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Melanins from two selected isolates of Pseudocercospora griseola grown *in-vitro*: Chemical features and redox activity

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ABSTRACT

Pseudocercospora griseola is the causal agent of Angular Leaf Spot (ALS), a disease of common bean. Due to its coevolution with beans, two major groups have been defined, "Andean" (*P. griseola f. griseola*) and "Mesoamerican" (*P. griseola f. mesoamericana*). The aim of this study was to characterize the dark pigment, melanin, synthetized by a selected isolate of each genic group of *P. griseola* when grown on Potato-dextrose broth. *P. griseola f. griseola* isolate S3b and *P. griseola f. mesoamericana* isolate T4 produced 1.7 ± 0.6 and 4.1 ± 0.9 mg of melanin per g of dry biomass, respectively. Although both melanins possessed similar UV–visible absorption spectroscopic pattern, *P. griseola f. mesoamericana* isolate T4 melanin had a lower UV–visible absorption, higher reducing activity and metal chelating ability than melanin from *P. griseola f. griseola* isolate S3b. However, when the size of the sample was 10 mg S3b melanin had a higher content of free phenolic groups. Furthermore, cell wall polysaccharides modified in melanin the availability of active phenolic groups, which was dependent on the fungal isolate and the size of the sample. Therefore, the amount and chemical features of melanin as well as its deposition in mycelium walls within isolates is different, which might explain the different pigmentation and physiological behaviours of these representatives of the two groups of *P. griseola*.

1. Introduction

Dark pigments known as melanin are complex polymers composed of aliphatic and indole- or phenol-type aromatic structures that have been related with pathogenesis and/or survival strategies of fungi [1–6]. Melanins are amorphous solids not amenable to crystallographic structural studies. Melanins are mostly insoluble in water and common organic solvents and can only be dissolved in alkaline solutions. They are located in cell walls mostly associated with other polymers and because of this, their extraction and purification demand the use of harsh chemical methods. Such procedures as well as the strategies used to solubilize these pigments might alter their structure, which complicates even further their characterization [4, 7, 8]. Since melanin cannot be defined based on aqueous solution-state or crystallographic techniques, alternative approaches must be followed in order to characterize it. Several techniques such as electron spin resonance (ESR), ultraviolet-visible absorption (UV–visible), and Fourier transform infrared (FTIR) spectroscopies, and chromatography technologies have been used to

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characterize melanins. In addition to this, specific chemical agents also have been employed to evaluate melanins' reactivity [8–10].

Pseudocercospora griseola (Sacc.) Crous and U. Braun (Mycosphaerellaceae, Capnodiales, Dothideomycetes) is the etiological agent of Angular Leaf Spot (ALS), a disease that severely affects common bean (*Phaseolus vulgaris* L.), provoking considerable reductions in yield [11, 12]. It is the most important endemic disease in northwestern Argentina [13]. This pathogen coevolved with its host, and like beans, has two major intraspecific groups, the *formae P. griseola f. griseola* (Andean group), and *P. griseola f. mesoamericana* (Mesoamerican group) [14]. Representatives of these groups differ in growth, pigmentation, thermal tolerance and range of virulence on bean varieties [14–16].

Saparrat et al. [17] reported that *P. griseola*, whether belonging to the Andean group or Mesoamerican one, synthesizes 1,8-dihydroxynaphthalene (DHN) melanin and suggested a possible link between melanin and growth. Recently, Bárcena et al. [16] found that tricyclazole (5-methyl-1,2,4-triazolo[3,4-*b*]benzothiazole), a specific inhibitor of 1,8-DHN melanin synthesis, affected growth, morphology and pigmentation of *P. griseola f. mesoamericana* isolate T4. Interestingly, as it has been shown for other fungi such as *Mycosphaerella fijiensis*, melanin is accumulated within cell walls [18]. However, to the best of our knowledge, there is no information regarding the synthesis, properties and roles of these dark pigments in *P. griseola f. griseola*, which is less pigmented than *P. griseola f. mesoamericana*.

Since the role of melanin within dematiaceous fungi, including pathogenic ones, is still under debate, we performed studies aimed at knowing more precisely the chemical nature and activity of melanin from *P. griseola* belonging to two pools of origin when grown *in-vitro*.

This study might provide the basis to understand the biological role of the melanin in this fungus and its contribution to the interaction between common bean and *P. griseola*. Therefore, the aim of this research was to characterize and compare isolated melanins and ones within biomass from cultures of *P. griseola f. griseola* and *P. griseola f. mesoamericana* grown under the same conditions.

2. Materials and Methods

2.1. Fungi

Pseudocercospora griseola belonging to the Andean (*P. griseola f. griseola*; isolates Ecu6, J2a, J1a, S3b and Ecu3) or Mesoamerican (*P. griseola f. mesoamericana*; isolates S6a, T2, PG1, PG15 and T4) group, such as reported by Stenglein and Balatti [19], were used in this study. They were kept at −20 °C on filter paper previously colonized by them. Fresh cultures of the isolates were grown for 7 days on a potato dextrose agar (PDA) medium at 24 ± 2 °C and maintained at 4 °C.

2.2. Screening for Colony Growth and Pigmentation on Agar Medium

Three replicates for ten isolates of *P. griseola* belonging to the Andean or Mesoamerican group were inoculated according to Bárcena et al. [16] on PDA plates, which were incubated in the dark at 24 ± 2 °C for a 21-day period. Colony growth and pigmentation, estimated as surface color, were measured. 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 analyzed with a grey scale using Adobe® Photoshop® 8.0.1 [20].

2.3. Inoculum Source, Fungal Cultures and Microscopical Analysis Using Selected Isolates

Cultures of each selected isolate grown on PDA medium were used as source of conidia and for microscopical study. Representative samples of mycelium of each fungus taken from three 21-day old cultures growing on PDA medium were processed and observed with transmission electron microscope (TEM) according to Bárcena et al. [16]. A conidial suspension (2.0×10^4 conidia ml^{-1}) was used to inoculate 1000 ml Erlenmeyer flasks containing 200 ml of potato dextrose (PD) broth, which were incubated at 24 ± 2 °C in the dark, in a shaker at 150 rev. min^{-1} for 21 days. The experiment was a completely randomized design with two treatments (S3b and T4) and the number of replicates per treatment was three Erlenmeyer flasks.

2.4. Fungal Biomass and Melanin Extraction

Mycelia were collected by filtration from 21 days old cultures, washed extensively with hot water and then were dried in an oven at 80 °C, until constant weight. This biomass was used to estimate growth and then was frozen with liquid nitrogen and ground in a mortar. The powder material was stored at 25 °C.

Melanin was extracted from 100 mg aliquots of dry fungal biomass, which was suspended in 5 ml of 1 M NaOH and heated at 121 °C for 20 min [17]. The sample was centrifuged at 5000 ×*g* for 10 min, the supernatant collected and the pH adjusted to 2 by adding 3 M HCl. In order to precipitate melanin, the supernatant was incubated overnight at 4 °C, and the pigment was recovered by centrifugation at 5000 ×*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.

2.5. UV–Visible and ESR Spectroscopies, Redox and Ion Chelating Activity of Melanins

Melanins were dissolved in 0.1 M NaOH to a final concentration of 0.1 mg ml^{-1} and their UV–visible absorption spectrum was measured in a Shimadzu spectrophotometer UV–visible (UV-160) PC. The UV–visible region scanned was 200–800 nm. The relationship between log absorbance and wavelength was determined.

The electron spin resonance (ESR) spectrum of melanins from isolates S3b and T4 either in dry state or in alkaline solution was taken in a Bruker EMX-Plus spectrophotometer at room temperature, under the following parameters: microwave frequency, 9.87 GHz; microwave power, 3 mW; and field modulation, 0.5 G [21, 22].

