Production and chemical characterization of pigments in filamentous fungi

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Production of pigments by filamentous fungi is gaining interest owing to their use as food colourants, in cosmetics and textiles, and because of the important biological activities of these compounds. In this context, the objectives of this study were to select pigment-producing fungi, identify these fungi based on internal transcribed spacer sequences, evaluate the growth and pigment production of the selected strains on four different media, and characterize the major coloured metabolites in their extracts. Of the selected fungal strains, eight were identified as Aspergillus sydowii (CML2967), Aspergillus aureolatus (CML2964), Aspergillus keveii (CML2968), Penicillium flavigenum (CML2965), Penicillium chermesinum (CML2966), Epicoccum nigrum (CML2971), Lecaniscllum aphanocladii (CML2970) and Fusarium sp. (CML2969). Fungal pigment production was influenced by medium composition. Complex media, such as potato dextrose and malt extract, favoured increased pigment production. The coloured compounds oosporein, orevactaene and dihydrotrichodimerol were identified in extracts of L. aphanocladii (CML2970), E. nigrum (CML2971), and P. flavigenum (CML2965), respectively. These results indicate that the selected fungal strains can serve as novel sources of pigments that have important industrial applications.

INTRODUCTION

Filamentous fungi are known to produce a wide variety of secondary metabolites, which play an important role in the diversification and adaptation of these micro-organisms to various ecological niches (Fox & Howlett, 2008). These metabolites have attracted immense interest from various researchers owing to their potential for biotechnological applications, such as drug development, cosmetics and food (Shwab & Keller, 2008).

Among the metabolites produced by fungi, pigments have been highlighted owing to their biological activity or to their potential use as dyes (Celestino et al., 2014). Filamentous fungi secrete diverse classes of pigments as secondary metabolites, including carotenoids, melanins, flavins, phe-nazines and quinones (Dufosse et al., 2014; Mapari et al., 2010). These natural compounds can serve as potential...
replacements and alternatives to synthetic pigments, which exhibit disadvantages including toxicity to health or the environment, and mutagenic and carcinogenic potential (Lopes et al., 2013). In addition, many pigments have important biological properties, such as antibacterial, antifungal and herbicidal activities, which make them important compounds for numerous biotechnological applications (Geweely, 2011; Premalatha et al., 2012; Teixeira et al., 2012).

Exploration of secondary metabolites produced by these micro-organisms can be readily accomplished because isolates may grow rapidly and produce high yields of the desired product using optimized culture conditions (Mapari et al., 2005). Studies have shown that changes in medium composition and/or culture conditions can result in enhanced pigment production. Thus, work has been aimed at selecting media or their components to increase the yield of these compounds (Mukherjee & Singh, 2011; Pradeep et al., 2013; Quereshi et al., 2010). Moreover, the identification of fungi and the chemical characterization of their extracts are important steps to select and direct such strains for various applications (Mapari et al., 2009).

Therefore, the objectives of this study were to screen and to identify pigment-producing fungi isolated from Brazilian caves and preserved in a research culture collection in Brazil, to evaluate the influence of different media on pigment production, and to characterize the major coloured metabolites produced by the selected strains.

