and three replica plates per treatment were made (including control plates free of inhibitors) and were incubated in the dark at 25 ± 2 °C for 7 days. Colony growth and pigmentation, estimated as surface color, were measured. While growth was estimated by determining colony diameter, color was measured in two different ways. An estimation of darkness (k) of the colonies was based on the analysis of the grey scale of their scanned images by means of Adobe® Photoshop® 9.0.2 [24]. The other method was to use a colorimeter (Minolta, Model CR-400, Osaka, Japan) to measure parameters such as L*: lightness values from 0 (absolute black) to 100 (absolute white), a*: negative (green) and positive (red) values and b*: negative (blue) and positive (yellow) values. Data were

Table 1 FTIR bands identities of pigment spectra

Wave number (cm^{-1})	Functional group	References
3440–3300	Stretching vibrations of free O–H and N–H	Babitskaya et al. [22], Tavzes et al. [23]
2920–2850	Stretching vibrations of various C–H groups in methyl and methylene groups	Babitskaya et al. [22], Tavzes et al. [23]
1800–1740	C=O stretching in free carboxylic acids	Tavzes et al. [23]
1740–1730	C=O stretching in aliphatic aldehydes, ketones, and carboxyls not conjugated with benzene ring	Tavzes et al. [23]
1700	C=O ketones, carboxylic acids esters	Saparrat et al. [11]
1670–1600	Changes in conjugated double bonds (C=C and C=O; and C=O in the composition of secondary amines). Amide I: C=O stretching in amides. Aldehydes, ketones, and carboxyls conjugated with C=C or benzene ring, conjugated quinone structures. This band, typical of a conjugated quinoid structure, is believed to be of considerable importance for identification of melanin	Babitskaya et al. [22], Tavzes et al. [23]
1500	Bonds aromatic C-C. Amide II: C–N and N–H deformation in amides	Saparrat et al. [11], Tavzes et al. [23]
1480–1350	Amide and amine groups and deformational changes in NH groups of secondary amines, CH_2 groups of aliphatic radicals, CH groups adjacent to COOH and OH groups, and C=O of quinones	Babitskaya et al. [22], Tavzes et al. [23]
	C–H deformation vibrations of CH_3 and CH_2	
1280–1250	CO groups of acid, ester and phenol groups	Babitskaya et al. [22]
1100–1050	Alcoholic C–O	Tavzes et al. [23]

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.

Colony Growth and Pigmentation under Fungicide Stress

Growth and pigmentation on PDA medium amended with increasing concentrations of two commercial fungicides, Difenoconazole (Bogard 25 %, Syngenta Agro S.A., Argentina) and Chlorotalonil (Daconil 72F, Syngenta Agro S.A., Argentina), were also determined. Aliquots of 2.5×10^5 ppm stock solutions were added to sterile PDA medium to make up concentrations of 50, 100, 250, 500, 1,000, 2,500 and 5,000 ppm. Plates were inoculated by placing a 6-mm diameter mycelial plug, which was cut from a culture grown on PDA medium. Three replica plates per treatment (including fungicide free control plates) were incubated in the dark at 25 ± 2 °C for 10 days. Colony growth and pigmentation were compared to control plates. Growth was estimated as described. The concentration of fungicide that caused 50 % of growth inhibition (Ci_{50}) was estimated from the relationship between the percentage of growth inhibition and the log-concentration of the fungicide [25]. Colony color (darkness, k) was measured as described. Data were analyzed by means of an 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.

Preparation of Genomic DNA, PCR Amplification of Ribosomal DNA and Analysis of the Nucleotide Sequences

Fungal genomic DNA was isolated by the method described by Bornet and Branchard [26]. Primers ITS-4 (5′-AAGCTTTCCTCCGCTTATTGATATGC-3′) and ITS-5 (5′-GAATTCGGAAGTAAAAGTCGTA ACAAGG-3′) from White et al. [27] were used to prime the PCRs aimed at amplifying the 3′ end of the 18S rDNA, ITS1, 5.8S rDNA, ITS2 and the 5′ end of the 28S rDNA. Reactions contained 10× amplification buffer (InbioHighway, Argentina), 1.5 mM $MgCl_2$ (InbioHighway, Argentina), 30 ng of each primer, 200 μM of each deoxynucleoside triphosphate (InbioHighway, Argentina), 25 ng μL^{-1} of DNA template and 0.5 U of Taq DNA polymerase (InbioHighway, Argentina) in a 15 μL volume. Reactions were performed in a PTC-150 Mini Cycler™, programmed as follows: an initial step at 94 °C for 4 min, followed by 33 cycles of a denaturing step at 94 °C for 45 s, annealing at 56 °C for 45 s, extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. Control experiments lacked template DNA. Amplified fragments were visualized and compared to a 100–1,000 bp marker (Inbio-Highway-Tandil-Argentina) on 1 % agarose gels stained with ethidium bromide and documented by means of an image analyzer Syngene Inc. The amplified PCR product was purified and sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit and the automated ABI Prism 3730 DNA sequencer (Applied Biosystems). The sequence of the ITS was deposited in GenBank under the accession number JF949719.