The antioxidant capacity of melanins from isolates S3b and T4 was determined by the ammonium molybdate method [23]. Two milliliters of a suspension of melanin (0.5 mg ml^{-1}) in phosphate buffer (0.2 M, pH 6.6) were combined with 2 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The screw-capped glass tubes were incubated in a thermal block at 95 °C for 120 min. Each 30 min, three replicate tubes (aliquots of each suspension) were cooled to room temperature, centrifuged at 10,000 ×*g* for 10 min, and absorbance of the supernatant was measured at 695 nm. The antioxidant activity was expressed as the absorbance of the sample. Butylated hydroxytoluene (BHT, 0.5 mg ml^{-1} in ethanol) was used as reference compound. Measurements were performed in triplicate.

Chelation of ferrous ions by melanin was estimated by the method of Farhan et al. [24]. Different amounts of melanin (2.0–15.0 mg) were incubated with 0.6 mM ferrozine and 0.12 mM $FeSO_4$ for 10 min at room temperature. The mixture was centrifuged 10,000 ×*g* for 10 min. and the absorbance of the supernatant at 562 nm was measured. The ability of melanin to chelate ferrous ions was calculated in relation to control reactions that contained only ferrous ions and ferrozine. We used the formula: Chelating ability (%) = [(abs. Control–abs. sample)/(abs. control)]∗100. Measurements were performed in triplicate.

2.6. Analysis of Isolated Melanins and Ones within Biomass Using the Folin-Ciocalteu Method, Fluorescence and FTIR Spectroscopies

The content of active phenolic groups was determined on both fungal biomass and isolated melanin using the Folin-Ciocalteu method [25]. Each 1 and 10 mg sample was mixed with 100 µl of Folin-Ciocalteu reagent and 1300 µl of distilled water and stirred at 25 °C for

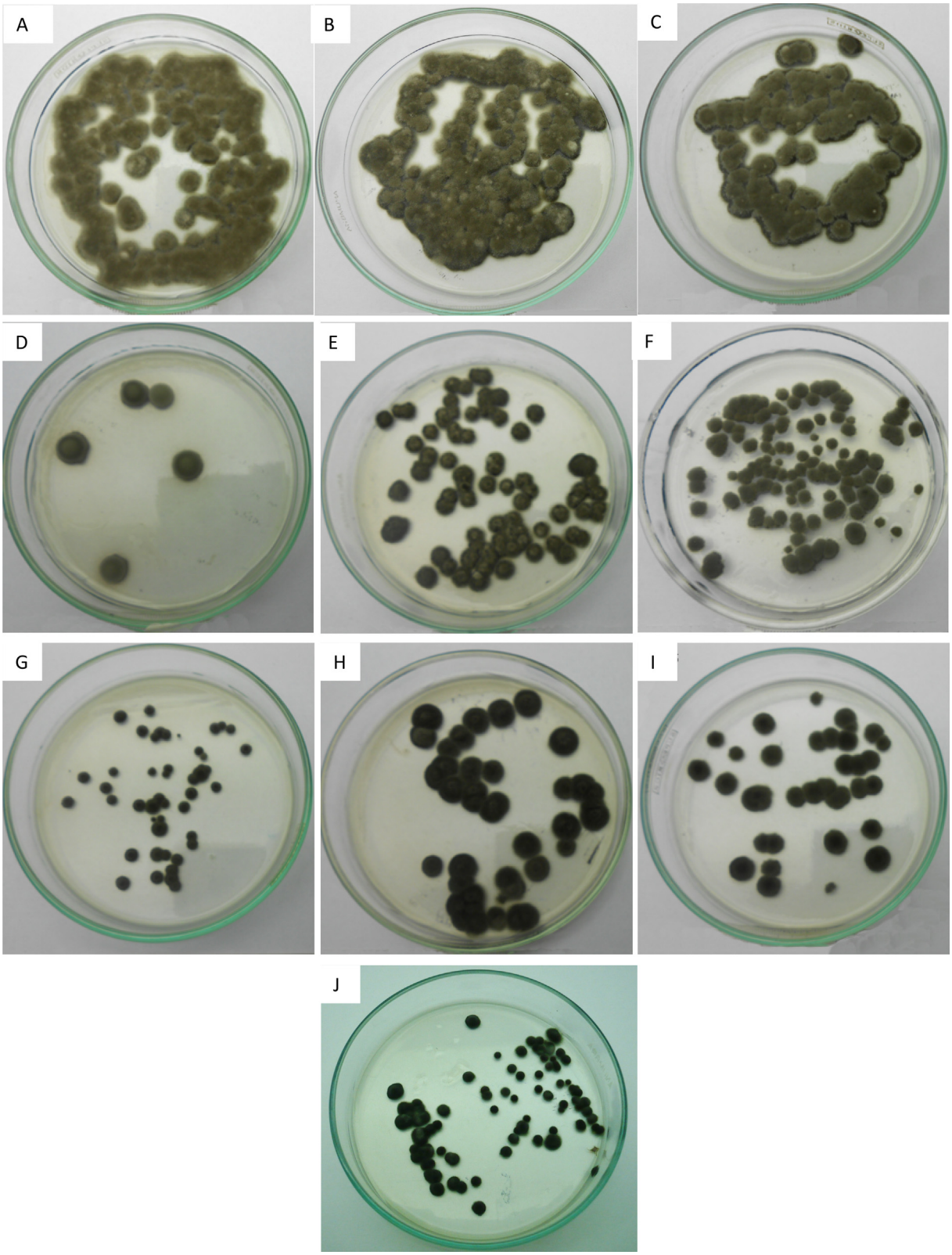

Fig. 1. Colonies of *P. griseola f. griseola* (isolates: Ecu6, A; J2a, B; J1a, C; S3b, D; and Ecu3, E) and *P. griseola f. mesoamericana* (isolates: S6a, F; T2, G; PG1, H; PG15, I; and T4, J) grown on PDA medium for a 21 days period at 24 ± 2 °C.

3 min. Then, 1500 µl of 2% Na_2CO_3 in 0.1 N NaOH was added, stirred and incubated at 25 °C for 1 h. The suspension was centrifuged at 10,000 × *g* for 10 min and absorbance was measured at 760 nm. In order to determine the total available phenolic content of each sample, calibration curves were prepared using standard solutions of tannic acid in a concentration range between 5.0 and 20.0 mg ml^{-1}. Controls consisted in reaction mixtures lacking sample. Results were calculated from the regression equation generated with a calibration curve and were expressed as µg of phenols per total amount of sample in mg. Assays were performed once with three replicates per treatment.

Melanin (10 mg for T4 isolate and < 1 mg for S3b one) or biomass (10 mg) of each fungus was exposed to H_2O_2 6 M and light for 15 min [26]. Excitation and emission spectra of alkaline solutions from each sample were measured in a spectrofluorometer (model RF-1501, Shimadzu Corporation, Kyoto, Japan) at a wavelength range of 200–800 nm, and at medium scanning speed. Controls consisted in melanin or biomass with no H_2O_2.

The chemical composition of biomass and melanin of S3b and T4 was analyzed by FTIR spectroscopy [27]. Samples were embedded in infrared grade KBr disk (2 mg 20 mg^{-1}) and scanned in a Perkin–Elmer–Instruments spectrometer (scanning range: 400–4000 cm^{-1}, resolution: 4 cm^{-1}, number of scans: 64). Spectra data were processed by means of the software OMNIC (Thermo Nicolet, Madison, WI) according to Saparrat et al. [28]. Measurements were performed twice with three replicates.

