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ORIGINAL PAPER

Characterization of melanin pigment produced by *Aspergillus nidulans*

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Abstract Although most of the Ascomycetes present DHN-melanin, some reports suggest that *A. nidulans* does not produce this type of melanin. In this study, we analyzed the pigment extracted from highly melanized strains (MEL1 and MEL2) of *Aspergillus nidulans* to determine the type of melanin present in this fungus. Our results showed that the pigment produced by MEL1 and MEL2 mutants possesses physical and chemical properties and UV- and IR-spectra very similar to synthetic DOPA-melanin. The characterization of this pigment in terms of its degradation products indicated the presence of indolic units, which were also found in synthetic DOPA-melanin. The analyses of the elemental composition showed that the pigment extracted from these mutants has a high percentage of nitrogen and, therefore, it cannot be DHN-melanin, which presents only trace of nitrogen. This observation was confirmed in the test with tricyclazole because this inhibitor of DHN-melanin biosynthesis did not suppress pigment production in the MEL1 and MEL2 strains. On the other hand, in a medium containing tropolone, an inhibitor of DOPA-melanin biosynthesis, the dark pigmentation of the colonies was not observed indicating that this compound inhibited melanin production in these strains. Taken together, the results obtained in this study indicate that melanin produced by these mutants is DOPA type, representing the first report on characterization of this type of melanin in *A. nidulans.*

Keywords *Aspergillus nidulans* · DOPA-melanin · Infrared and ultraviolet spectra · Tricyclazole · Tropolone

Abreviations

DOPA	3,4-dihydroxyphenylalanine
DHN	1,8-dihydroxynaphthalene
GDHB	Glutaminyl-3,4-dihydroxybenzene
UV	Ultraviolet
IR	Infrared
ESR	Electron spin resonance

Introduction

A number of fungi, including *Aspergillus nidulans*, produce dark-brown pigments known as melanins, which are present in cell walls associated with chitin and/or in the medium as soluble extracellular polymers (Bull 1970; Ellis and Griffiths 1974). This pigment appears to be essential for protection of the organisms against environmental stress. In vitro studies have shown that melanized fungi are more resistant than those no-melanized to UV light-induced and oxidant-mediated damages, temperature extremes, hydrolytic enzymes, heavy metal toxicity and antimicrobial drugs (Zhdanova et al. 1990; Bell and Wheeler 1986; Fogarty and Tobim 1996; Butler and Day 1998; Jacobson 2000; Garcia-Rivera and Casadevall

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2001; Gómez and Nosanchuk 2003; Dadachova et al. 2007; Singaravelan et al. 2008).

The effect of melanin enhancing the survival of fungi under adverse conditions can be mainly due to its powerful free radical scavenger properties, which can neutralize oxidants generated by the fungus in response to environmental stress (Wang and Casadevall 1994; Jacobson et al. 1995; Schnitzler et al. 1999). In early studies, we show that the pigment extracted from *A. nidulans* presents antioxidant activity for biological oxidants, as HOCl, and may be a promising material in cosmetic formulations to protect the skin against possible oxidative damage (Gonçalves and Pombeiro-Sponchiado 2005).

In contrast to great number of studies about to biological roles of melanin, there is little knowledge of their chemical structure. Melanins are difficult to study because they are amorphous and insoluble substances and the purification of this pigment requires harsh chemical methods, which can to modify its structure. Various techniques, including electron paramagnetic resonance (Enochs et al. 1993), X-ray diffraction (Crippa et al. 1989), infrared, ultraviolet and visible spectroscopy (Wilczok et al. 1984; Harki et al. 1997) and nuclear magnetic resonance (Duff et al. 1988; Youngchim et al. 2004) have been used to elucidate the melanin structure from different organisms. These studies have shown that fungi can synthesize different types of melanins by oxidative polimerization of phenolic or indolic compounds.

Melanin in cell walls of Basidiomycotina is derived from phenolics precursors, as glutaminyl-3,4-dihydroxybenzene (GDHB) or catechol. However, in *Cryptococcus neoformans*, a pathogenic basidiomycetous yeast, the type of produced pigment depends on the chemical structure of the exogenous substrate. This fungus is know to make DOPA-melanin when *o*-diphenolic compounds, such as 3,4-dihydroxyphenylalanine, are present in the culture medium (Chaskes and Tyndall 1975; Casadevall et al. 2000; Karkowska-Kuleta et al. 2009).