Identification of *pks* and Phylogenetic Analysis

By means of a set of degenerate primers, LC1/LC2c [28], which are homologous to the conserved keto-synthase (KS) condensing domain of fungal poliketide synthases (PKSs), we run amplification reactions with genomic DNA of isolate LPSC 1088 as template. Polymerase chain reactions contained 200 μM of dNTPs, 1 μM of each primer, 0.5 U Taq DNA polymerase (InbioHighway, Argentina) and 10 ng of genomic DNA as template. Amplifications were performed in a thermal cycler programed as follows: denaturation step of 5 min at 94 °C followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 10 min. PCR products were resolved in a 1 % agarose gel with 0.2 μg μL^{-1} ethidium bromide and were visualized under UV light and compared to a 100–1,000 bp DNA molecular marker (Inbio Highway-Tandil-Argentina). Gel scanning and analysis was performed on Gene Genius image analyzer (Syngene Inc). Sequencing of the fragment was performed by means of the BigDye Terminator Cycle Sequencing Ready Reaction kit and the automated ABI Prism 3730 DNA sequencer (Applied Biosystems) based on the dideoxy terminator reaction chemistry [29]. The DNA sequence was deposited in GenBank under the accession number JN205332 and identified by means the Blast (www.ncbi.nlm.nih.gov) with highly homologous sequences deposited in the NCBI GenBank.

A phylogenetic analysis was conducted using the Bioedit Sequence Alignment Editor (BioEdit versión 7.0.0) on the DNA sequence coding for a fragment of

KS domain of PKSs in *Cladosporium* LPSC 1088 and homologous sequences available for other ascomycetous fungi in public databases aligned in CLUSTAL W (version 1.83) [30] as well as that from *Streptomyces avermitilis*, which was used as an outgroup [31]. Phylogenetic trees were prepared by the neighbour-joining method. Boostrap values were calculated from 1,000 replications of the bootstrap procedure using the program Mega (version 5.01).

Results

Fungal Identification

Cladosporium sp. LPSC 1088 colonies reached a diameter of 3.9 cm ± 0.6 after a 10 days incubation period on PDA medium at 25 °C in the darkness. Colonies had defined margins and a green-olivaceous to olivaceous-grey pigmentation on the top and black