2.7. Statistical Analyses

Data of colony growth and its pigmentation as well as available phenolic groups, antioxidant activity, and metal chelating activity of the melanins were analyzed by means of a one-way analysis of variance (ANOVA) and means of the treatments were contrasted by the TukeyTest (at $P \leq 0.05$) using the Statistix 8.0 software for Windows. The means of thickness of hyphal walls of the selected fungi were contrasted by means of Student's *t*-test (Statistix 8).

3. Results

3.1. Colony Growth and Pigmentation of Isolates de Pseudocercospora griseola on Agar Medium

Fig. 1. shows colonies of several isolates of *P. griseola* f. *griseola* and *P. griseola* f. *mesoamericana* grown on PDA medium for a 21 days period at 24 ± 2 °C in the darkness. Although a variable range in growth and pigmentation was observed among agar cultures of the isolates tested, differences in these features were found among fungi belonging to each genic pool ($P < 0.05$, Table 1). Isolate S3b showed the highest growth among fungi belonging to Andean group. Although fungi PG1 and PG15 presented higher diameters compared to those revealed by other isolates tested belonging to the Mesoamerican group, differences were not always statistically significant. In relation to pigmentation, *P. griseola* f. *griseola* Ecu3 showed darker colonies compared to other isolates belonging to Andean group. However, T4, a Mesoamerican isolate, was the one showed higher darkness "k" level. Since S3b and T4 were the isolates belonging to different genic pools of *P. griseola* that exhibited a contrasting phenotype compared to the others tested, they were selected for further study.

3.2. Growth and Melanin Content of Selected Isolates of Pseudocercospora griseola in PD Broth

Pseudocercospora griseola f. mesoamericana (T4), which developed into smaller colonies, grew more than *P. griseola f. griseola* (S3b). T4 and S3b had a 2.41 ± 0.63 g and 0.27 ± 0.24 g dry biomass, respectively in PD broth after 21 days of incubation. Concomitantly with this, the amount of pigment synthetized by T4 cultures was higher than that produced by S3b cultures, which was 4.1 ± 0.9 and 1.7 ± 0.6 mg of melanin per g of dry material, respectively.

3.3. UV–Visible and ESR Spectroscopies, Redox and Ion Chelating Activity of Melanins

The UV–visible (200–800 nm) absorption spectra of S3b and T4 melanin presented a characteristic intense absorption peak, at λ_{max} = 230 nm, and two other broad peaks that look like shoulders of very low intensity at *ca.* 300 and *ca.* 400 nm, which were superimposed on the tail of the first peak (Fig. 2). Absorbance measured of melanin from T4 was lower than that of melanin isolated from S3b, even though melanin concentration was the same. The slopes of the $\log_{10}$ plot of absorbance (300–600 nm) *vs.* the wavelength were − 0.0039 and − 0.0049 for S3b and T4 respectively (Inset in Fig. 2).

Table 1
Growth and pigmentation of colonies of several isolates of *P. griseola* on PDA medium for a 21-day-period. Values are means of three replicates. Data in each column corresponding to each genic group and followed by the same letter are not significantly different (Tukey test, $P < 0.05$).

Genic group	Isolate	Diameter (mm)	Pigmentation (k)
Andean	Ecu6	5.81 ± 1.18^{a}	42 ± 1^{a}
	J2a	5.38 ± 0.23^{a}	46 ± 2^{a}
	J1a	6.06 ± 0.32^{a}	46 ± 4^{a}
	S3b	8.41 ± 0.78^{b}	49 ± 1^{a}
	Ecu3	6.16 ± 0.65^{a}	56 ± 3^{b}
Mesoamerican	S6a	4.60 ± 0.36^{ab}	55 ± 5^{a}
	T2	3.84 ± 0.09^{a}	64 ± 1^{b}
	PG1	7.09 ± 1.23^{c}	69 ± 1^{b}
	PG15	6.36 ± 0.60^{bc}	69 ± 4^{b}
	T4	4.40 ± 0.20^{a}	79 ± 1^{c}

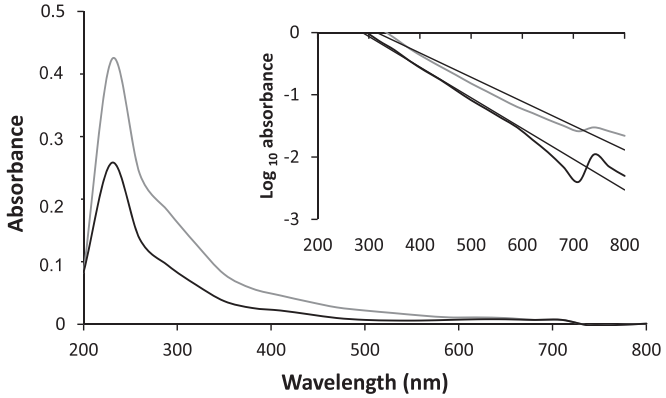

Fig. 2. Ultraviolet–visible spectra of melanin (concentration: 0.1 mg ml^{-1}, in 0.1 M NaOH). Grey line, *P. griseola f. griseola* (isolate S3b). Black line, *P. griseola f. mesoamericana* (isolate T4). The inset shows the linear graph with the negative slopes.

The ESR spectra of pigments from both isolates - in solid phase and alkaline solution - revealed the presence of stable free radicals, with g value equals to 2.006 (Fig. 3).

Melanin isolated from cultures of T4 had a higher antioxidant activity than that of S3b, but both were comparatively lower than that of BHT (Fig. 4).

The competitive ability of melanins from both isolates to immobilize iron (II) was compared with that of ferrozine. While 15 mg of melanin from T4 had a 55% of chelating activity, an equal amount of S3b melanin sequestered only 3% iron (Fig. 5).

3.4. Analysis of Isolated Melanins and Ones within Biomass Using the Folin-Ciocalteu Method, Fluorescence and FTIR Spectroscopies

Biomass and melanin content of phenolic groups was a function of the fungal isolate and the size of the sample (Table 2). It was greater in the biomass of T4 than that of S3b whether the sample consisted in 1 or 10 mg. When phenol equivalents were estimated in 1 mg-samples of melanin, T4 and S3b presented similar levels (517 ± 48 μg for T4 and 582 ± 53 μg for S3b). However, in 10 mg-samples a slightly higher amount of phenols was found in S3b melanin (1870 ± 37 μg) than in T4 one (1513 ± 82 μg). Provided the amount of sample was the same, either 1 or 10 mg, we found more free phenol groups in melanin

