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Original Article

Isolation of *Fonsecaea pedrosoi* from the Shell of the Babassu Coconut (*Orbignya phalerata* Martius) in the Amazon Region of Maranhão Brazil

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Abstract

Fonsecaea pedrosoi, a dematiaceous fungus and the main causative agent of chromoblastomycosis, has been isolated in worldwide from different natural sources in regions where the disease is endemic. In the Amazon region of Maranhão, Brazil, where the disease is prevalent, the breaking of the babassu coconut (*Orbignya phalerata* Martius) represents an important agricultural activity. In order to determine the presence of this fungus on this plant and on other natural substrates, material was collected in the Fortaleza Village Municipality of Pinheiro, Maranhão, in April and September 2002. A total of 68 samples, including 18 (26.5%) obtained from the shell of the babassu coconut, were analyzed. Samples were cultured using a standard method. Isolates were identified based on macromorphological aspects of the colonies on Sabouraud dextrose agar and based on the micromorphology of the conidia after growth on potato dextrose agar. *Exophiala* sp. was the most prevalent fungus isolated from the different natural substrates analyzed, while *Cladophialophora* sp. was only isolated from decomposing wood. *Fonsecaea pedrosoi* was isolated from one sample of babassu coconut shell suggesting that this coconut represents an important source of infection of chromoblastomycosis during extraction of the plant product in this region.

Key words: babassu coconut, *Fonsecaea pedrosoi*, chromoblastomycosis, dematiaceous fungus, Amazon region, Maranhão, Brazil

Introduction

Fonsecaea pedrosoi is a dematiaceous fungus of the family *Herpotrichiellaceae* and is considered to be one of the main etiological agents of chromoblastomycosis in the world[1, 2, 3]. This mycosis found worldwide is prevalent in countries with tropical and subtropical climates, mainly affecting men aged 30 to 60 years[4, 9]. Infection probably occurs through transcutaneous trauma and mainly affects the lower limbs, with the disease being more prevalent among rural workers[5, 6, 10, 11].

In Brazil, large numbers of cases of the disease have been reported in the North and South region of the country[5, 8, 9]. The Amazon region is considered to be the main area affected by the disease, with the largest number of cases being reported in this region[8]. This

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high incidence of chromoblastomycosis is believed to be mainly due to the climatic conditions, including a mean annual rainfall of 1500-1700 mm, a temperature of 24-26°C and a relative annual humidity of about 83%, which seem to contribute to the development of the fungus in nature[8, 12]. The State of Maranhão, which is part of this region, occupies third place in the Brazilian reported series, with most cases being reported in the "Baixada Maranhense"[6, 11, 13].

The number of dematiaceous fungal species implicated as agents of chromoblastomycosis is still a matter of debate. In addition to the classical agents that frequently cause the