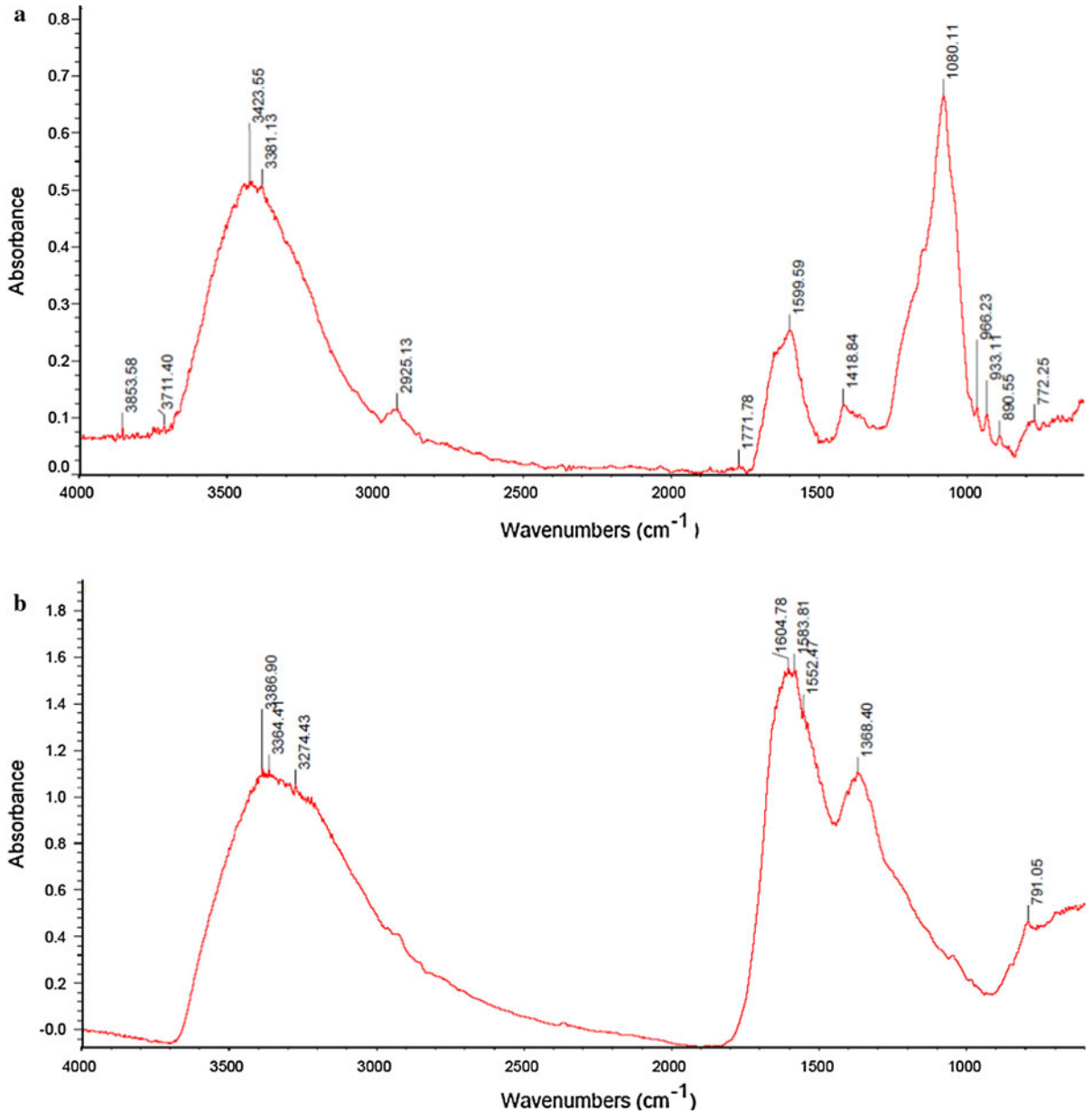

Fig. 1 FTIR spectrum of melanin-like compound isolated from *in-vitro* cultures of *C. cladosporioides* (**a**) and standard one (**b**)

on the reverse of the plates. The fungus presented typical morphological characteristics of the *Cladosporium* complex, such as branched straight and smooth conidiophores of an olivaceous-brown to olivaceous colour arising terminally from ascending hyphae, conidiogenous cells integrated usually terminal of 16–38 μm long and 1–2 μm of diameter, numerous conidia 3–6 μm long and 2–2.5 μm wide, catenate, cylindrical or ellipsoid, with 0–2 septa and an olivaceous-green colour. Both morphological characteristics as well as the ITS sequence (Genbank accession JF949719) confirmed that the isolate was *C. cladosporioides*.

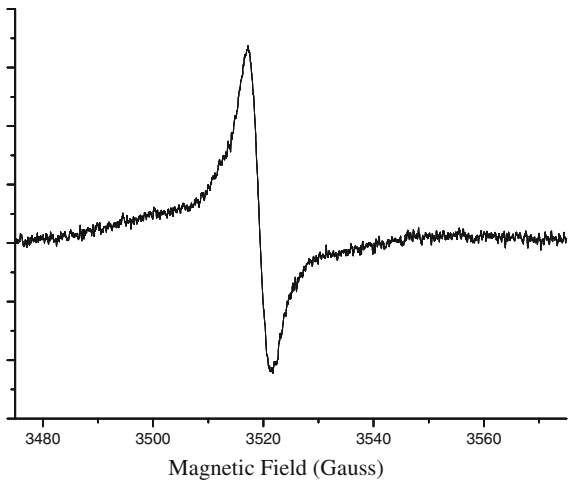

Fig. 2 ESR spectrum of dark pigment isolated from *in-vitro* cultures of *C. cladosporioides*, which was performed in dry state. Ordinate is the derivative of the ESR absorption in arbitrary units