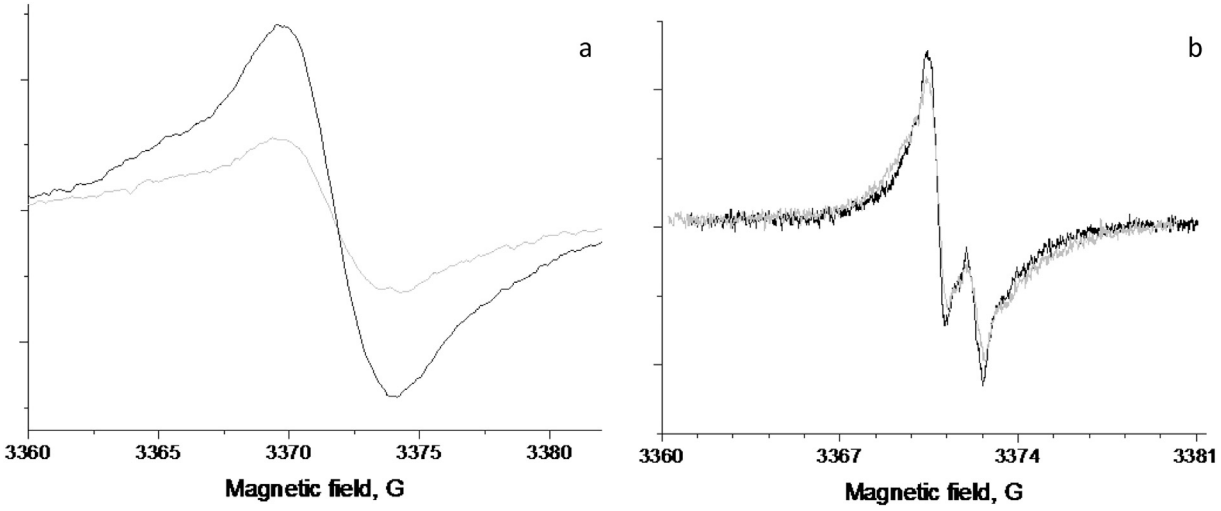

Fig. 3. ESR spectra of dark pigments isolated from *in-vitro* cultures of *P. griseola f. griseola* (isolate S3b, grey line) and *P. griseola f. mesoamericana* (isolate T4, black line), which were performed in dry state (a), and in alkaline solution (b). *Ordinate* is the derivative of the ESR absorption in arbitrary units.

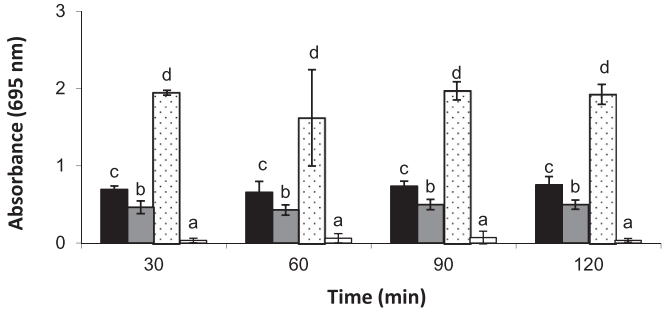

Fig. 4. Antioxidant activity of 0.5 mg ml^{-1} melanin from *P. griseola f. mesoamericana* T4 (black bars) and *P. griseola f. griseola* S3b (grey bars) and BHT (as reference; dotted bars). White bars correspond to the control. Bars with different letter are significantly different (Tukey test, $P < 0.05$).

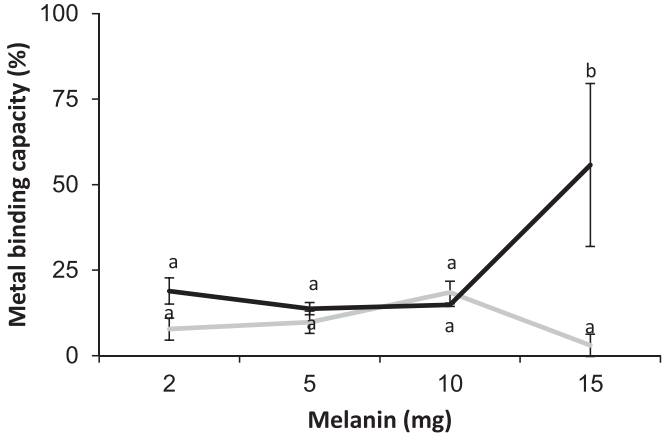

Fig. 5. Metal binding capacity of *P. griseola f. mesoamericana* T4 melanin (black line), and *P. griseola f. griseola* S3b melanin (grey line). Data with the same letter are not significantly different (Tukey test, P < 0.05).

isolated than in the biomass samples of isolate S3b, a pattern that was not reproduced in T4.

The alkaline-soluble products of the oxidative degradation of S3b and T4 biomass as well as their melanin were analyzed by fluorescence spectroscopy (Fig. 6). The alkaline solution of the oxidative reaction mixture containing the T4 biomass had an excitation peak at 362 nm and an emission peak at 433 nm (Fig. 6a). T4 melanin did not fluoresce, unless samples were diluted 10 times. Under such conditions, there was an excitation peak at 469 nm and an emission peak at 529 nm (Fig. 6b). Regarding isolate S3b, the oxidative reaction mixture containing the biomass presented an excitation peak at 431 nm and an emission peak at 494 nm (Fig. 6c), while the S3b melanin presented an excitation peak at 391 nm and an emission peak at 470 nm (Fig. 6d).

Table 2
Total phenolic content of *P. griseola* f. *griseola* (isolate S3b) and *P. griseola* f. *mesoamericana* (isolate T4) expressed in µg per total amount of sample in mg. Data with different letter are significantly different (Tukey test, P < 0.05).

Isolate	Sample		Phenol content
	Type	(mg)	(µg)
T4	Biomass	1	425 ± 43^{b}
		10	1956 ± 47^{e}
	Melanin	1	517 ± 48^{b}
		10	1513 ± 82^{d}
S3b	Biomass	1	136 ± 16^{a}
		10	909 ± 120^{c}
	Melanin	1	582 ± 53^{b}
		10	1870 ± 37^{e}

The FTIR spectra of melanin and dry biomass of S3b and T4 are shown in Fig. 7. Both melanins had a conserved FTIR pattern with stretching vibrations of –OH and –NH groups (3364 cm^{-1}), > CH_2 and $-CH_3$ stretching (2852 and 2925 cm^{-1}) and stretching vibrations by conjugated double bonds (C═C and C═O) in aromatic ring (1644–1656 cm^{-1}) that are typical of conjugated quinoid structures. These spectra also had deformations of C−N and N−H groups on amides II (1536–1553 cm^{-1}), CH groups adjacent to COOH and OH groups and C═O of quinones (1370–1410 cm^{-1}) as well as C−O deformation vibrations of aliphatic alcohols (1050–1100 cm^{-1}). The FTIR spectra of fungal isolates biomass presented some differences when compared with the spectrum of melanin such as C═O stretches in C−O esters and stretches of phenol group of the aromatic ring as well as of the acetyl group of polysaccharides (1746 and 1241–1246 cm^{-1}). Though the ratio between intensity at 1650 cm^{-1} and at 1746 cm^{-1} was similar within melanin samples of both isolates (0.48), the ratio was higher when the biomass was analyzed, being higher for S3b (1.48) than for T4 (0.80).