**METHODS**

**Screening and identification of pigment-producing fungi.** Twenty strains isolated from Brazilian caves that currently are a part of the collection at the Bioprospecting and Genetics of Fungi Laboratory (Biogen) of the Federal University of Lavras, Brazil, were inoculated on PDA medium [potato (200 g l⁻¹), glucose (20 g l⁻¹) and agar (20 g l⁻¹)] and were incubated at 25 °C for 7 days. Strains demonstrating visual pigment production on PDA were selected and submitted for species identification based on the molecular phylogenetics of the internal transcribed spacer (ITS)-5.8S region of rDNA. Fungal DNA was extracted from mycelium scraped from PDA plates using the MO BIO UltraClean microbial extraction kit according to the manufacturer’s instructions. The ITS region was amplified: ITS 1 (5'-TCCGTAAGTGAACCTGCGG-3') was the forward primer and ITS 4 (5'-TCCTCCGGCTTTATATGC-3') was the reverse primer (White et al., 1990). Each 30 μl PCR mix contained 15 μl Qiagen TopTag Master Mix Kit 250 (containing 250 U TopTag DNA Polymerase in total, 10 × Coralload Concentrate, and RNase-free water), 12 μl ultrapure water, 1 μl each primer (10 pmol) and 1 μl genomic DNA. Thermal profiles for PCR were as follows: initial denaturation at 95 °C for 2 min, 35 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, and a final extension for 7 min at 72 °C. Samples were sequenced by Macrogen in South Korea. The sequences were edited using the SeqAssem v. 0.7/2008 software. Search for homologous sequences in GenBank was performed using Blast (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/blast) (Altschul et al. 1997). The ITS sequences of reference strains (Table S1) of fungal species that matched the Blast searches were downloaded and aligned with sequences of the pigment-producing fungi using Muscle, as implemented in the Megias software (Tamura et al., 2011). The alignment, containing 592 bp, including gaps, was subjected to Bayesian phylogenetic analysis using MrBayes 3.2 (Ronquist et al., 2012). The SYM + I + G model of sequence evolution was estimated using jModelTest (Darriba et al., 2012) and applied to the Bayesian analysis. Two independent analyses were run in parallel for 2.5 × 10⁶ generations in MrBayes, and sampled every 500 generations. The convergence between runs was assessed by examining the sd of split frequencies. One 50 %-majority-rule consensus tree was then generated after discarding 25 % of the initial trees. The sequences generated in this work were deposited in GenBank and the selected and identified fungi were deposited in Coleção Micológica de Lavras (CML) of the Phytopathology Department, Lavras Federal University, Brazil.

**Liquid culture on different media for pigment production.** Pigment-producing fungi belonging to different species were selected and cultivated in 250 ml Erlenmeyer flasks containing 100 ml of one of the four media: potato dextrose (PD) (Sharma et al., 2012); malt extract (ME) (Mapari et al., 2008); defined medium (MD) (Velmurugan et al., 2010), and Czapek–Dox (CZP) (Méndez et al., 2011, with modifications: 3 % sucrose; 0.3 % NaNO₃; 0.1 % KH₂PO₄; 0.05 % MgSO₄·7H₂O; 0.05 % KCl; 0.01 % FeSO₄·7H₂O). Shake-flasks were inoculated with two mycelial plugs approximately 9 mm in diameter from a 1-week-old culture grown on PDA medium. Incubation was carried out at 30 °C in the dark on a rotary shaker at 150 r.p.m. for 7 days. Pigment and biomass production in each medium were investigated in triplicate and mean values were reported. The R statistical software environment was used to compare the data using the Scott–Knott test (P < 0.01) (Scott & Knott, 1974).

**Extracellular pigment and biomass estimation.** Estimation of extracellular pigment content and biomass was performed as described by Velmurugan et al. (2010). Pigment production was expressed in absorbance units [at the wavelength (λ) of maximum absorption for each pigment] multiplied by the dilution factor (AU × df). Biomass was expressed as mycelial dry weight (g l⁻¹).

**Chemical characterization of pigment produced by selected fungi.** For chemical characterization, pigment production was performed in a volume of 1 l of the medium selected for each strain for 7 days at 30 °C and 150 r.p.m. The cultures were filtered and the supernatant was extracted twice with 0.5 vol ethyl acetate. Extracts were concentrated in a rotary evaporator (RV10 digital; IKA) and evaluated using TLC. Polar extracts were fractionated by chromatography on a Sephadex LH-20 (Sigma-Aldrich) column using methanol as eluent. The collected fractions were analysed on a Xevo TQ-S (Waters) system for direct insertion-mass spectrometry (DI-MS) and for the MS/MS experiments [collision-induced dissociation (CID)]. The nonpolar extract was fractionated by chromatography on a silica gel stationary phase column (35–70 μm; Acros). For elution, mixtures of hexane : ethyl acetate and ethyl acetate : methanol with increasing polarity were employed. The collected fractions were analysed by TLC on silica plates. Fractions showing the same chromatographic pattern were pooled and analysed on a Xevo TQ-S (Waters) system for ID-MS. The pooled fractions that showed precursor ions of the metabolites of interest were separated by HPLC using a Shimadzu system equipped with a C18-20A system control, LC-6AD pump, and UV–visible detector SPD-20A (λ, 301 and 370 nm) on an Eclipse XDB-C₁₈ semi-preparative column (9.4 × 250 mm, 5 μm; Agilent). For elution, a mixture of acetonitrile : water (50 : 50) was used with a flow rate of 3 ml min⁻¹.

