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ISOLATION OF HERPOTRICHIELLACIOUS FUNGI FROM THE ENVIRONMENT

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ABSTRACT

Herpotrichiellaceous fungi, common agents of chromoblastomycosis and phaeohyphomycosis, were searched in samples of rotten wood, leaf littler, bark and soil of the rhizosphere, collected in the Centro Nacional de Pesquisas de Florestas/EMBRAPA, Colombo, PR, Brazil. Morphological analyses of macro, optic and scanning electron microscopy, as well as the determination of the nutritional pattern of the isolated strains were carried out for a taxonomical study. In a total of 17 colonies, 3 isolates (17.6%) were identified as species of medical relevance: *Cladophialophora bantiana* (Sacc.) de Hoog *et al.*, *Fonsecaea pedrosoi* (Brumpt) Negroni and *Phialophora verrucosa* Medlar. The identifications were based on the results of the analyses and on the comparison with CBS (Centraal Bureau voor Schimmelcultures) reference strains. This investigation revealed the saprophytic existence of species known as agents of chromoblastomycosis and phaeohyphomycosis. These diseases are considered autochthonous in the studied area.

Key words: Chromoblastomycosis; fungal isolation; Herpotrichiellaceae; phaeohyphomycosis

INTRODUCTION

Herpotrichiellaceae is a family of a wide group of mostly ascomycete anamorphs whose main characteristic is the presence of melanized hyphae and conidia. They are also called black fungi and are able to endure non-optimal growth conditions easily causing a variety of infections (2). Their medical representatives are known as black yeasts, i.e., mitosporic fungi with dark budding cells during at least one phase of their development. The yeast-form, however, is not a pre-exigency because black fungi may only express the filamentous-form with erect conidiophores, specially at low levels of moisture content (1, 10).

It is said that melanized fungi are fast growing saprophytes, but a surprising number of species able to cause chronic mycosis has increased this last decade (2). Their medically important species have become a source of debates including discussions on identification, species concept and taxonomical position due to the morphological plasticity and wide ecological amplitudes they present (1, 2). These species are known as causative agents of chromoblastomycosis, phaeohyphomycosis and true mycetoma (10, 11). Nowadays, the genera *Fonsecaea, Phialophora, Exophiala, Cladophialophora* and *Rhinocladiella* are considered the most important ones of this group (2, 5, 10).

Human chromoblastomycosis is a chronic infection characterized by cauliflower-like lesions limited to the skin and subcutaneous tissue, where muriform cells occur (10, 13). The disease is mainly found in agricultural male labourers of tropical and sub-tropical areas. In Brazil, the infection can be found over the State of Paraná, principally caused by plant fragment traumatisms (13). The genus *Fonsecaea*, with two species, is the most common and studied agent (10, 11, 13).

Phaeohyphomycosis is meant for a group of primary and opportunistic mycoses, which may result in a chain of inflammatory responses, while the presence of mycotic granules in the infected tissues is the characteristic of true mycetomas.

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The latter can be roughly classified depending on whether the grains are white, red or black (2, 3, 10, 18).

Thus, all pathogenic black yeasts are supposed to belong to the family Herpotrichiellaceae; most of their available cultures are from clinical cases. The isolation of environmental strains is more difficult and the identification even harder due to their pleomorphic condition and slow growth. There are several papers reporting these agents in nature (6, 7, 8, 11, 12), and some discuss their ecological niche (1, 11, 15, 16).

The aim of this paper was the isolation and the study of Herpotrichiellaceous fungi from environmental samples taken from places where chromoblastomycosis and phaeohyphomycosis are considered autochthonous. Such approach is of great relevance because it raises useful information for a more appropriated taxonomical definition and a better understanding on the natural ecology of this group.