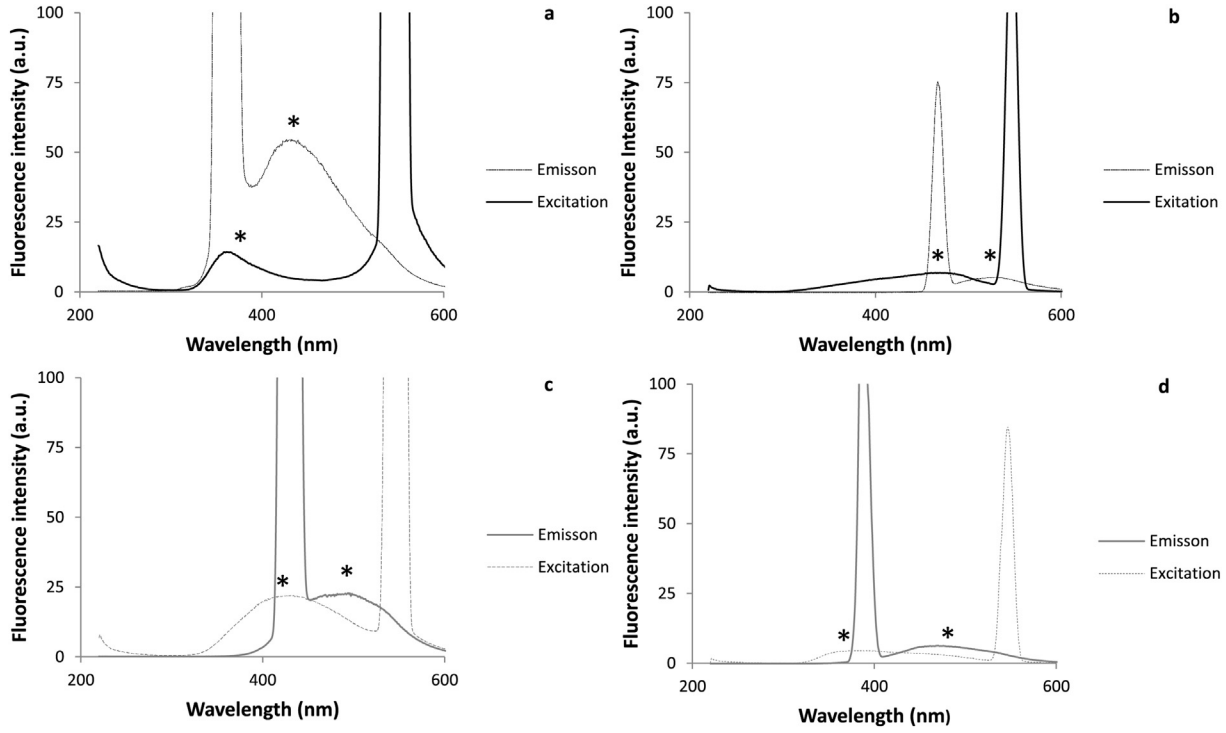

Fig. 6. Fluorescence excitation and emission spectra of oxidized biomass and melanin of *P. griseola f. mesoamericana* isolate T4 (a and b respectively), and of *P. griseola f. griseola* isolate S3b (c and d respectively). The amount of biomass exposed to oxidation was 10 mg, but the amount of melanin was 10 and < 1 mg for T4 and S3b respectively. Fluorescence intensity is shown in arbitrary units (a.u.). The asterisk (*) on the plot indicates the excitation and emission peaks. The excitation wavelength for each emission spectrum was 362 (a), 469 (b), 431 (c) or 391 (d) nm, and the emission wavelength for the excitation spectra was 543 nm.

4. Discussion

Melanin is a secondary metabolite made up of a complex array of heterogeneous polymers. The properties of these dark pigments are a function of their chemical structure and their source of origin. Furthermore, melanin is an insoluble heterogeneous structure and so it is particularly difficult to characterize. Several types of melanins are synthesized by fungi, even within the same fungal species, and they can play different biological roles such as in morphogenesis, stress resistance, virulence and energy transduction [6]. In this work, we isolated melanins from a selected isolate of each genic group of *P. griseola* and compared their interaction with several electromagnetic radiations as well as their content in phenolic groups, antioxidant and metal binding activity.

Bárcena et al. [16] and Saparrat et al. [17] found that *P. griseola* produces DHN-melanin, which was additionally related to fungal growth. They estimated melanin only based on data of absorbance, so more accurate results regarding the chemical characteristics are needed. Therefore, in this work we quantified melanin gravimetrically [29, 30]. As reported before on cultures grown on PDA medium [17], T4 developed smaller colonies than S3b when they were grown on PD broth. However, on this liquid medium T4 grew more and had a higher content of melanin than S3b. Also an analysis with TEM using material from agar cultures (Fig. S1) showed that the thickness of hyphal walls from mycelium of T4 (371.2 ± 91.3 nm) was higher than that found in the isolate S3b (147.5 ± 35.4 nm; Student's t, $P < 0.05$). However, both fungi revealed to have a similar stratification of the hyphal wall, showing an inner electron-transparent layer and an outer electron-dense one. This latter layer has been previously suggested to be the place of deposition of melanin in the hyphal wall of isolate T4 [16]. The fact that the T4 biomass has 2.45 times more melanin per unit of biomass than S3b explains the darker pigmentation of T4 cultures. The UV–visible absorption spectra of both melanins (Fig. 2) presented a characteristic intense absorption band in the UV region, then the absorbance decreased progressively when the wavelength increased beyond 230 nm and spread up to the visible region. Similar absorption spectra have been observed using dark pigments obtained from *Aspergillus bridgeri*, *A. flavus*, *A. niger*, *A. tamarii, A. terreus*, *A. tubingensis*, *Lachnum singerianum*, *Mycosphaerella fijiensis* and *Phyllosticta capitalensis* [18, 31–34]. This is a highly unusual feature; most biological pigments exhibit distinct absorption bands. The broadband spectrum might be due to the presence of many complex conjugated structures within melanin molecules. Melanin consists of many chemically distinct species, so most probably the broadband spectrum reflects superposition of the spectral bands of these species. This is the so called “chemical disorder model” [35]. These spectral properties provide additional evidence of the recognition of the dark pigments as melanin, which in general present an unusually broadband absorption spectrum that increases monotonically as the wavelength decreases. This is consistent with the dark color that indicates that all visible wavelengths are absorbed. The melanin extracted from S3b had a higher absorption than that of T4. Aromatic rings of phenolic compounds absorb radiation in the UV-region, though substitutions in aromatic rings influence the conjugated system, altering the UV absorption spectrum [36]. Also, Kumar et al. [33] suggested that absorption in the UV region of melanin is associated with the presence of phenolic groups that in aqueous alkaline medium (0.1 M NaOH) will be partially dissociated. The different absorbance of samples containing the same amount of pigments might have alternative explanations. First, Wolbarsht et al. [37] noted that Rayleigh scattering might be the main contributor to the broadband spectrum and optical density of melanin at short wavelengths, but it might not explain the differences found between melanins isolated from S3b and T4, since both melanins had the same particle size in solution. Riesz [38] stated that when pigments are extracted from materials frozen with liquid nitrogen and spectroscopic solutions are appropriately prepared, scattering of light is negligible, therefore absorption reflects the pigments ability to absorb UV–visible radiation. Besides, absorption might be due to a structural portion within each pigment