Dark Pigments from *C. cladosporioides*

Pigments were dark gray in solid phase and turned to a dark-brown color when they were heated at 121 °C for 20 min in 1 M NaOH solution. They were insoluble in 3 N HCl, water and organic solvents such as chloroform or ethanol as well as decolorized with H_2O_2 and also turned to a reddish orange colour when treated with $FeCl_3$. The peak of UV–visible absorption was at 240 nm and there was a gradient of $\log_{10}$ absorbance (240–500 nm) versus wavelength plots of −0.0041. It showed a conserved FTIR pattern of absorption at 3,440–3,300, 2,925–1,599, 1,418, 1,100 and 1,050 cm^{-1}, which corresponded to –OH and –NH bonds, –O–CH_3 and –CH_2 groups, conjugated double bonds (C=C and C=O; and C=O in the composition of secondary amines), amide and amine groups and deformational changes in NH groups of secondary amines, CH_2 groups of aliphatic radicals, CH groups adjacent to COOH and OH groups, and C=O of quinones and C–O deformation vibrations of aliphatic alcohols, respectively (Fig. 1). In summary, the pigment had a typical conjugated quinoid structure as well as a melanoprotein component, which are both characteristic features of melanins. Though its comparison with the FTIR spectrum of a standard melanin (absorption bands between 3,400–3,200, 1,605–1,550, 1,368 and 791 cm^{-1}) exhibited a somewhat similar pattern, it was different since a peak around 1,080 cm^{-1} was only found in *C. cladosporioides* pigment. Furthermore, the ESR analysis revealed the presence of stable free radicals in dry pigment from *C. cladosporioides* LPSC no. 1088 (Fig. 2).

Fig. 3 Colonies of *C. cladosporioides* LPSC no. 1088 grown on PDA in absence (Control) and presence of 10 and 100 ppm of tricyclazole (T) for a 7 days period

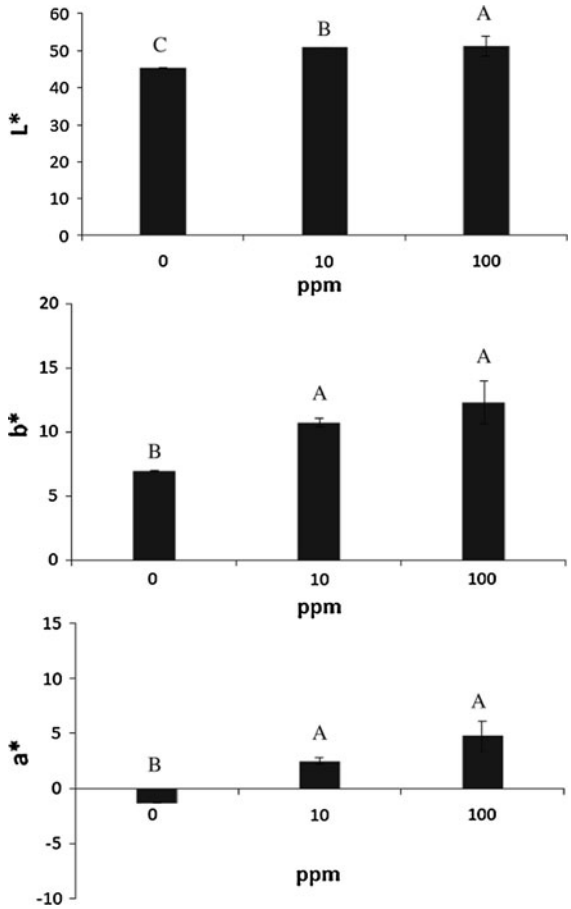

Fig. 4 Pigmentation of colonies of *C. cladosporioides* LPSC no. 1088 grown on PDA in absence (0) and presence of 10 and 100 ppm of tricyclazole for a 7 days period expressed as L^*, a^* and b^*. Values are means of three replicates; *error bars* correspond to SD. *Bars* with the *same capital letter* are not significantly different ($P < 0.05$)

As expected the addition of tricyclazole provoked a reduction in fungal growth (Fig. 3), which was a function of its concentration (at 100 ppm a 33.6 % growth reduction, $P < 0.05$). Furthermore, the fungicide also provoked changes in colonies colour that turned to a brown-orange, which resulted in a reduction of the darkness "k" and an increase in the L*, a* and b* colour components (Fig. 4). Microscopic observations of the fungus grown in the presence of tricyclazole compared to control cultures confirmed also the inhibition of the dark pigmentation of hyphae and conidia of *C. cladosporioides* when it is grown on PDA medium (Fig. 5). On the other hand, growth and pigmentation did not change in response to the addition of kojic acid (data not shown).