MATERIALS AND METHODS

Sampling area

The woods belonging to the Centro Nacional de Pesquisas de Florestas – CNPF/EMBRAPA, located in Colombo town, metropolitan surroundings of Curitiba, PR, Brazil. Geographical coordinates are 25º 20' S and 49º 14' W, with maximum altitude of 915 m. The predominant tree is the pinus *Podocarpus lambertii* Klotzsch ex Endl., followed by *Gognathia polymorpha* (Lessing) Cabrera (known as Cambará), *Araucaria angustifolia* (Bertoloni) (also called Pinus of Paraná) Otto Kuntze and *Syagrum romanzoffianum* (Cham.) Glassm. (Jerivá). The soil is humic, clay texture. The first sampling was made on October, 1997 and the atmospheric condition data, obtained from the EMBRAPA's Meteorological Station, in that month were: rainfall 166.64 mm; 16.7ºC mean temperature; 88% mean air humidity and 77.2 hours of total luminosity (2.5 h/day). A second sampling, one year later, was done under very similar conditions: rainfall of 192.9 mm; 16.5ºC mean temperature; 88% mean air humidity and 105.4 hours of total luminosity (3.4 h/day). Thirty samples of rotten wood, leaf litter of the predominant trees, bark and soil of the Cambara's rhizosphere were collected. Two other collections occurred on May 1998 and January 1999, however only strains of the saprophytic *Cladosporium* species were found.

Fungal isolation

Ten grams of each sample were added to flasks containing 100 mL sterile salt solution, 200U penicilin, 200 µg/L streptomicin, 200 µg/L chloranphenicol and 500 µg/L cycloheximide. These were incubated for 30min. at room temperature. Then, 200 mL of sterile mineral oil were dispensed into the flasks and the solution vigorously shaked for 5 min. After 20 min., the interfase was collected and innoculated on Mycosel agar (DIFCO) at 36ºC for 4 weeks (6, 9). The growing black cultures were transferred and stocked in slants for analysis.

Fungal analyses

All strains studied are listed in Table 1. Stock cultures were maintained on slants of malt extract agar (MEA). For morphological observations, strains were grown on Saboraud and Potato Dextrose Agar using slide cultures in moist chambers. For scanning electron microscopy (SEM), samples were placed over carbon tape and gold sputtered for 180s. Observations were made in a Zeiss DSM 940 A microscope, operated at 5kV.

Nutritional physiology

Growth and fermentative abilities were tested in duplicate and with blanks, according to van der Walt and Yarrow modified (4). Assimilation tests were performed in liquid medium with tubes incubated in a nearly horizontal position, aerated through slow rocking (50 rpm approximately) and weekly examined for 21 days. Halotolerance was tested in liquid medium at 2.5, 5 and 10% (w/v) NaCl and $MgCl_2$. Cycloheximide tolerance was observed in liquid medium at 0.01, 0.05 and 0.1% (w/v) (4).

Table 1. Fungal strains studied.

Strain number	Fungus	Nomenclature	Source	Geographical origin
FE9	*Cladophialophora bantiana* (Sacc.) de Hoog *et al.*		Bark of Jerivá's palm	Brazil
FE5p4	*Fonsecaea pedrosoi* (Brumpt) Negroni		Cambara wood in decomposition	Brazil
FE3	*Phialophora verrucosa* Medlar		Soil from root of fallen Cambara tree	Brazil
CBS 271.37	*Fonsecaea pedrosoi* (Brumpt) Negroni	NT	Human	South America

FE = Fungus from EMBRAPA, Colombo-PR-Brazil; CBS = Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. T = type strain; NT = neotype strain.