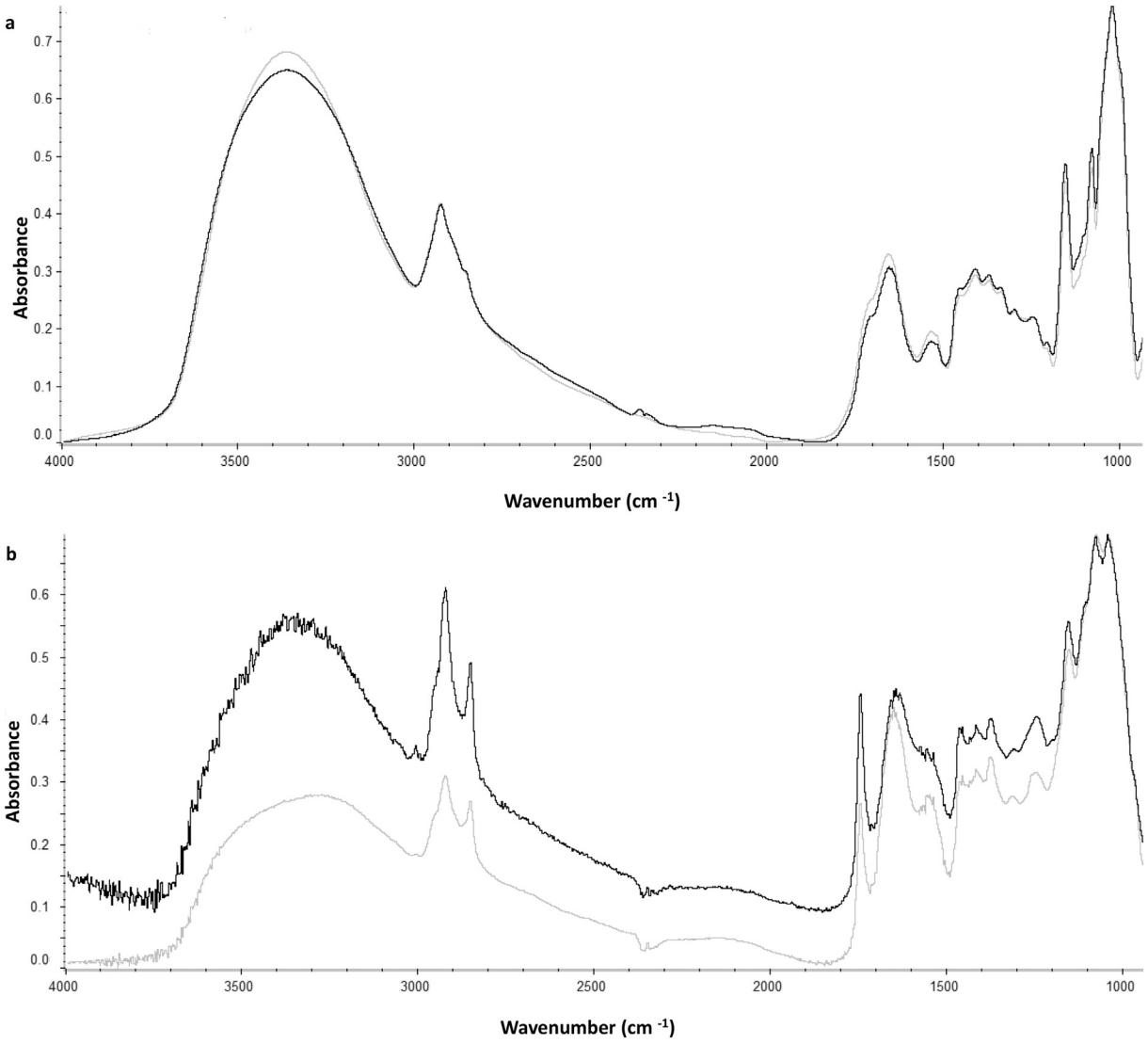

Fig. 7. FTIR spectra of melanin (a) and biomass (b) of *P. griseola f. mesoamericana* (isolate T4; grey line) and *P. griseola f. griseola* (isolate S3b; black line).

that might be repeated many times since it is a polymer. If the number of conjugated structures increase, absorption would shift towards longer wavelengths. S3b and T4 melanin gave similar spectra that differ only in the intensity of the absorption, so probably these structures are not conjugated to each other or the degree of conjugation is the same for both melanins. Also melanin from S3b must have a greater number of structural portions than T4 melanin, since absorbances at the same wavelength are added together. In other words, it appears that melanin from S3b is formed by a larger number of structural units than T4 melanin. Interestingly, each structural portion of melanin contains an electronic conjugated π-system, but the different structural units are not conjugated between them, probably due to a lack of co-planarity of the electronic conjugated π-system. Under these conditions the second monomer should rotate out of the plane to reduce steric hinderance. Such an explanation suggests a higher molecular weight for S3b-melanin than for that of T4. However, we have no experimental data confirming this and there are no reports in the literature. Other explanations of the different absorbance between melanins might be that they differ in particular chemical groups such as phenolics, not only in their type but also in their amount, which might contribute to a particular absorption intensity within certain wavelengths. Regarding this, 10 mg melanin samples from S3b had more phenolic groups than a similar amount of T4 melanin. However, when the melanin sample was 1 mg there was not difference between melanins in the amount of available phenol groups. This might be related with a technical limitation to detect phenols in small samples. Additionally, the technique used (Folin-Ciocalteu one) might lack specificity and therefore also might indicate the presence of other non-phenolic compounds, *e.g.*, melanoidins. Furthermore, secondary reactions might lead either to over- or underestimate such compounds as reported by Blainski et al. [39] and Opitz et al. [40]. But the FTIR analysis revealed the presence of other functional groups within melanin of *P. griseola* such as conjugated quinoid structures, that as reported by Bell and Wheeler [1], Llorente et al. [22], Pal et al. [34] and Raman et al. [41] are typical characteristics of melanin. The presence of some groups with nitrogen in their structure suggest that cell wall proteins might be complexed with DHN-melanin [18]. Therefore, additional elemental analysis of melanins in *P. griseola* are necessary to confirm these results. However, this demands to have a high amount of melanin to enable an accurate estimation and probably because of this, information yet is unknown.

We also analyzed the redox reaction characteristics of both melanins of *P. griseola*. As reported by Kumar et al. [33], Pal et al. [34], Llorente et al. [22], Rosas et al. [42], Nosanchuk and Casadevall [4], Gomez et al. [43] and Morris-Jones et al. [44] for other fungal melanins, a peak in the ESR spectrum was found, which is typical of melanin and is therefore considered diagnostic [45]. The presence of this peak

confirmed the radical nature of melanins isolated from S3b and T4. Specifically, ESR signals found at a g value equals to 2.006 are compatible with the presence of o-semiquinone free radicals in both melanins [46]. This g value is typical of a structure with carbon-centred organic free radicals conjugated with oxygen containing functional groups [47], which is in accordance with the quinoid structures of melanins of *P. griseola* inferred by FTIR analysis.

Two other features of *P. griseola* melanins were analyzed such as the antioxidant activity and the ability to chelate the ferrous ion. The reducing capacity of a compound is a reliable index of its potential antioxidant activity [48]. Although *P. griseola* melanin isolated either from S3b or T4 had the ability to inactivate radical species as well as to chelate iron (II), such abilities were higher for T4 melanin than for S3b one. These antioxidant and chelating capacities have been associated to a protective role against oxidative stress [49]. Goncalves and Pombeiro-Sponchiado [50] reported that in *Aspergillus nidulans* melanins protected the fungus against oxidizing agents. Also in other fungi such as *Aspergillus bridgeri* [33], *Alternaria alternata, Aspergillus carbonarius, Paecilomyces variotii, lnonotus obliquus* and *Phellinus robustus* [51], melanins proved to have antioxidant activity. Therefore, these results raise a question regarding the role melanin plays against oxidative stress in *P. griseola*.

Melanins are poor fluorophores [16, 52–54], therefore in order to characterize them further, we analyzed fluorescence of the byproducts of the oxidatively degraded melanins and their pigmented biomass, from where they were isolated, by hydrogen peroxide. Although the excitation and emission maxima of the samples analyzed were different probably due to differences in the composition, incidentally their spectra followed the fluorescence pattern described for several oxidized melanins (Fig. 6). This is to say that the excitation and emission maxima revealed by oxidation of melanin from isolate T4 by hydrogen peroxide were similar to those found by Bárcena et al. [16] on other melanin preparations from the same fungus and by Kayatz et al. [26] when using synthetic melanin and isolated bovine melanosomes. Therefore, this methodology might have applicability to detect and/or quantify melanin associated to fungal biomass such as reported by Fernandes et al. [54] on zebrafish embryos and human hair.

Several reports describe the complexity and polydispersity of melanins [6], and those isolated from representatives of *P. griseola* are not an exception. The slope of the $\log_{10}$ absorbance *versus* the wavelength of S3b- and T4-melanin was different from those reported before [17]. Ellis and Griffiths [55] reported that such slope might change probably due to an oxidative alteration of melanin in an alkaline solution. This confirms that melanins migh be heterogeneous in their behaviour in UV–visible range, which depends upon the type of culture and the conditions of incubation as well as the extraction procedure, since both studies used the same isolates. We also found differences within the infrared spectra of both melanins and the spectra reported by Saparrat et al. [17].