Colony Growth and Pigmentation under Fungicide Stress

Chlorothalonil and Difenoconazole inhibit fungal growth though the latter one with a higher efficiency (IC_{50} values were 246.4 and 121.1, respectively). Furthermore, both fungicides provoked a reduction in conidia production (data not shown) and an increment in the darkness "k" component of the colony color, at concentrations higher than 500 ppm of Difenoconazole and 250 ppm of Chlorothalonil (Fig. 6).

pks Identification

By means of primers LC1/LC2c we visualized on a gel a ~700 bp amplified PCR product (*pks*,

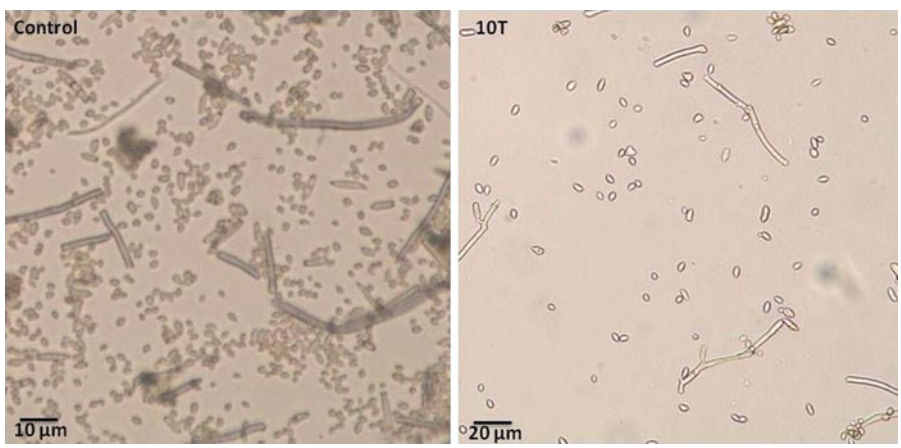

Fig. 5 Mycelia and conidia of *C. cladosporioides* LPSC no. 1088 grown on PDA in absence (Control) and presence of 10 ppm of tricyclazole (10 T) for a 7 days period

Fig. 7), which according to the published sequence of PKS enzymes involved in DHN-melanin biosynthesis was of the expected size. The DNA sequence of the amplified fragment, which had 651 bp, was highly homologous to those of other *pkss* such as *pks*1 of fungal endophyte sp. CR61 (84 %), *Arpks1* of *Ascochyta rabiei*, *Mppks1* of *Ascochyta pinodes*, *pks*1 of *Bipolaris oryzae* and *pks*18 of *Cochliobolus heterostrophus* (83 %). A phylogram based on the partial nucleotide sequences of several fungal *pkss* and that of *Streptomyces avermitilis* generated a tree where the *pks* sequence of *C. cladosporioides* was grouped with sequences that corresponded to PKSs involved in aflatoxin and pigments synthesis, including DHN-melanin (Fig. 8).