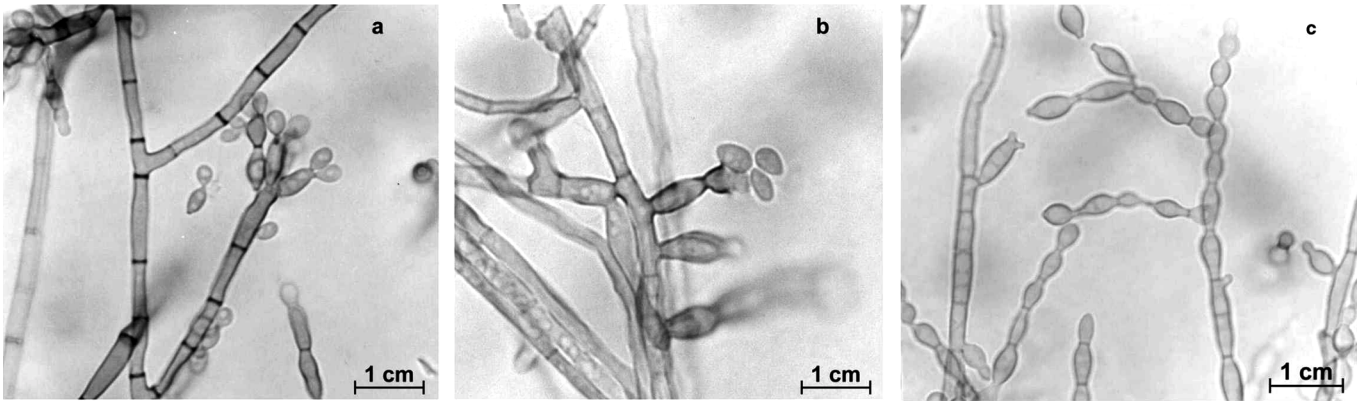

Figure 1. (a) *F. pedrosoi* (FE5p4): septate hyphae and proliferation of primary and secondary 1-celled conidia from the conidiogenous cell; (b) *P. verrucosa* (FE3): flask-shaped phialides with funnel-shaped collarettes; (c) C. *bantiana* (FE9): conidia in long poorly branched chain on undifferentiated hyphae. Photomicroscopy x1000.

RESULTS AND DISCUSSION

Among seventeen cultures of black fungi isolated from the samples studied, three were Herpotrichiellaceous of medical importance: *Cladophialophora bantiana* (FE9); *Fonsecaea pedrosoi* (FE5p4) and *Phialophora verrucosa* (FE3) (Table 1). Their morphologies are detailed in Figs. 1 and 2. The Jerivá and the Cambará trees were considered interesting substrates for recovering this group of fungi, and more extensive investigations on them seem to be promising.

Table 2 summarizes the results of the physiological tests of both environmental and reference strains. Some compounds were readily assimilated and scored as positive, (+); not assimilated (negative score, -); poorly assimilated (indicated by "w" for weak), and w/+ or w/- indicate a variable weak. From 65 tests, sixteen were positive for all fungi, although sometimes weak: D-glucose, D-galactose, D-arabinose, maltose, sucrose, α-α-Trehalose, cellobiose, arbutin, melezitose, glycerol, D-mannitol, nitrate, ethylamine, L-lysine, cadaverine and growth at 30ºC. On the other hand, no strain was able to grow at 40ºC, ferment glucose, or degrade inulin or methanol. The results above are in agreement with another author who found them relevant to characterize medically important Herpotrichiellaceous fungi (3). The "in vitro" metabolism has shown to be an important tool for the understanding of the biology and ecology of these agents (1, 4, 5, 17).

C. bantiana (FE9) differed from the strains studied due to a weak growth in L-rhamnose, galactitol and nitrate. The results of the nutritional tests were compared with a key to species of *Cladophialophora* and similar taxa, showing significant similarities (17). Its ability to assimilate ethanol, lactose and citrate is mentioned as characteristic of this species (3). However the growth absence at 40ºC may indicate a better adaptation to a saprophytic condition.