Future investigations should be aimed at studying with additional tools the fine chemistry of DHN-melanins and their main molecular substructures. This will allow us to establish if the fungi that belong to each genic pool synthetize different melanins. Furthermore, our results give preliminary information about the usefulness of rapid methodologies using pigmented fungal biomass to detect melanin features avoiding an extraction procedure, which might simplify the diagnostic assessments of fungi and their melanization.

5. Conclusion

The Andean and Mesoamerican groups of *P. griseola* differ in the growth and pigmentation of their colonies. Melanins are deposited within mycelial walls of *P. griseola* f. *griseola* isolate S3b and *P. griseola* f. *mesoamericana* isolate T4 while being synthetized. The melanin of *P. griseola* has antioxidant properties and chelates iron. This result raises a question regarding the role melanin plays against oxidative stress in this fungus. Chemical groups in melanin such as phenolics as well as their reactivity are a differential character in *P. griseola* S3b and *P. griseola* T4 such as also evidenced at the level of colony pigmentation. In fungal biomass, melanin was partially characterized only by some of the technologies used. Even though we used different approaches to characterize melanin fraction there is still room for improvement. However, this information can contribute to develop new fungicides so as to control dematiaceous fungi, including pathogenic ones, by blocking the synthesis of DHN-melanin.

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References

[1] A.A. Bell, M.H. Wheeler, Biosynthesis and functions of fungal melanin, Annu. Rev. Phytopathol. 24 (1986) 411–451.
[2] M.L. Elliott, Effect of melanin biosynthesis inhibiting compounds on *Gaeumannomyces* species, Mycologia 87 (1995) 370–374.
[3] J.M. Henson, M.J. Butler, A.W. Day, The dark side of mycelium: melanins of phytopathogenic fungi, Annu. Rev. Phytopathol. 37 (1999) 447–471.
[4] J.D. Nosanchuk, A. Casadevall, The contribution of melanin to microbial pathogenesis, Cell. Microbiol. 5 (2003) 203–223.
[5] R.J. Cordero, A. Casadevall, Functions of fungal melanin beyond virulence, Fungal Biol. Rev. 31 (2017) 99–112.
[6] A.V. Toledo, M.E.E. Franco, S.M.Y. Lopez, M.I. Troncozo, M.C.N. Saparrat, P.A. Balatti, Melanins in fungi: types, localization and putative biological roles, Physiol. Mol. Plant Pathol. 99 (2017) 2–6.
[7] R.C.R. Goncalves, H.C.F. Lisboa, S.R. Pombeiro-Sponchiaso, Characterization of melanin pigment produced by *Aspergillus nidulans*, World J. Microbiol. Biotechnol. 28 (2012) 1467–1474.
[8] J.D. Nosanchuk, R.E. Stark, A. Casadevall, Fungal melanin: what do we know about structure? Front. Microbiol. 6 (2015) 1463, https://doi.org/10.3389/fmicb.2015.01463.
[9] G.A. Duff, J.E. Roberts, N. Foster, Analysis of the structure of synthetic and natural melanins by solid-phase NMR, Biochemistry 27 (1988) 7112–7116.
[10] H.C. Eisenman, A. Casadevall, Synthesis and assembly of fungal melanin, Appl. Microbiol. Biotechnol. 93 (2012) 931–940.
[11] H.F. Schwartz, G.E. Gálvez, Problemas de Producción Del Frijol: Enfermedades, Insectos, Limitaciones Edáficas y Climáticas de *Phaseolus vulgaris*, Centro Internacional de Agricultura Tropical (CIAT), Cali, 1980.
[12] G. Ddamulira, C. Mukankusi, R. Edema, P. Sseruwagi, P. Gepts, Distribution and variability of *Pseudocercospora griseola* in Uganda, J. Agric. Sci. 6 (2014) 16–29.
[13] O.N. Vizgarra, P.A. Balatti, S.A. Stenglein, C.M. Espeche, L.D. Ploper, TUC 550: nueva variedad de poroto negro (*Phaseolus vulgaris* L.) con tolerancia a la mancha angular (*Phaeoisariopsis griseola*), Rev. Agríc. Tucumán 87 (2010) 55–61.
[14] P.W. Crous, M.M. Liebenberg, U. Braun, J.Z. Groenewald, Re-evaluating the taxonomic status of *Phaeoisariopsis griseola*, the causal agent of angular leaf spot of bean, Stud. Mycol. 55 (2006) 163–173.
[15] A. Bárcena, Estudio de las Melaninas de *Pseudocercospora griseola*, Agente Causante de la Mancha Angular del Poroto y rol Biológico, Doctoral Thesis, Universidad Nacional de La Plata, 2016.
[16] A. Bárcena, G. Petroselli, S.M. Velasquez, J.M. Estévez, R. Erra-Balsells, P.A. Balatti, M.C.N. Saparrat, Response of the fungus *Pseudocercospora griseola f. mesoamericana* to tricyclazole, Mycol. Prog. 14 (2015) 1–11.
[17] M.C.N. Saparrat, G.E. Fermoselle, S.A. Stenglein, M.B. Aulicino, P.A. Balatti, *Pseudocercospora griseola* causing angular leaf spot on *Phaseolus vulgaris* produces 1,8-dihydroxynaphthalene-melanin, Mycopathologia 168 (2009) 41–47.
[18] M.J. Beltrán-García, F.M. Prado, M.S. Oliveira, D. Ortiz-Mendoza, A.C. Scalfo, A. Pessoa, M.H.G. Medeiros, J.F. White, P. Di Mascio, Singlet molecular oxygen generation by light-activated DHN-melanin of the fungal pathogen *Mycosphaerella fijiensis* in black Sigatoka disease of bananas, PLoS ONE 9 (2014) e91616, , https://doi.org/10.1371/journal.pone.0091616.
[19] S.A. Stenglein, P.A. Balatti, Genetic diversity of *Phaeoisariopsis griseola* in Argentina as revealed by pathogenic and molecular markers, Physiol. Mol. Plant Pathol. 68 (2006) 158–167.
[20] G.W. Griffith, G.L. Easton, A. Detheridge, K. Roderick, A. Edwards, H.J. Worgan,