In the Ascomycota fungi, melanin pigment is generally synthesized from the pentaketide pathway in which 1,8-dihydroxynaphthalene (DHN) is the immediate precursor of the polymer. However, some species of this class, including *Cladosporium* (*Amorphotheca) resinae*, *Epicoccum nigrum*, *Hendersonula toruloidea*, *Eurotium echinulatum*, *Humicola grisea* and *Hypoxylon archeri*, appear don't produce this type of pigment. (Ellis and Griffiths 1974; Bell and Wheeler 1986; Saiz-Jimenez, 1995; Henson et al. 1999; Nosanchuk and Casadevall 2003; Wu et al. 2008).

In the genus *Aspergillus*, some members as *A. fumigatus* synthesizes DNH-melanin (Tsai et al. 2001; Langfelder et al. 2003) and also are able to produce a second type of melanin, pyomelanin, by the tyrosine degradation pathway (Schmaler-Ripcke et al. 2009) while in other species, as *A. nidulans*, does not been identified this type of melanin (Bell and Wheeler 1986). Bull (1970) observed that *A. nidulans* mutants defective in the production of melanin in hyphal cell walls accumulated pink and purple pigments, identified as dopachrome (indole 5,6-quinone 2-carboxilic acid) and melanochrome (indole 5,6-quinone, or more, probably, a small oligomer of the latter) which are intermediates in the DOPA melanin pathway. Previous studies demonstrated the indolic nature of the melanin produced by *A. nidulans*, although DOPA was not detected as an intermediate (Rowley and Pirt 1972; Bull 1970; Bell and Wheeler 1986). In *A. nidulans* strains was identified at least four phenoloxidase isoenzymes that mediates the formation of the melanin, and one of which showed to be a tyrosinase based on its substrate specificity (DOPA substrate) and susceptibility to inhibitors (Bull and Carter 1973; Martinelli and Bainbridge 1974; Martinelli, 1994). Watanabe et al. (1999) reported that *A. nidulans* synthesizes a heptaketide naphthopyrone, as a precursor for its green conidial pigment, but it does not use the DHN pentaketide pathway. Although these studies suggest that *A. nidulans* may produce DOPA-melanin, so far no direct evidence was presented to support this hypothesis.

In this context, the aim of the present study was to analyze the pigment produced by MEL1 and MEL2 mutants of *A. nidulans* using various physico-chemical and spectrophotometric techniques. In addition, the effect of specific inhibitors of DHN- and DOPA-melanin pathways (respectively tricyclazole and tropolone) was also evaluated to determine the type of pigment produced in this fungus.

Materials and methods

Strains and growth conditions

In the present study were utilized mutants of *Aspergillus nidulans*, denominated MEL1 and MEL2, which present overproduction of brown pigment. These mutants were isolated by Pombeiro (1991) after NQO (4-nitroquinoline-1-oxide) mutagenesis in a strain of *A. nidulans*. These mutants were grown in 250 ml Erlenmeyer flasks containing 100 ml of liquid minimal medium (Cove 1966) supplemented with 55 mM glucose (as the carbon source), 70 mM sodium nitrate (as nitrogen source) and nutritional requirements necessary for the growth of these strains such as inositol (20 μg ml^{-1}) for MEL1 and biotin (0,2 μg ml^{-1}) and *ρ*-aminobenzoic acid (5 mM) for MEL2. This medium was inoculated with 10^6 conidia/ml and incubated at 37°C in a rotary shaker at 220 rpm. After growth for 72 h, the mycelium was collected by vacuum filtration and the pigment present in the mycelium and culture medium was then extracted.

Pigment extraction

The pigment present in the culture medium was extracted using the method described by Paim et al. (1990). The culture were acidified to pH 1.5 with 6 M HCl and allowed to stand overnight to precipitate the pigment, which was recovered by centrifugation at 4,500*g* for 15 min and lyophilized. The amount of produced pigment was approximately 175 μg per ml medium.