The final pigment characterization was performed by MS/MS and ¹H NMR. The MS parameters were as follows: desolvation of N₂ gas at 300 °C; scan time 0.5 s; ion source temperature 120 °C; capillary 3.2 kV and cone 60 V for ESI⁺, and capillary −3.2 kV and cone −60 V for ESI⁻. The collision energies ranged between 10 and 50 eV, depending on the analyte. The software used to analyse the data was...
MassLynx v. 4.1 (Waters). 1H-NMR experiments were performed using a Bruker DRX-400 spectrometer. The samples were dissolved in CD3OD or CDCl3 (Sigma-Aldrich), depending on their solubility.

RESULTS

Screening and identification of pigment-producing fungi

Of the 20 fungal strains evaluated, 12 had the capacity to produce pigments in PDA medium; these 12 were selected and identified. The phylogenetic tree constructed based on the ITS sequences is shown in Fig. 1. The identified fungi consisted of one isolate of Aspergillus sydowii (CML2967), two of Aspergillus aureoletus (CML2964 and E.4.1), two of Aspergillus keveii (CML2968 and ON175), three of Penicillium flavigenum (CML2965; E.2.7 and 3.1.a), one of Penicillium chermesinum (CML2966), one of Epicoccum nigrum (CML2971), one of L. aphanocladii (CML2970) and one of Fusarium sp. (CML2969). The origin of each isolate and its GenBank accession number are presented in Table 1.

Influence of different culture media on pigment production in liquid culture

Of the identified fungi, eight strains belonging to different species were deposited in CML. These strains were selected to evaluate the influence of different media on growth and production of extracellular pigments in liquid culture. The visible colours of the pigments produced by the selected fungi on the various media are presented in Fig. 2. Each filtrate was scanned at 400–700 nm to determine the maximum absorption wavelength (λ) of each pigment. Subsequently, the supernatant was read at 400 nm for the yellow pigments of A. sydowii (CML2967), A. aureolatus (CML2964), A. keveii (CML2968), P. flavigenum (CML2965), P. chermesinum (CML2966), and Fusarium sp. (CML2969), at 430 nm for the orange–yellow pigment of E. nigrum (CML2971), and at 500 nm for the red pigment of L. aphanocladii (CML2970).

The ME medium was the most favourable medium to mycelial growth of all fungal species tested (Fig. 3). However, pigment production in this medium was significantly lower only to the following isolates: A. keveii (CML2968), P. flavigenum (CML2965) and Fusarium sp. (CML2969) (P<0.01) (Table 2). For other fungal species evaluated, including A. sydowii (CML2967), A. aureolatus (CML2964), P. chermesinum (CML2966), E. nigrum (CML2971), and L. aphanocladii (CML2970), the amount of biomass did not necessarily result in higher pigment production (Fig. 3 and Table 2).

As shown in Table 2, maximal yellow–orange pigment production for the E. nigrum (CML2971) isolate was observed in PD medium (UA430nm, 8.19 ± 3.04). On average, yellow–orange pigment production for the E. nigrum (CML2971) isolate in PD medium was 37 times higher than that of the other media tested (P<0.01). However, growth in this medium was the lowest (7.09 ± 1.61 g·l⁻¹) (Fig. 3). This behaviour was also observed for the isolate of P. chermesinum (CML2966), which produced pigments only in the CZP medium, where its growth was lower than in other media (3.98 ± 0.72 g·l⁻¹). Moreover, A. sydowii (CML2967) and P. chermesinum (CML2966) did not produce pigments in the ME medium, despite showing a higher biomass production in this medium (Fig. 3 and Table 2).