Comparing the *F. pedrosoi* (FE5P4) isolate with CBS (Centraal Bureau voor Schimmelcultures) reference strain, 80% similarity was observed among their physiological results, differing in the assimilation of L-sorbose, melibiose, ribitol, xylitol, *myo*-inositol, glucono-δ-lactone, D L lactate, succinate, nitrite, urease and tolerance to 5.0% NaCl. The culture *F. pedrosoi* (FE5P4) is very close to CBS 271.37 with characteristics like conidiogenous cells loosely branched and cylindrical, with clusters of prominent denticles in sympodial arrangement (Fig. 1a, Fig. 2a,b), what

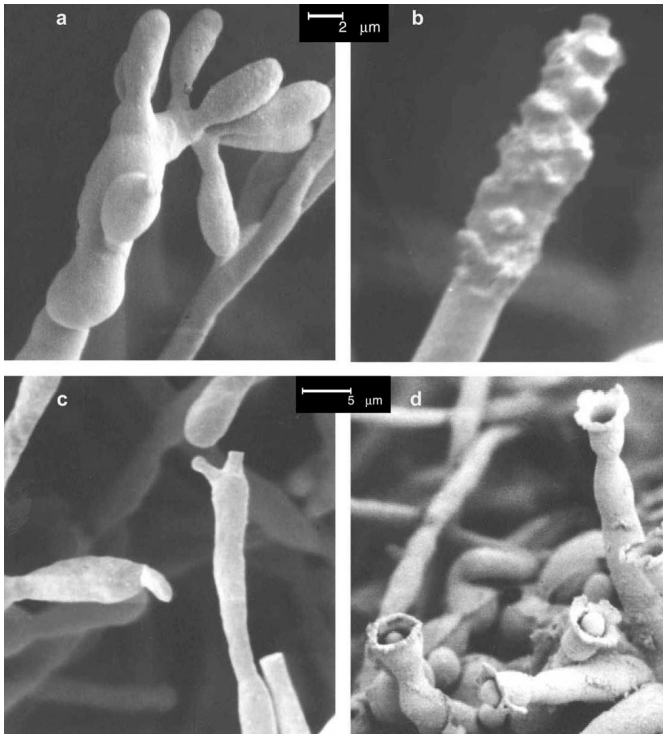

Figure 2. *F. pedrosoi* (FE5p4): (a) loose conidial arrangement, (b) protruding conidial denticles in sympodial arrangement; C. *bantiana* (FE9): (c) undifferentiated hyphae; *P. verrucosa* (FE3): (d) funnel-shaped collarettes.

Table 2. Physiological profiles of studied strains.

	FE9	FE5 P4	FE3	271.37
D Glucose	+	+	+	+
D Galactose	+	+	+	+
L Sorbose	+	+	+	w
D Glucosamine	w	w	w/+	w
D -Ribose	w	w/-	-	w/-
D -Xylose	+	+	+	+
L -Arabinose	+	+	+	+
D -Arabinose	+	w	+	w/-
L -Rhamnose	w	+	+	+
Sucrose	+	+	+	+
Maltose	+	+	+	+
’ -Trehalose	+	+	+	+
methyl- - D -Glucoside	+	w	w	w/-
Cellobiose	+	+	+	+
Salicin	+	+	+	+
Arbutin	+	+	+	+
Melibiose	+	+	-	w/-
Lactose	+	w	-	w/+
Raffinose	+	+	+	+
Melezitose	+	+	+	+
Inulin	-	-	-	-
Sol. starch	w	-	w	w/-
Glycerol	+	w	+	w/+
meso-Erythritol	+	w	+	w/-
Ribitol	w	w	+	+
Xylitol	+	w	-	+
L -Arabinitol	+	+	w	w/+
D -Glucitol	+	+	+	+
D -Mannitol	+	+	+	+
Galactitol	+	w	w	w/+
myo-Inositol	+	+	+	-
Glucono- -lactone	+	+	+	w
D -Gluconate	w	+	+	w/+
D -Glucuronate	w	+	+	w/+
D -Galacturonate	+	w	+	w/-
D L -Lactate	+	w	+	+
Succinate	w	w	+	+
Citrate	w	w	+	w/-
Methanol	-	-	-	-
Ethanol	w/+	w	w/+	w/-
Nitrate	w/+	+	+	+
Nitrite	w/+	w	+	+
Ethylamine	w	w	+	w/+
L -Lysine	w	w	+	w
Cadaverine	+	+	+	+
Creatine	w	w	+	w
Creatinine	w/	w	+	w
2.5% $MgCl_2$	+	+	+	+
5.0% $MgCl_2$	+	+	+	+
10 % $MgCl_2$	+	+	w	+
2.5% NaCl	+	+	+	+
5.0% NaCl	+	+	w	w
10 % NaCl	-	-	-	-
0.1 % Cycloheximide	+	+	+	+
0.05 % Cycloheximide	+	+	+	+
0.01 % Cycloheximide	+	+	+	+
Urease	-	w	-	+
30ºC	+	+	+	+
37ºC	+	+	+	+
40ºC	-	-	-	-
Fermentation	-	-	-	-
Acid production	-	-	-	-
Mycosel	+	+	+	+
Gelatine	-	w	-	-
DNAse	-	-	-	-