Extraction and purification of the brown pigment from the mycelium were performed according to the procedure described by Sava et al. (2001) with minor modifications. Firstly, the mycelial mass, treated with 2 M NaOH (pH 10.5) for 36 h, was centrifuged at 4,000*g* for 15 min and the supernatant was acidified with 2 M HCl to pH 2.5. After incubation for 2 h at room temperature, the mixture was again centrifuged at 4,000*g* for 15 min and the precipitate obtained was purified by acid hydrolysis using 6 M HCl at 100°C for 2 h to remove carbohydrates and proteins and it was also treated with an organic solvent (chloroform, ethyl acetate and ethanol) to wash away lipids. The residue obtained was then dried at room temperature, re-dissolved in 2 M NaOH and centrifuged at 4,000*g* for 15 min. The supernatant was precipitated by adding 1 M HCl, washed with distilled water and lyophilized. From this procedure, about 94.5 μg of pigment per mg mycelial mass was yielded.

Characterization of the melanin pigment

The pigment extracted from MEL1 and MEL2 strains was characterized by certain chemical properties used to define a fungal pigment as melanin, described by Thomas (1955), such as insolubility in water and organic solvents, resistance to degradation by concentrated acids, bleaching by oxidizing agents, solubilization by hot alkali solutions and positive reaction for polyphenols.

The identification of melanin degradation products was done as described by Ellis and Griffiths (1974) by adding 0.5 g of the dried pigment to 10 g of pure fused KOH. This mixture was brought to the boil and cooled, and the resulting dark solid mixture was dissolved in a mixture of distilled water and a comparable volume of saturated sodium chloride solution with diethyl ether. The indole nature of the pigments was determined using Erlich's indole reagent (4 g of *p*-dimethylaminobenzaldehyde dissolved in 380 ml of ethanol and 80 ml of concentrated HCl), where the addition of a few drops of reagent produces a coral pink or magenta color.

The elemental composition of the pigments extracted from the MEL1 and MEL2 mutants were determined by a CHNS-O CE elemental analyser model EA 1110.

The spectrophotometric analysis of the pigment obtained from MEL1 and MEL2 mutants was carried out from ultraviolet (UV)-visible and infrared (IR) spectra. To obtain the UV absorption spectrum, a Varian Carry 50 CONC was used at a wavelength of 200–600 nm and the samples were prepared in 0.5 M sodium hydroxide at a final concentration of 0.5 mg/ml. The infrared spectra of the pigment were recorded on an IR spectrometer Nicolet Impact 400, using KBr pellets obtained by pressing under vacuum uniformly prepared mixtures of 2 mg of pigment sample and 150 mg of spectrometry grade KBr.

The electron spin resonance (ESR) spectrum of the pigment obtained from MEL1 was recorded in a solid state at room temperature in quartz tubes on a BRUKER mod. Ellexys—E580 ESR spectrometer operating at 100 kHz modulation frequency and the g-value of the sample was measured at 25°C.

Synthetic DOPA melanin (Sigma ref. M8631) was used to calibrate the results of the study.

Test with tricyclazole and tropolone

The effect of the tricyclazole and tropolone, respectively biosynthesis inhibitors of DHN- and DOPA-melanin, on pigmentation of colonies was evaluated after fungal growth for 72 h at 37°C in solid minimum medium containing 0.1, 1 and 10 μg ml^{-1} of these inhibitors (final concentration).

Results and discussion

The dark brown pigments produced by MEL1 and MEL2 strains of the *A. nidulans* were analyzed in relation to certain chemical properties, which are used to define a fungal pigment as being melanin. The results summarized in Table 1 show that the intra and extracellular pigments extracted from the mutants were soluble in Na_2CO_3 and hot NaOH, but were insoluble in water, organic solvents (acetone, chloroform and ethanol) and cold NaOH; they precipitated in concentrated HCl, decolorized in the presence of H_2O_2 and gave positive reactions for polyphenols by producing flocculent brown precipitates with $FeCl_3$. These characteristics were also exhibited by the synthetic DOPA-melanin (Table 1) and are common to various melanins described in literature (Gadd 1982; Bell and Wheeler 1986; Harki et al. 1997).