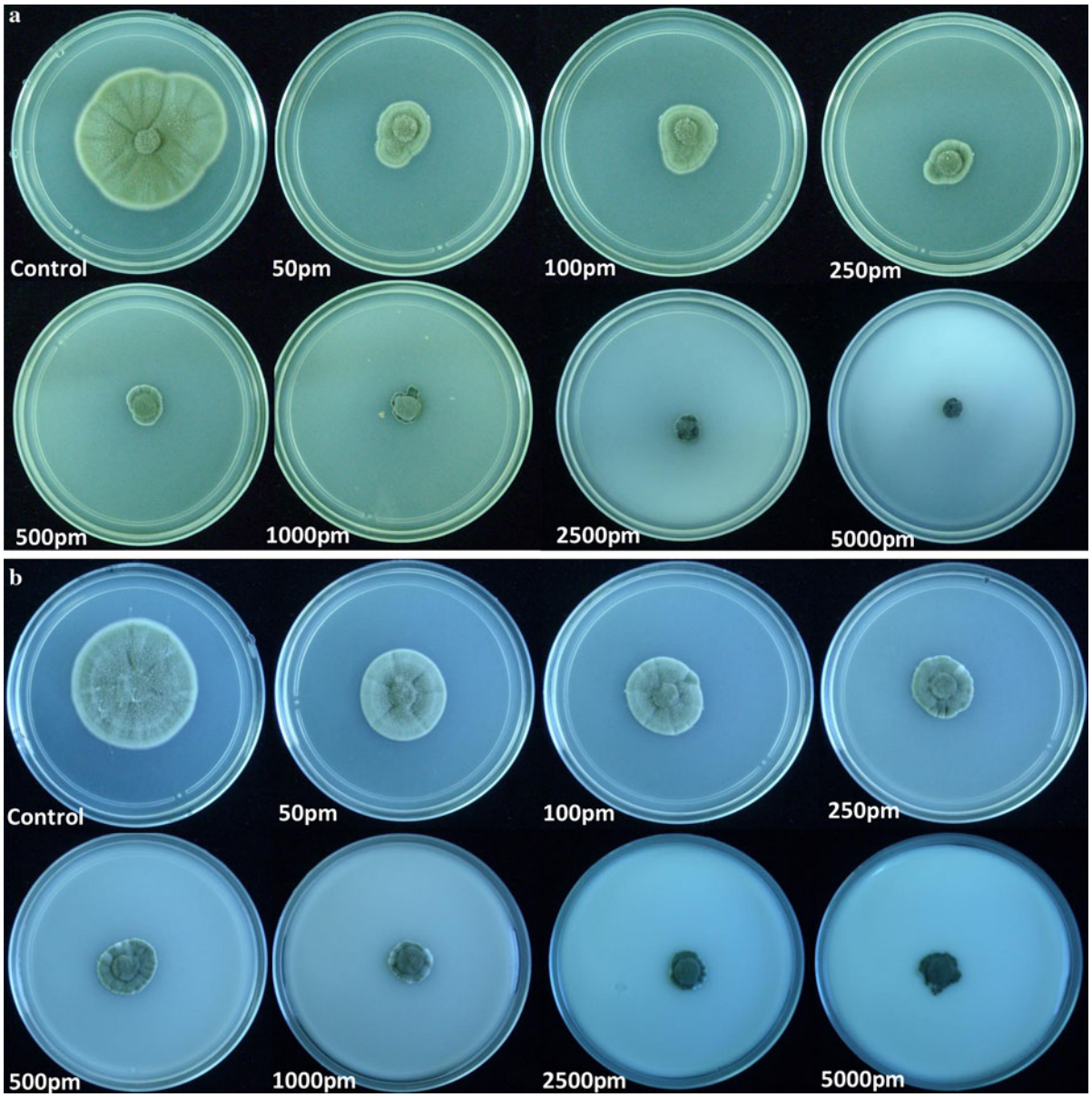

Fig. 6 Colonies of *C. cladosporioides* grown on PDA medium (control) and in the presence of different concentrations of fungicides Difenoconazole (**a**) and Chlorothalonil (**b**) for a 10 days incubation period

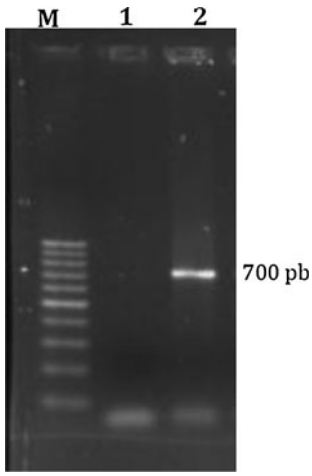

Fig. 7 Fragment amplified by PCR with primers LC1/LC2c on the genomic DNA of *C. cladosporioides* LPSC 1088. *Lane 1* control PCR without DNA, *Lane 2* PCR amplification product. *M* molecular weight marker 100–1,000 bp (Highway)

Discussion

Cladosporium cladosporioides is a dematiaceous fungus that is widely distributed and most probably plays relevant roles in several areas such as the agronomical, industrial and medical one. The colour of both mycelium and conidia of *C. cladosporioides* as well as that of other fungi belonging to the family *Davidiellaceae* is due to the presence of dark pigments known as melanins [3, 6, 7]. Since the nature of the melanins found in *C. cladosporioides* complex is controversial [7, 32, 33], here we analyzed the dark pigments synthetized by *Cladosporium* sp. LPSC no. 1088. Physico-chemical properties such as UV-visible absorbance and ESR spectrum, behaviour in several solvents and other qualitative diagnostic tests suggested that the pigment synthetized by isolate 1088 is most probably a melanin-like compound. The FTIR analysis indicated that the extracted main pigment might be DNH-melanin since it shared characteristic signals of melanins found in *Amorphotheca resinae* Parbery (syn. *Cladosporium resinae* Vries) [23]. Interestingly, the FTIR pattern of the melanin-like compound produced by isolate 1088 was different from that of standard melanin from *Sepia officinalis* (DOPA-melanin, a copolymer of 5,6-dihydroxyindole and 5,6-dihydroxyindole 2-carboxylic acid) [34]. Melanins such as DHN- and DOPA-ones have already been found in *C. cladosporioides* [7, 12, 13, 15, 35]. However, a reliable taxonomy identification of the isolates used as pigment source is still needed. Also, *C. sphaerospermum*, a species phylogenetical-related to *C. cladosporioides*, contained both DHN- and DOPA-melanins [16]. Therefore, our results and those of others suggest that melanins such as DHN- and DOPA-ones are widespread within the genus *Cladosporium*.