L. aphanocladii (CML2970) and A. sydowii (CML2967) showed maximal pigment production on PD medium. L. aphanocladii (CML2970) produced an intense reddish colour (UA500nm, 0.68 ± 0.6), and A. sydowii (CML2967) produced a yellow colour (UA400nm, 0.98 ± 0.66). For the A. aureolatus (CML2964) isolate, there was no significant difference in pigment production on PD or ME medium at 400 nm. Additionally, A. aureolatus (CML2964) produced a dark green colour in PD medium, with an absorption peak at a wavelength of 730 nm (UA730nm, 0.17 ± 0.12) (Fig. 2).

Chemical characterization of pigments produced by selected fungi

Eight of the selected species were cultured in a volume of 11 medium selected for each isolate: PD medium for L. aphanocladii (CML2970), E. nigrum (CML2971), A. aureolatus (CML2964), and A. sydowii (CML2967); ME medium for P. flavigenum (CML2965), A. keveii (CML2968) and Fusarium sp. (CML2969); and CZP medium for P. chermesinum (CML2966). The extraction of pigments using ethyl acetate was only possible for the filtrate of isolates of L. aphanocladii (CML2970), E. nigrum (CML2971) and P. flavigenum (CML2965). The pigments of the other isolates were insoluble in ethyl acetate.

Extract of these three strains, whose pigments were soluble in ethyl acetate solution, was concentrated in a rotary evaporator and evaluated using TLC. According to the retention observed on the silica plate, it was observed that the extracts of L. aphanocladii (CML2970) and E. nigrum (CML2971) were predominantly constituted of polar compounds. In order to prevent loss of compounds of interest, preliminary purification on a Sephadex column was performed. In contrast, because of the nonpolar character of P. flavigenum (CML2965) extract, this was fractionated on a silica column.

Fifteen and 12 fractions from extract produced by L. aphanocladii (CML2970) and by E. nigrum (CML2971), respectively, were recovered from fractionation on the Sephadex column. Fractions that showed colour (5–13 of CML2970 extract and 3–9 of CML2971 extract) were directly analysed by mass spectrometry.

By analysing the mass spectra of fractions 6–9 of L. aphanocladii (CML2970) extract (Fig. S1), the precursor ions (m/z 307 [M + H⁺] and 329 [M + Na⁺]) of oosporein, a metabolite commonly produced by related species, were found (Fig. 4a). These fractions were pooled based on spectral analysis, and produced a red precipitate (8.5 mg) that
Fig. 1. Fifty per cent-majority-rule consensus tree based on ITS sequences of pigment-producing fungi and reference strains of identified species and species groups. Posterior probabilities ≥0.9 are given near the nodes. *Peziza ampelina* KH 00.011 (C) was used as the outgroup.
Table 1. Origin of the isolates selected in this study and their GenBank accession numbers

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>City/state/country</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. aphanocladii (CML2970)</td>
<td>Gruta do Cavaliño/air</td>
<td>Pains/Minas Gerais/Brazil</td>
<td>KR261451</td>
</tr>
<tr>
<td>E. nigrum (CML2971)</td>
<td>Gruta do Honoração/soil</td>
<td>Iuiu/Bahia/Brazil</td>
<td>KR261452</td>
</tr>
<tr>
<td>A. keveii (CML2968)</td>
<td>Gruta Toca Cora de Frade/air</td>
<td>Coronel José Dias/Piauí/Brazil</td>
<td>KR261449</td>
</tr>
<tr>
<td>P. chermesinum (CML2966)</td>
<td>Lab contaminant/air</td>
<td>Lavras/Minas Gerais/Brazil</td>
<td>KR261447</td>
</tr>
<tr>
<td>A. sydowii (CML2967)</td>
<td>Gruta dos Morcegos</td>
<td>Delmiro Gouveia/Alagoas/Brazil</td>
<td>KR261448</td>
</tr>
<tr>
<td>P. flavigenum (CML2965)</td>
<td>Gruta Lapa Nova/soil</td>
<td>Vazante/Minas Gerais/Brazil</td>
<td>KR261445</td>
</tr>
<tr>
<td>A. aureolatus (CML2964)</td>
<td>Gruta Lapa Nova/soil</td>
<td>Vazante/Minas Gerais/Brazil</td>
<td>KR261445</td>
</tr>
<tr>
<td>Fusarium sp. (CML2969)</td>
<td>Lab contaminant/air</td>
<td>Lavras/Minas Gerais/Brazil</td>
<td>KR261450</td>
</tr>
<tr>
<td>P. flavigenum (3.1.a)</td>
<td>Gruta Lapa Nova/soil</td>
<td>Vazante/Minas Gerais/Brazil</td>
<td>KR261455</td>
</tr>
<tr>
<td>P. flavigenum (E.2.7)</td>
<td>Gruta Lapa Nova/soil</td>
<td>Vazante/Minas Gerais/Brazil</td>
<td>KR261456</td>
</tr>
<tr>
<td>A. aureolatus (E.4.1)</td>
<td>Gruta Lapa Nova/soil</td>
<td>Vazante/Minas Gerais/Brazil</td>
<td>KR261454</td>
</tr>
<tr>
<td>A. keveii (ON175)</td>
<td>Gruta Toca Cora de Frade/air</td>
<td>Coronel José Dias/Piauí/Brazil</td>
<td>KR261453</td>
</tr>
</tbody>
</table>