In addition to the chemical tests, the pigments obtained from the MEL1 and MEL2 mutants were also submitted to UV-visible spectrophotometric analysis because, among biological pigments, only melanins absorb all visible wavelenghts, this characteristic being responsible for the dark color of the pigment (Bell and Wheeler 1986). The

Table 1 Chemical analysis of pigment extracted from MEL1 and MEL 2 mutants of *A. nidulans* and the synthetic DOPA-melanin

Tests	MEL1 pigment[a]		MEL2 pigment[a]		Synthetic DOPA-melanin[a]
	IM[b]	EM[c]	IM[b]	EM[c]	
Solubility in H_2O at 25°C	−	−	−	−	−
Solubility in organic solvents (chloroform, ethanol and acetone)	−	−	−	−	−
Solubility in Na_2CO_3	+	+	+	+	+
Solubility in NaOH at 20°C	−	−	−	−	−
Solubility in NaOH at 50°C	+	+	+	+	+
Decolorization by oxidants agents (H_2O_2)	+	+	+	+	+
Precipitation with HCl	+	+	+	+	+
Reaction for polyfenols with $FeCl_3$	+	+	+	+	+
Indol test	+	+	+	+	+

[a] (+) positive and (−) negative response

[b] IM (intracellular melanin): melanin extracted from mycelium

[c] EM (extracellular melanin): melanin extracted from culture medium

UV-visible spectra of these pigments showed a profile very similar to that of synthetic DOPA-melanin, having greater absorption in the UV region and no maximal or minimal in the visible ranges (Fig. 1). These results are typical of the absorption profile of aromatic compounds, such as melanin, which absorbs strongly in the UV region and decreases progressively as the wavelength is increased (Bell and Wheeler 1986). As a consequence of this behavior, the plot of the logarithm of the absorbance of the pigment against wavelength (between 400 and 600 nm) gave straight lines with negative gradients (Spiegel-Adolf 1937). In this study, the intra and extracellular pigments from MEL1 and MEL2 strains exhibited negative slopes varying between −0.0031 and −0.0037 and these values were very similar to those obtained for synthetic DOPA-melanin (−0.0032) (Fig. 1).

In the present study, the pigment extracted from MEL 1 mutant exhibited a electron spin resonance (ESR) spectrum similar to the synthetic DOPA-melanin, with G value equals to 2,007 (Fig. 2). The presence of stable paramagnetic species is a characteristic of the melanin pigment (Enochs et al. 1993) and similar G values were obtained for melanin extracted from *A. glaucus*, *A sydowi* (Senesi et al. 1987) and *A. fumigatus* (Youngchim et al. 2004).

Although, the results relating to chemical and spectral analysis showed that the pigment produced by *A. nidulans* is melanin, they didn't allow to determine the type of melanin present in this fungus. For this reason in the present study, the infrared spectrometric analyses of the pigments extracted from MEL1 and MEL2 mutants were also done to obtain information on the distinct functional groups prevailing in the structure of this pigment.

As can be seen in Fig. 3, the IR spectra of the intra and extracellular pigments from these mutants were more similar and some absorption peaks were also common to