Tricyclazole, a specific inhibitor of DHN-melanin synthesis, affected growth and pigmentation of *in-vitro* cultures of isolate 1088. This suggests that it produces DHN-melanin as the main chromophore, which most probably is responsible of colony pigmentation, as described for other typical fungi from Ascomycota [11]. Latgé et al. [12] found that this fungicide inhibited melanin synthesis in *C. cladosporioides* LCP 404 and also induced diffusion of coloured shunt products of the pentaketide pathway to the medium. In addition to this, Bensch et al. [3] found that cultures of *C. perangustum* growing on PDA medium, without tricyclazole, synthetize orange soluble pigments that are occasionally released to the agar. Therefore, these results further support that colonies of *Cladosporium* species are mostly pigmented mainly by melanin, though these fungi may also synthesize other pigments, including water soluble ones that might diffuse to the media.

Melanins are considered secondary metabolites that may protect fungi from environmental stresses such as UV-light, predation, desiccation as well as xenobiotics and metals [8, 9, 11]. Therefore we hypothetized that melanin synthesis should be affected by a chemical stress such as the presence of Chlorothalonil and Difenoconazole. Both fungicides resulted in the development of darker colonies of *C. cladosporioides* confirming that pigment synthesis increased due to the stress provoked by the chemicals, and therefore might protect fungal cells against the stress. Griffith et al. [24] found that copper, a heavy-metal with fungicide activity, induced the development of a dark pigmentation in *in-vitro* cultures of several fungi, including *C. herbarum*. Furthermore, pigmentation has been found to increase and most probably protect fungi such as *C. phlei*, *Pneumocystis carinii* and *Rhizoctonia solani* to oxidative stress, UV-irradiation, desiccation and fungicides [36–38]. Although we did not determine the amount of melanin synthesized in the tissue of stressed cultures of isolate 1088, it was evident that melanization was enhanced. Singaravelan et al. [39] found that melanins are not essential for fungal