was dissolved in CD3OD for 1H-NMR analysis. The data from 1H-NMR were consistent with previous data on the characterization of oosporein (Nagaoka et al., 2004), exhibiting only one chemical shift at δ 1.88 (s) (6H, s) and confirming the identity of this pigment (Fig. 5a).

The mass spectra of fractions 5–7 of E. nigrum (CML2971) extract (Fig. S2) showed the ion precursor ([M–H]−) of orevactaene (Fig. 4b). The presence of this metabolite was confirmed by the fragmentation profile (MS/MS) of ion 611, which showed characteristic ions at m/z 403, 447, 491 and 521 in negative mode (Fig. 5b), similar to previously reported patterns (Shu et al., 1997). These fragments were a result of the cleavage of the hydroxylated side chain moiety, followed by the cleavage of the dihydroxypyran ring and by decarboxylation (Shu et al., 1997).

Fifty fractions from extracts produced by P. flavigenum (CML2965) were obtained from the silica gel column separation. Fractions that showed the same chromatographic profile were pooled and analysed by MS. By analysing the mass spectra of the pooled fractions 8–18 of P. flavigenum (CML2965) extract (Fig. S3), the precursor ions (m/z 499 [M + H+] and 521 [M + Na+] ) of dihydrotrichodimerol, a metabolite produced by species of the genera Penicillium and Trichoderma, were detected (Fig. 4c). This combined fraction (8–18) was purified by HPLC. Of the 19 recovered fractions, fractions 9 and 10, which showed ions of m/z at
499 and 521, were pooled and yielded a yellow precipitate (4.43 mg) that was dissolved in CDCl₃ for ¹H-NMR analysis. The presence of dihydrotrichodimerol was confirmed by the ¹H-NMR data, particularly because of the presence of signals with chemical shifts at δ 5.49 (m), 6.14 (d), 6.2 (m), 6.3 (m) and 7.33 (dd) (Fig. S4). These chemical shifts agree with previous characterization of dihydrotrichodimerol by Lee et al. (2005), confirming the identity of this compound.

### DISCUSSION

Fungal identification of the isolates selected in this work used molecular phylogenetics of the ITS-5.8S region of the rDNA. As this region is rapidly evolving, methods based on its analysis have been widely used to discriminate between closely related species (White et al., 1990). In addition, sequence variation within this region has been useful in phylogenetic studies of many fungi (Bastola et al., 2004).