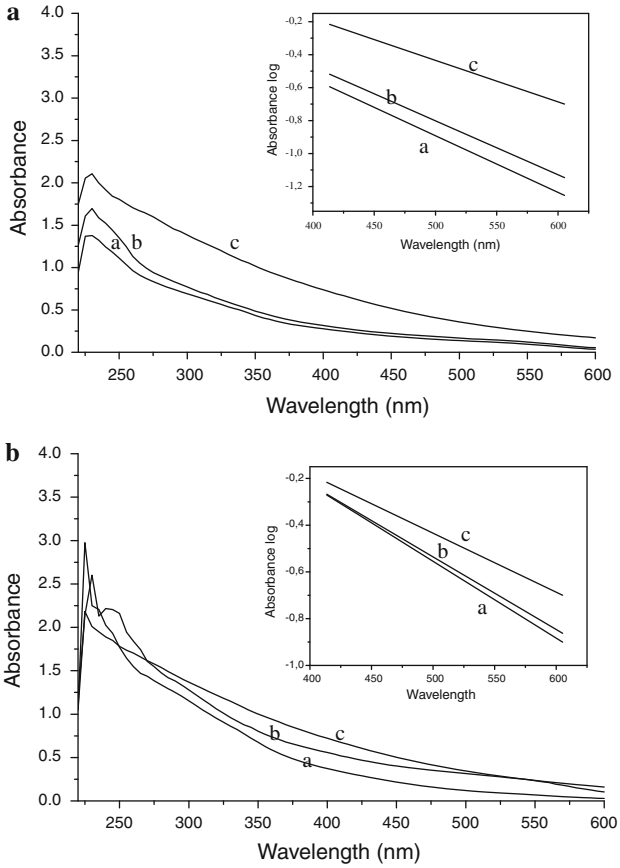

Fig. 1 UV and visible spectra of pigments extracted from culture medium (*a*) and mycelium (*b*) of MEL1 (**a**) and MEL2 (**b**) mutants in comparison with synthetic melanin (*c*). *Inset graph*: *Linear plots* with negative slopes given by pigments from the culture medium (*a*) and mycelium (*b*) of MEL1 (**a**) and MEL2 (**b**) mutants and synthetic melanin (*c*)

synthetic melanin, as the strong and broad bands in the 3,400–3,200 cm^{-1} region, which can be attributed to OH and NH_2 stretching (Bonner and Duncan 1962). On the other

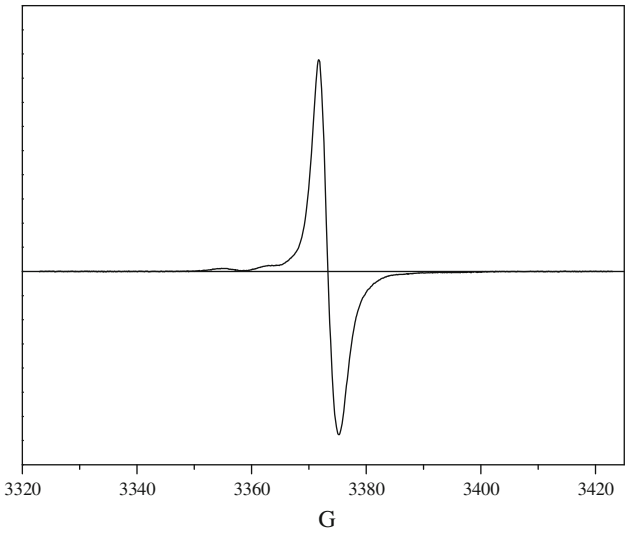

Fig. 2 ESR spectral analysis of extracellular melanin from MEL1

hand, in the 2,930–2,860 cm^{-1} region, assigned to CH_2 and CH_3 stretching (Senesi et al. 1987), the fungal spectra have a more differentiated absorption compared to synthetic DOPA-melanin (Fig. 3). This result, in conjunction with bands at 1,460–1,450 cm^{-1}, attributed to CH_2 and CH_3 bending (Bilinska 1996, Bridelli et al. 1999), indicates that the pigment from the mutants have a higher content of aliphatic groups (Fig. 3). A substantial part of this aliphaticity could be due to contamination of cell wall carbohydrates since the band at 1,030 cm^{-1}, assigned to C–O stretching of polysaccharides (Paim et al. 1990), displayed the greatest intensity in the spectra of the intracellular pigment from the MEL1 and MEL2 mutants (Fig. 3).

The spectra of the synthetic and fungal melanins also exhibited a strong absorption at 1,650–1,620 cm^{-1} (Fig. 3), which was attributed to stretching vibrations of aromatic C=C or C=O groups (Bonner and Duncan 1962). This absorption may be indicative of the compounds containing carbonyl groups conjugated with a benzene ring, which corresponds to the typical quinone structure of DOPA-melanin (Bridelli et al. 1999). A difference observed in these IR spectra is the sharp band at 1,720 cm^{-1} presents in the spectra of the synthetic melanin, attributed to COOH groups (Senesi et al. 1987), which appears as a shoulder in the pigment from MEL1 and MEL2 mutants (Fig. 3).