J. Nicholson, W.T. Perkins, Copper deficiency in potato dextrose agar causes reduced pigmentation in cultures of various fungi, FEMS Microbiol. Lett. 276 (2007) 165–171.
[21] P. Selvakumar, S. Rajasekar, K. Periasamy, N. Raaman, Isolation and characterization of melanin pigment from *Pleurotus cystidiosus* (telomorph of *Antromycopsis macrocarpa*), World J. Microbiol. Biotechnol. 24 (2008) 2125–2131, https://doi.org/10.1007/s11274-008-9718-2.
[22] C. Llorente, A. Bárcena, J. Vera Bahima, M.C.N. Saparrat, A.M. Arambarri, M.F. Rozas, M.V. Mirifico, P.A. Ballati, *Cladosporium cladosporioides* LPSC 1088 produces the 1,8-dihydroxynaphthalene-melanin-like compound and carries a putative pks gene, Mycopathologia 174 (2012) 397–408.
[23] S. Huang, Y. Pan, D. Gan, X. Ouyang, S. Tang, S.I.N. Ekunwe, H. Wang, Antioxidant activities and UV-protective properties of melanin from the berry of *Cinnamomum burmannii* and *Osmanthus fragrans*, Med. Chem. Res. 20 (2010) 475–481.
[24] H. Farhan, H. Rammal, A. Hijazi, H. Hamad, A. Daher, M. Reda, B. Badran, *In-vitro* antioxidant activity of ethanolic and aqueous extracts from crude *Malva parviflora* L. grown in Lebanon, Asian J. Pharma. Clin. Res. 5 (2012) 234–238.
[25] R.D. Arrua, J.F. Basbus, M.C. Strumia, C.I. Alvarez, M.A. Nazareno, Synthesis of macroporous polymers with radical scavenging properties by immobilization of polyphenolic compounds, React. Funct. Polym. 72 (2012) 807–813.
[26] P. Kayatz, G. Thumann, T.T. Luther, J.F. Jordan, K.U. Bartz-Schmidt, P.J. Esser, U. Schraermeyer, Oxidation causes melanin fluorescence, Invest. Ophthalmol. Vis. Sci. 42 (2001) 241–246.
[27] K. Lammers, G. Arbuckle-Keil, J. Dighton, FTIR study of the changes in carbohydrate chemistry of three New Jersey pine barrens leaf litters during simulated control burning, Soil Biol. Biochem. 41 (2009) 340–347.
[28] M.C.N. Saparrat, J.M. Estevez, M.I. Troncozo, A.M. Arambarri, P.A. Balatti, *In-vitro* depolymerization of *Scutia buxifolia* leaf-litter by a dominant Ascomycota *Ciliochorella* sp, Int. Biodeterior. Biodegrad. 64 (2010) 262–266.
[29] D.N. Hu, Methodology for evaluation of melanin content and production of pigment cells *in-vitro*, Photochem. Photobiol. 84 (2008) 645–649.
[30] A. Mejía-Caballero, R. Anda, G. Hernández-Chávez, S. Rogg, A. Martinez, F. Bolívar, Biosynthesis of catechol melanin from glycerol employing metabolically engineered *Escherichia coli*, Microb. Cell Factories 15 (2016) 1–8.
[31] M. Ye, Y. Wang, M. Qian, X. Chen, X. Hu, Preparation and properties of the melanin from *Lachnum singerianum*, Int. J. Basic Appl. Sci. 11 (2011) 51–58 ISSN: 2077-1223.
[32] T.S. Suryanarayanan, J.P. Ravishankar, G. Venkatesan, T.S. Murali, Characterization of the melanin pigment of a cosmopolitan fungal endophyte, Mycol. Res. 108 (2004) 974–978.
[33] C.G. Kumar, P. Mongolla, S. Pombala, A. Kamle, J. Joseph, Physicochemical characterization and antioxidant activity of melanin from a novel strain of *Aspergillus bridgeri* ICTF-201, Lett. Appl. Microbiol. 53 (2011) 350–358.
[34] A.K. Pal, D.U. Gajjar, A.R. Vasavada, DOPA and DHN pathway orchestrate melanin synthesis in *Aspergillus* species, Med. Mycol. 52 (2014) 10–18, https://doi.org/10.3109/13693786.2013.826879.
[35] P. Meredith, B.J. Powell, J. Riesz, S.P. Nighswander-Rempel, M.R. Pederson, E.G. Moore, Towards structure property function relationships for eumelanin, Soft Matter 2 (2006) 37–44.
[36] A. Connors, A. García Asuero, M. Guzman Chozas, Curso de Análisis Farmacéutico (ensayo del medicamento), Reverté, España, 1980 (ISBN: 84-291-7113-4).
[37] M. Wolbarsht, A. Walsh, G. George, Melanin, a unique biological absorber, Appl. Opt. 20 (1981) 2184–2186.
[38] J. Riesz, The spectroscopic properties of melanin, Doctoral Dissertation, University of Queensland, 2007.
[39] A. Blainski, G.C. Lopes, J.C. Palazzo De Mello, Application and analysis of the Folin Ciocalteu method for the determination of the total phenolic content from *Limonium brasiliense* L, Molecules 18 (2013) 6852–6865.
[40] S.E. Opitz, S. Smrke, B.A. Goodman, M. Keller, S. Schenker, C. Yeretzian, Antioxidant generation during coffee roasting: a comparison and interpretation from three complementary assays, Foods 3 (2014) 586–604.
[41] N.M. Raman, S. Ramasamy, Genetic validation and spectroscopic detailing of DHN-melanin extracted from an environmental fungus, Biochem. Biophys. Rep. 12 (2017) 98–107.
[42] A.L. Rosas, J.D. Nosanchuk, M. Feldmesser, G.M. Cox, H.C. McDade, A. Casadevall, Synthesis of polymerized melanin by *Cryptococcus neoformans* in infected rodents, Infect. Immun. 68 (2000) 2845–2853.
[43] B.L. Gomez, J.D. Nosanchuk, S. Diez, S. Youngchim, P. Aisen, L. Cano, A. Restrepo, A. Casadevall, A.J. Hamilton, Detection of melanin-like pigments in the dimorphic fungal pathogen *Paracoccidioides brasiliensis in vitro* and during infection, Infect. Immun. 69 (2001) 5760–5767.
[44] R. Morris-Jones, S. Youngchim, B.L. Gomez, P. Aisen, R.J. Hay, J.D. Nosanchuk, A. Casadevall, A.J. Hamilton, Synthesis of melanin-like pigments by *Sporothrix schenckii in-vitro* and during mammalian infection, Infect. Immun. 71 (2003) 4023–4026.
[45] W.S. Enochs, M.J. Nilges, H.M. Swartz, A standardized test for the identification and characterization of melanins using electron paramagnetic resonance (EPR) spectroscopy, Pigment Cell Res. 6 (1993) 91–99.
[46] M. Zdybel, B. Pilawa, E. Buszman, T. Witoszyńska, EPR studies of *Cladosporium cladosporioides* complexes with amphotericin B, Nukleonika 58 (2003) 401–405.
[47] N.M. Atherton, J.S.S. Willder, EPR and ENDOR of free radicals formed during the aerobic oxidation of chlorogenic acid and of caffeic acid in strongly alkaline solution, Res. Chem. Intermed. 19 (1993) 787–795.
[48] S. Meir, J. Kanner, B. Akiri, S. Philosoph-Hadas, Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves, J. Agric. Food Chem. 43 (1995) 1813–1819.
[49] L. Hong, J.D. Simon, Current understanding of the binding sites, capacity, affinity, and biological significance of metals in melanin, J. Phys. Chem. A 111 (2007) 7938–7947.
[50] R.D. Goncalves, S.R. Pombeiro-Sponchiado, Antioxidant activity of the melanin pigment extracted from *Aspergillus nidulans*, Biol. Pharm. Bull. 28 (2005) 1129–1131.
[51] V.V. Shcherba, V.G. Babitskaya, V.P. Kurchenko, N.V. Ikonnikova, T.A. Kukulyanskaya, Antioxidant properties of fungal melanin pigments, Appl. Biochem. Microbiol. 36 (2000) 491–495.
[52] S.D. Kozikowski, L.J. Wolfram, R.R. Alfano, Fluorescence spectroscopy of eumelanins, IEEE J. Quantum Electron. 20 (1984) 1379–1382.
[53] J.M. Gallas, M. Eisner, Fluorescence of melanin-dependence upon excitation wavelength and concentration, Photochem. Photobiol. 45 (1987) 595–600.
[54] C. Fernandes, R. Prados-Rosales, B.M. Silva, A. Nakouzi-Naranjo, M. Zuzarte, S. Chatterjee, R.E. Starkd, A. Casadevalle, T. Gonçalves, Activation of melanin synthesis in *Alternaria infectoria* by antifungal drugs, Antimicrob. Agents Chemother. 60 (2016) 1646–1655.
[55] D. Ellis, D.A. Griffiths, The location and analysis of melanins in the cell walls of some soil fungi, Can. J. Microbiol. 20 (1974) 1379–1386.