Table 2. Pigment production of isolates in different media

<table>
<thead>
<tr>
<th>Isolate</th>
<th>λ (nm)</th>
<th>PD</th>
<th>ME</th>
<th>CZP</th>
<th>MD</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. aphanocladii (CML2970)</td>
<td>500</td>
<td>0.68 ± 0.06 a</td>
<td>0.22 ± 0.13 b</td>
<td>0.06 ± 0.02 b</td>
<td>0.12 ± 0.09 b</td>
</tr>
<tr>
<td>E. nigrum (CML2971)</td>
<td>430</td>
<td>8.19 ± 3.04 a</td>
<td>0.22 ± 0.08 b</td>
<td>0.22 ± 0.06 b</td>
<td>0.29 ± 0.09 b</td>
</tr>
<tr>
<td>A. keveii (CML2968)</td>
<td>400</td>
<td>0.20 ± 0.06 b</td>
<td>1.79 ± 0.06 a</td>
<td>0.37 ± 0.11 b</td>
<td>0.10 ± 0.07 b</td>
</tr>
<tr>
<td>P. chermesinum (CML2966)</td>
<td>400</td>
<td>–</td>
<td>–</td>
<td>1.58 ± 0.07 a</td>
<td>–</td>
</tr>
<tr>
<td>A. sydowii (CML2967)</td>
<td>400</td>
<td>0.98 ± 0.66 a</td>
<td>–</td>
<td>0.09 ± 0.03 b</td>
<td>–</td>
</tr>
<tr>
<td>A. flavigenum (CML2965)</td>
<td>400</td>
<td>1.56 ± 0.25 b</td>
<td>3.38 ± 0.35 a</td>
<td>0.16 ± 0.16 c</td>
<td>0.37 ± 0.03 c</td>
</tr>
<tr>
<td>A. aureolatus (CML2964)</td>
<td>400</td>
<td>0.68 ± 0.24 a</td>
<td>0.72 ± 0.17 a</td>
<td>0.08 ± 0.08 b</td>
<td>0.14 ± 0.05 b</td>
</tr>
<tr>
<td>Fusarium sp. (CML2969)</td>
<td>400</td>
<td>0.54 ± 0.13 b</td>
<td>2.03 ± 0.05 a</td>
<td>–</td>
<td>0.23 ± 0.1 b</td>
</tr>
</tbody>
</table>

Values (UA × df) are means of triplicates ± SD and those with different letters are significantly different at P<0.01.

Fig. 4. Chemical structure of compounds: oosporein (a) (Nagaoka et al., 2004), orevactaene (b) (Shu et al., 1997), and dihydrotrichodimerol (c) (Lee et al., 2005).
**E. nigrum**, one of the species herein identified, is a recognized producer of a variety of secondary metabolites, including pigments such as carotenoids (Gribanovski-Sasu & Foppen, 1967), flavonoids (Șoptica & Bahrim, 2005) and/or polyketides (Shu et al., 1997), of red, orange, and yellow hues. Consequently, **E. nigrum** should be considered as a potential source of pigments (Mapari et al., 2008).

Two pigment-producing species belonging to genus Penicillium were identified as **P. flavigenum** (CML2965; E.2.7 and 3.1.a) and **P. chermesinum** (CML2966). In a previous study by Huang et al. (2011), **P. chermesinum** (ZH4-E2) was isolated from mangroves and reported as a producer of the new azaphilones (chermesinones). **P. flavigenum** belongs to section Chrysogena, which is known to have member strains capable of producing anthraquinones and other yellow polyketides (Frisvad & Samson, 2004). Moreover, antibiotic xanthocillins have been found in two species in Chrysogena (**P. chrysogenum** and **P. flavigenum**; Frisvad et al., 2004). According to Mapari et al. (2009), the species **P. flavigenum** should be investigated as a possible source of pigments for industrial applications.

One isolated strain of the genus Fusarium was identified among the evaluated isolates in the present study. According to previous studies, species belonging to this genus [such as **F. oxysporum** (Nagia & El-Mohamedy, 2007), **F. verticilloides** (Boonyapranai et al., 2008) and **F. moniliforme** (Premalatha et al., 2012)] are sources of different bioactive metabolites, including pigments such as the anthraquinones.

![Fig. 5. (a) $^1$H-NMR spectra of oosporein ($\delta=1.88, 6$H, s). (b) Fragmentation profile of ion at m/z 611 (negative mode) showing characteristic ions at m/z 403, 447, 491 and 521, confirming the structure of orevactaene.](image)
A. aureolatus, also identified in this study, has been reported as a parasitic fungus of Agaricus sp. and of Sphaerotheca fuliginea (Heijwegen, 1989). In addition, it is known both for being entomogenous (Péciulyté & Kačergius, 2012) and as a potential biological control agent against aphids (Zare & Mohammadi, 2006). This species has been confused with Aphanocladium album (Preuss) and the use of this name in most of the literature refers to L. aphanocladii. An important characteristic of this fungus is the capability possessed by most of its strains to produce red pigments in agar (Zare & Gams, 2001). This trait was also observed in the present study.