The MEL1 and MEL2 spectra (Fig. 3) displayed bands at 1,520–1,530 cm^{-1} (N–H bending), which together with the absorption at 1,320–1,390 cm^{-1} (C–N stretching) are strong suggestive of a substantial amount of pyrrole and/or indole groups in the structure of the pigment (Bridelli et al. 1999). This hypothesis was supported by results obtained from the indol test carried out with the pigment extracted from MEL1 and MEL2 mutants, in which the addition of some drops of

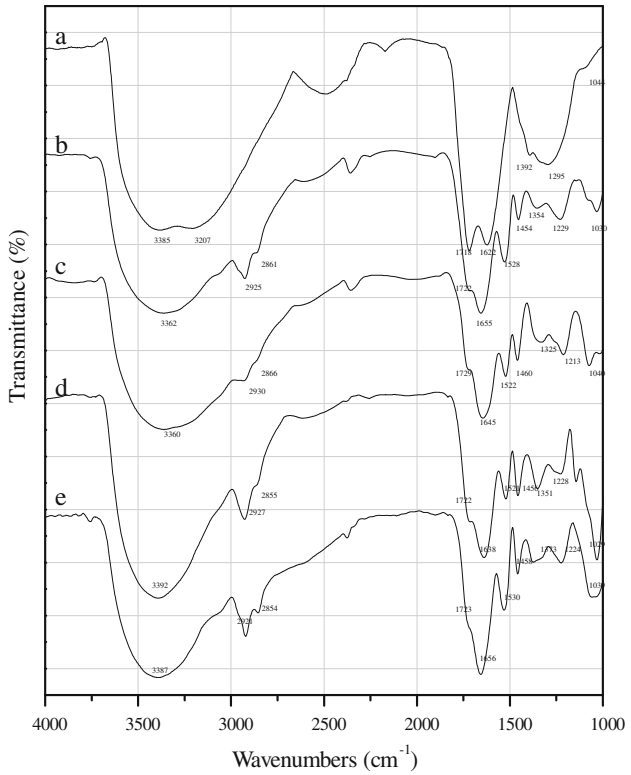

Fig. 3 Infrared spectra of *A. nidulans* and synthetic melanins: *a* synthetic DOPA-melanin; *b* intracellular melanin from MEL1 mutant; *c* extracellular melanin from MEL1; *d* intracellular melanin from MEL2 mutant; *e* extracellular melanin from MEL2 mutant

Table 2 Elemental composition of the pigments obtained from the culture medium (EM) and mycelium (IM) of MEL1 and MEL2 mutants of *A. nidulans* and other fungal melanins

Samples	Nitrogen %	Carbon %	Hidrogen %	Sulfur %
MEL1-EM	5.7	52.6	5.0	0
MEL1-IM	6.7	50.4	5.5	0
MEL2-EM	5.2	48.1	5.6	0
MEL2-IM	5.1	53.5	5.9	0
DOPA-Melanin	6.2	44.4	3.2	0
GHB-melanin*	10.3	53.4	5.2	0
Catecol-melanin[a]	Trace	57.9	3.8	0
DHN-melanin[a]	Trace	ND[b]	ND[b]	ND[b]

* as reported by Stussi and Rast (1981)

[a] as reported by Piatelli et al. (1963)

[b] *ND* Not determined

Ehrlich's reagent resulted in the appearance of a pink color, characteristic of indole derivatives, which were also found in synthetic DOPA-melanin (Table 1). This result confirmed the observations made by Bull (1970) that the melanin produced by *A. nidulans* appears to be a heteropolymer

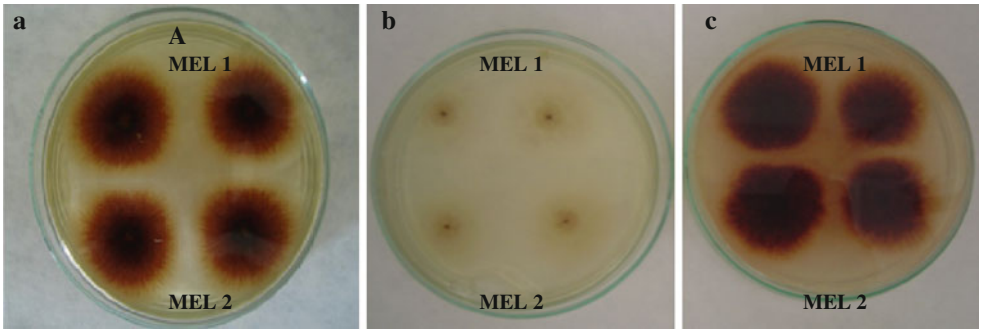

Fig. 4 Pigmentation of the MEL1 and MEL2 mutants grown in the absence (**a**) and in the presence of biosynthesis inhibitors of DOPA-melanin and DHN-melanin, being tropolone (**b**) and tricyclazole (**c**) respectively