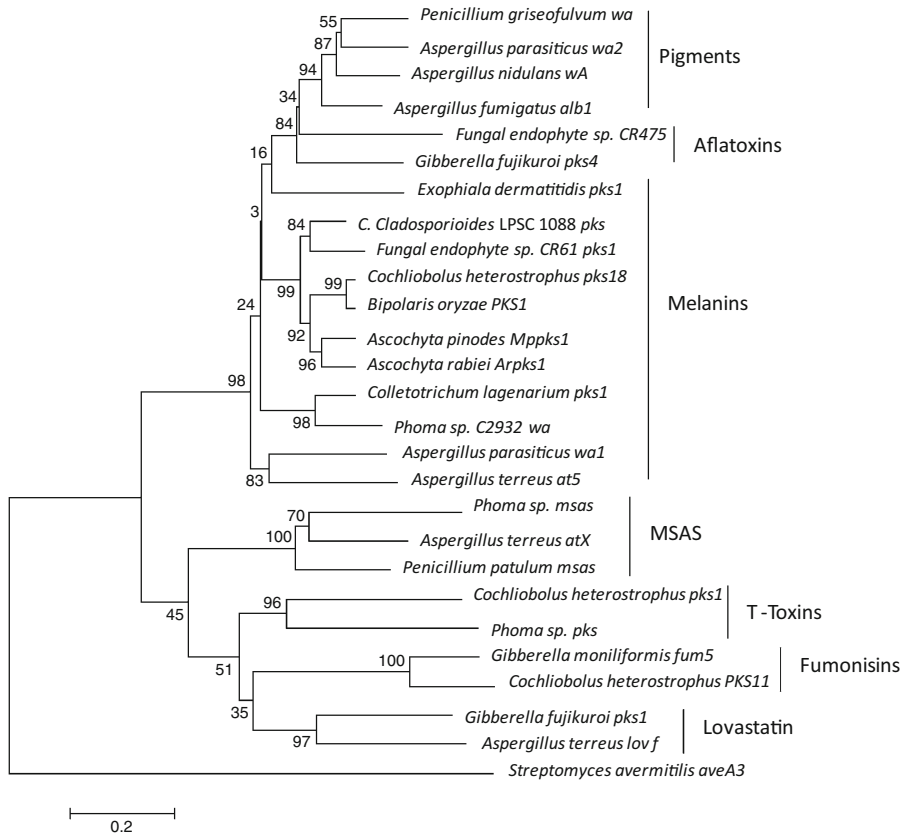

Fig. 8 Neighbor-joining tree based on the phylogenetic analysis of nucleotide sequences belong to fungal *pkss* and their role. MSAS, 6-methylsalicylic acid synthases. Source of sequences (GenBank accession number) used: *Ascochyta pinodes Mppks1* (ACS74443), *Ascochyta rabiei Arpks1* (ACS74449), *Aspergillus fumigatus alb1* (AAC39471), *Aspergillus nidulans wA* (CAA46695), *Aspergillus parasiticus wA1* (CAB44698) and *wA2* (CAB44699), *Aspergillus terreus at5* (BAB88752), *atX* (BAA20102) and *lovF* (AAD34559), *Bipolaris oryzae pks1* (BAD22832), *Cochliobolus heterostrophus pks1* (AAB08104), *pks11* (AAR90266) and *pks18* (AAR90272), *Colletotrichum lagenarium pks1* (BAA18956), *Exophiala dermatitidis pks1* (AAD31436), *Fungal endophyte* sp. CR 475 *pks1* (AAP68701), *Fungal endophyte* sp. CR61 *pks1* (AAP68704), *Gibberella fujikuroi pks1* (CAC44633) and *pks4* (CAD19100), *Gibberella moniliformis fum5* (AAD43562), *Penicillium griseofulvum wA* (CAB44712), *Penicillium patulum msas* (CAA39295), *Phoma* sp. *msas* (CAB44720), *pks* (AAO62426) and *wa* (CAB44719) and *Streptomyces avermitilis aveA3* (BAC68652) as outgroup

growth, but they enhance survival and competitiveness of fungi under stressful environments. Our findings suggest that melanin synthesis was enhanced by the fungicides mostly due to the stress provoked, therefore melanins might also protect *C. cladosporioides* cells against other stresses including environmental ones.

PKSs are key multi-enzymes that participate in the biosynthesis of DHN-but not DOPA-melanin [40]. We found within genomic DNA of *C. cladosporioides* a 651 bp sequence coding for PKS, which was highly homologous to the *pks* of the fungal endophyte CR61 of the wild cranberry (*Vaccinium macrocarpum*) plants, which belongs like *Cladosporium* to the Class Dothideomycetes (Order Dothideales) [41]. A phylogenetic analysis was run with *psk* sequences using as outgroup the *pks* sequence corresponding to *Streptomyces avermitilis*. The partial sequence of *pks* of isolate 1088 belongs to the non-reducing type I PKSs that are mostly involved in biosynthesis of pigments such as DHN-melanin and other spore pigments and also secondary metabolites such as aflatoxins, which is a sister group of the reducing PKSs [42, 43]. Clustering also showed the close relationship of the *pks* sequence of isolate 1088 with those of representatives of the Class Dothideomycetes, which had been associated with DHN-melanin. Schmitt et al. [44] also

found a relationship between clusters from *pks* sequences and taxonomical entities in the lichenized Ascomycota. All this evidence led us to concluded that isolate 1088 has a pentaketide-pathway for synthesis of melanin, that is most probably responsible of fungal pigmentation. To our knowledge, this is the first report about the isolation of a partial sequence of a gene coding for enzymes involved in the melanin biosynthesis in the genus *Cladosporium.* Furthermore, the availability of data on a *pks* in *C. cladosporioides* provides an opportunity to further investigate the biosynthesis of melanins in this fungus and its relationship with fungal response to fungicides.

In conclusion, *C. cladosporioides* isolate 1088 is an opportunistic fungus that was isolated from tomato leaf lesions, that look like typical leaf mold symptoms. The fungus produces dark mycelium and conidia and this was due to the synthesis of DHN-melanin like compounds, which most probably are responsible main of pigmentation of the fungus. Melanization of *C. cladosporioides* LPSC no. 1088 through a pentaketide pathway might play a protecting role against stresses imposed either by chemical compounds such as fungicides or the environment.

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