Three species of the genus Aspergillus were herein identified: A. aureolatus (CML2964 and E.4.1), A. kevëi (CML2968 and ONI75) and A. sydowi (CML2967). There are no other reports about the potential of these species as pigment producers. Among the fungi belonging to the genus Aspergillus, only some species have been reported as possible sources of pigments, such as A. glaucus, A. cristatus and A. repens, which were reported to produce known yellow and red hydroxyanthraquinoid pigments, such as emodin and physcion (yellow pigments), quentin (yellow to orange–brown) and erythroglauca, catenarin and rubrocistin (red) (Caro et al., 2012).

Most of the species identified in this work have hardly been studied for the production of pigments and therefore may compose new sources of colourants and/or metabolites with important biotechnological applications.

After screening and identification of the pigment-producing filamentous fungi, eight strains, belonging to different species, were selected to evaluate the influence of different media on growth and production of extracellular pigments in liquid culture. The media were selected based on the available literature for pigment production by filamentous fungi in liquid cultures. Only the extracellular production of pigments was evaluated in this study; we preferred to study these pigments because of their solubility in culture media, and the ease and low cost of downstream processing (Mapari et al., 2009).

Of the media evaluated, ME medium was significantly favoured for pigment production and growth of three isolates, A. kevëi (CML2968), P. flavigenum (CML2965) and Fusarium sp. (CML2969). ME medium is composed of glucose, malt extract and peptone. The malt extract is a source of vitamins and coenzymes that can promote the growth and production of pigments by these fungi (Pradeep & Pradeep, 2013). Furthermore, it has been reported that various types of peptone, commonly used in culture media as a source of nitrogen, increased the pigment production of many fungal species (Celestino et al., 2014; Pradeep et al., 2013; Quareshi et al., 2010; Velmuru-gan et al., 2010). Peptone provides many nutrients such as peptides and amino acids to the broth and it seems to be easily metabolized by most fungi, which can lead to increased production of their metabolites, including pigments (Celestino et al., 2014).

The increased biomass did not necessarily result in higher pigment production for A. sydowi (CML2967), A. aureolatus (CML2964), P. chermesinum (CML2966), E. nigrum (CML2971) and L. aphanocladii (CML2970) in the media evaluated. In general, the pigments produced by filamentous fungi are secondary metabolites whose production usually commences late in the growth of the micro-organisms, especially when entering the stationary phase (Calvo et al., 2002). However, the metabolic versatility of the micro-organisms allows a multiplicity of responses according to different environments and nutritional conditions. Another aspect that should be considered is the presence of inducers of pigment production by micro-organisms (Celestino et al., 2014). Therefore, the biomass and pigment-producing ability of filamentous fungi may not be positively correlated in liquid cultures.

The strain A. aureolatus (CML2964) produced a dark green pigment in PD medium that was not observed in other media. This result indicates that PD medium favoured the production of other pigments by this isolate. Species of the genus Monascus also produce pigments of different colours depending on the medium’s composition, and the production of one pigment may be favoured over the others (Mukherjee & Singh, 2011).

In this study, seven of the eight species evaluated showed higher pigment production in media of undefined composition, such as ME and PD. These media contain glucose, which is a carbon source widely reported to induce the production of pigments (Chatterjee et al., 2009; Mukherjee & Singh, 2011; Pradeep et al., 2013). Also, these media contain other components such as malt extract, peptone and starch, which provide a series of nutrients including vitamins, coenzymes, peptides, amino acids, sulfur, carbon and nitrogen. These nutrients can regulate the expression of genes of interest and activate metabolic pathways important for the production of pigments (Pradeep et al., 2013). Thus, depending on the selected species, some sources of carbon and nitrogen can be more easily assimilated and promote higher yields of the desired product (Celestino et al., 2014).