Abbreviations used: + = growth; w = weak growth ; – = no growth; FE9 = *C. bantiana*; FE5P4 = *F. pedrosoi* ; FE3 = *P. verrucosa*; 271.37 = *F. pedrosoi.*

strongly suggests the identification as *F. pedrosoi*. There are other reports of its occurrence in nature (4, 11).

The species *P. verrucosa* (FE3) was not able to assimilate lactose, ribose and inulin, but was positive for L-lysine assimilation as observed for the type strain (4, 5). Microscopical features like the presence of a typical funnel-shaped collarette (Fig. 2d) is also an important character in the taxonomy of this fungus (2, 4), as observed in the isolated culture.

The isolation of the strains reported in this paper was considered of great relevance since two species, *F. pedrosoi* (FE5P4) and *P. verrucosa* (FE3) are widely known as agents of chromoblastomycoses (3, 10, 11, 13) and *C. bantiana* (FE9), agent of phaeohyphomycoses (2, 3, 18). The diseases have already been detected in many clinical cases in the surroundings of the sampling area (13).

Since dematiaceous fungi are able to grow in extreme conditions, submerged cultures under different osmotic pressures and the addition of cicloheximide are parameters commonly evaluated (17). The high adaptation ability of these fungi indicates a pathogenic potential (19).

The infections caused by this group of fungi, frequently resulted from a traumatism may reflect the existence of adaptative factors in their natural niches, which favors their survival in the tissues of the host (1, 16). It was stated that the ability to live in very adverse conditions may result in a cell protection against the macrophage attack (14). Other research showed the survival capability and the development of *F. pedrosoi* cells inside macrophages of the peritoneum of a mouse (7). The strains isolated in this study were very resistant to stressing conditions. Further studies are necessary for final conclusions about their pathogenicity.

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RESUMO

Isolamento de fungos herpotriquieláceos do ambiente

Fungos da família Herpotrichiellaceae, agentes comuns de cromoblastomicose e feohifomicose, foram isolados de amostras de solo e de material vegetal em decomposição, durante duas coletas realizadas na propriedade do Centro Nacional de Pesquisa de Florestas/EMBRAPA, localizada em Colombo, PR, Brasil. Após o isolamento, os fungos foram submetidos às análises óticas macro e microscópicas e eletrônica de varredura, além do estudo de seus padrões

nutricionais. Das 17 colônias de fungos isoladas, 3 (17,6%) foram identificadas como espécies de importância clínica, tais como: *Cladophialophora bantiana* (Sacc.) de Hoog *et al*, *Fonsecaea pedrosoi* (Brumpt) Negroni e *Phialophora verrucosa* Medlar. As identificações basearam-se nos resultados dos testes fisiológicos e na comparação da morfologia dos isolados com linhagens referência do CBS (Centraal Bureau voor Schimmelcultures). Esta investigação revelou a existência sapróbia de espécies de fungos conhecidas como agentes de cromoblastomicose e feohifomicose. Tais doenças são tidas como autóctones na região estudada.

Palavras-chave: Cromoblastomicose; feohifomicose; Herpotrichiellaceae; isolamento de fungos

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