containing indolic residues characteristic of DOPA-melanin. In addition, in the spectra of the MEL1 and MEL2 pigment are also observed bands at 1,210–1,230 cm^{-1} (Fig. 3), which may be assigned to C–OH stretching and OH deformation of alcoholic and carboxylic groups (Senesi et al. 1987; Bridelli et al. 1999). Thereby, these results suggest that the pigment from MEL1 and MEL2 strains is at least partially composed of dihydroxyindole and indolequinone, including their carboxilated forms (Plonka and Grabacka 2006; Tran et al. 2006), being that these units are present in the structure of DOPA-melanin proposed by Raper-Mason pathway (Bertazzo et al. 1999).

In the present study was also carried out the elemental composition analysis of the pigment extracted from MEL1 and MEL2 strains to verify the correlation between the nitrogen content in this pigment and the absorption at 1,540 cm^{-1} (N–H bending), as suggested by Senesi et al. (1987). The data showed in the Table 2 confirmed that the pigment obtained from mutants has a high percentage of nitrogen (varying from 5.1 to 6.7%), which was similar to synthetic DOPA-melanin (around 6%) and very different from DHN- melanin that presents only trace of N. The percentage of nitrogen present in melanin extracted from MEL1 and MEL2 mutants is in according with the values obtained for *A. nidulans* melanin (varying from 3.92 to 5.82%) described by Bull (1970). Comparing with data in literature relating to other fungal melanin, found that the N content for melanin extracted from *A. sydowi* was 3% (Paim et al. 1990) and from *Epicocum nigrum* and *Stachybotrys chartarum* was 3,5% (Filip et al. 1974), suggesting that these ascomycete fungi does not produce DHN-melanin.

Although results obtained had shown that pigment extracted from mutants of *A. nidulans* presents chemical properties, IR and UV spectra and elemental composition very similar to synthetic DOPA-melanin, they are not sufficient to assure that the melanin present in this fungus is DOPA-type. For this reason, in this study, compounds that specifically inhibit the biosynthetic pathway of the DOPA- and DHN-melanins were tested. Tricyclazole, pyroquilon, fthalide and chlobenthiazone inhibit the enzymatic reduction of two hydroxynapthalene compounds to scytalone and vermelone, intermediates in melanin production from DHN while tropolone, kojic acid, 2-mercaptobenzimidazole and diethyldithiocarbamate inhibit tyrosinase, a enzyme responsible for production of DOPA-melanin from tyrosine (Wheeler and Greenblatt 1988; Wheeler and Klich 1995).

The assay in the presence of tricyclazole (DHN-melanin inhibitor) showed that pigmentation of the MEL1 and MEL2 strains was not inhibited at the various concentrations tested (Fig. 4c) confirming that the pigment produced by these strains cannot be DHN-melanin. On the other hand, the colonies of the MEL1 and MEL2 mutants grown on the various concentrations of tropolone (inhibitor of DOPA-melanin biosynthesis) did not exhibit the usual dark-brown pigmentation (Fig. 4b) when compared to the colonies grown without the inhibitor (Fig. 4a), indicating that this compound inhibited melanin production in these strains.

Thus, as judged from the results obtained in this study, we show that brown pigment produced in MEL1 and MEL2 strains is not of type DHN but DOPA-melanin. This study contributed to the literature on fungal melanins, because it represents the first report on characterization of this type of melanin in *Aspergillus nidulans*.

In addition, growth tests with the MEL1 and MEL2 mutants from *A. nidulans* could be used to screen activators or inhibitors substances of the biosynthesis of DOPA-melanin applied in therapy.

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