The purpose of using different media in these experiments was to increase pigment production in order to facilitate the downstream process of extraction and characterization. Moreover, these results mark the start of a discussion about the nutritional conditions for pigment production by these fungi, which can help in the selection of substrates to optimize future work.

Among the pigments characterized in this study, oosporein (2,5-dihydroxybenzoquinone), identified in the extract produced by A. aphanocladii (CML2970), is a mycotoxin originally isolated from the basidiomycete Oospora colorans (Kogl & van Wessem, 1944). It has also been found in other fungi like Chaetomium trilaterale, Verticillium psalliotae, Beauveria sp., and Chaetomium cupreum (Cole et al., 1974; Luo et al., 2015; Mao et al., 2010; Nagaoka et al., 2004). This compound has important biological activities,
including inhibition of growth in plants and phytotoxic effects (Cole et al., 1974). Moreover, oosporein showed antifungal activity against *Phytophthora infestans* (Nagaoka et al., 2004), *Rhizoctonia solani*, *Botrytis cinerea* and *Pythium ultimum*, and the potential to inhibit proliferation of tumour cell lines (Mao et al., 2010). This compound has also been shown to possess antiviral activity (Terry et al., 1992), while demonstrating toxic effects in various poultry including broiler chickens and turkeys, resulting in gout, kidney damage and even death (Cole et al., 1974; Pegram et al., 1982).

The pigment orevactaene, identified in an extract produced by *E. nigrum* (CML2971), is a yellow antioxidant compound and has great potential as a food colourant (Mapari et al., 2010). Moreover, orevactaene has potential for inhibition of HIV replication, as it was found to inhibit binding of the HIV-1 regulatory protein Rev and its viral RNA-binding site (Shu et al., 1997).

The compound dihydrotrichodimerol, identified in *P. flavigenum* (CML2965) extract, is a bisorbicillinoid polyketide with an open-ended cage structure. These kinds of compounds are rare in nature and are unique metabolites found in various terrestrial and marine fungi. The bisorbicillinoids have attracted interest owing to their structural complexity and biological activities, such as antioxidant and antimicrobial activity (Abe et al., 1998; Lee et al., 2005; Liu et al., 2005).

Dihydrotrichodimerol has been isolated from species including *Penicillium terrestre* (Liu et al., 2005) and *Trichoderma citrinoviride*. This compound reduced the feeding preference of the aphid *Schizaphis graminum*, one of the most important pests of cereal crops, suggesting a potential for the development of new agrochemicals for control of this pest (Evidente et al., 2009). Moreover, dihydrotrichodimerol previously isolated from *P. terrestre* and from unidentified fungal strains, showed, respectively, cytotoxic effects to cancer cell lines and effects on the activation of PPARγ (peroxisome proliferator-activated receptor γ), a nuclear receptor with therapeutic potential in the treatment of type 2 diabetes, inflammatory disease, and certain cancers (Lee et al., 2005; Liu et al., 2005). To the best of our knowledge, this is the first time that this compound has been isolated from the species *P. flavigenum*.

**CONCLUSIONS**

Considering the results of this study, it was concluded that the evaluated fungi may have potential in the production of pigments with biotechnological applications. Among the 20 fungi evaluated, 12 were able to synthesize coloured compounds, and the molecular identification of these strains resulted in identification of 8 different species belonging to the genera *Penicillium*, *Aspergillus*, *Epichoccum*, *Lecanicillium* and *Fusarium*. In this study, the growth and pigment-production capabilities of these strains differed depending on the fungal species tested and were shown to be influenced by the composition of the medium. Complex media, PD and ME, seemed to favour the production of these metabolites. Coloured compounds produced by these fungi were identified by MS/MS and NMR techniques as: oosporein [red mycotoxin in the *L. aphanoocladii* (CML2970) extract], orevactaene [orange antioxidant in the *E. nigrum* (CML2971) extract] and dihydrotrichodimerol [yellow compound in the *P. flavigenum* (CML2965) extract]. The presence of coloured compounds with antioxidant potential in extracts of *E. nigrum* (CML2971) and *P. flavigenum* (CML2965) indicates the possibility of using these isolates for the production of pigments with industrial applications.

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