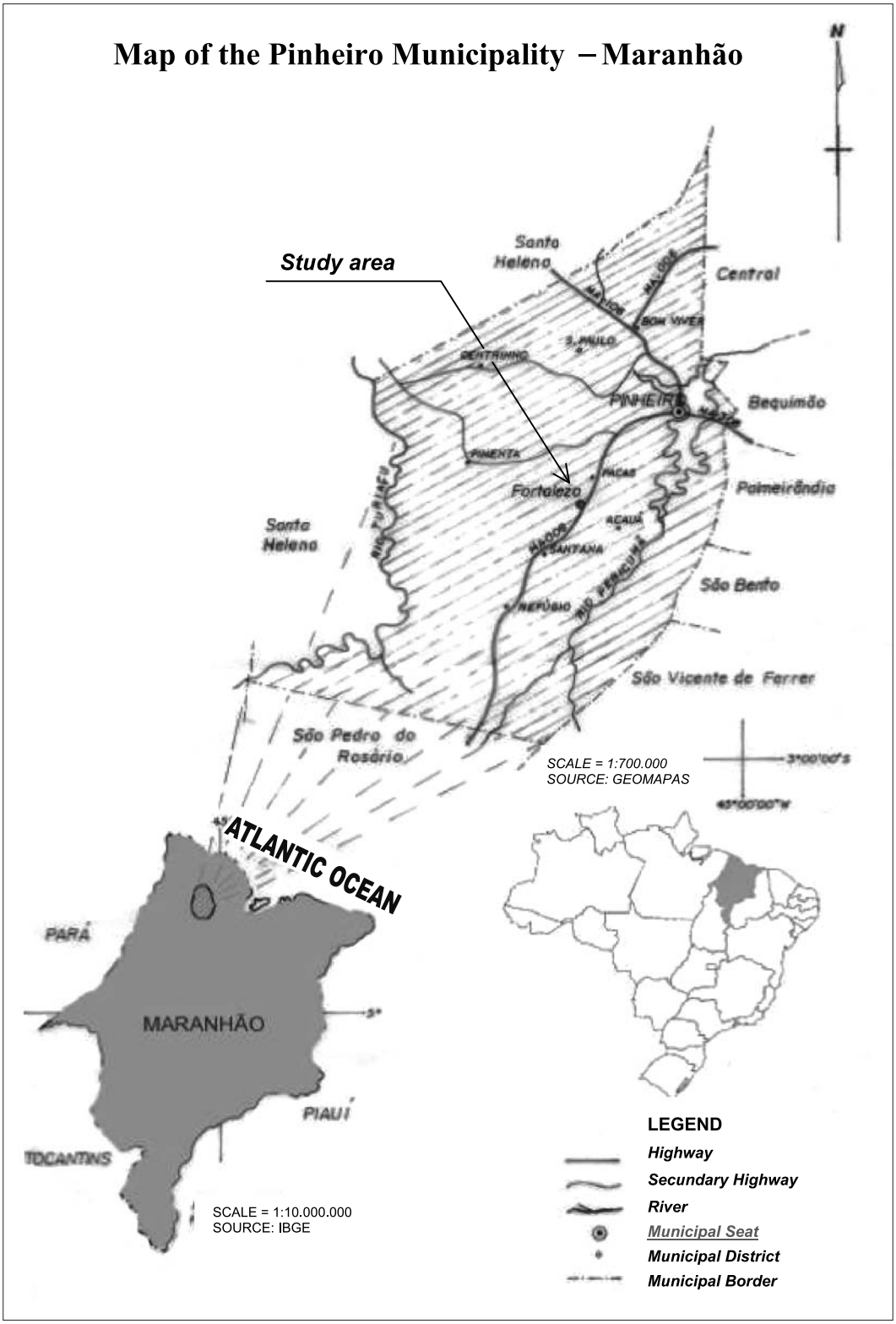

Fig. 1. Location of the Fortaleza Village, Municipality of Pinheiro, Maranhão, Brazil.

disease, i.e., *F. pedrosoi*, *Cladophialophora carrionii* and *Phialophora verrucosa*[3, 14], other species have sporadically been recognized as agents of chromoblastomycosis based on the presence of muriform elements in the lesions. These species include *F. compacta*, *Rhinocladiella aquaspersa*, *Exophiala jeanselmei*, *E. spinifera*, *E. castellanii* and *Aureobasidium pullulans*[9, 15, 21].

Knowledge about the distribution of dematiaceous fungi in nature is incomplete and reports regarding the isolation of these agents from natural sources, especially in areas where the disease occurs, are rare. Studies carried out in different countries have demonstrated the isolation of *P. verrucosa*, *F. pedrosoi* and *C. carrionii* from natural sources such as decomposing wood and plant matter, soil and other substrates[1, 2, 22, 24]. In Brazil, investigations on the isolation of dematiaceous fungi from natural sources, including the causative agents of chromoblastomycosis, have only been carried out in the States of Amazonas and Paraná[25, 26].

Recently, two cases of chromoblastomycosis caused by *F. pedrosoi* have been reported in the State of Maranhão, with the lesions being located in the gluteal region and with the only occupational activity of the patients being the breaking of the babassu coconut (*Orbignya phalerata*)[6]. The authors called attention to the possibility that infection had occurred during extraction of this plant product.

Babassu is a palm tree native to the Brazilian Midnorth which forms a belt in the northeast-southeast direction of Maranhão, called the coconut forest or babassu zone. Maranhão contains 54.2% of the Brazilian babassu palm trees, with two thirds of them being located in the "Baixada Maranhense". Approximately 300,000 rural families are estimated to be involved in the extraction of babassu seeds, a manual process during which the individual sits on the ground or on the coconut shell, hitting the fruit with a wooden stick against the inverted blade of an ax, a procedure that frequently causes injuries[27, 29]. About 76.9% of patients with chromoblastomycosis originate from this region, with their main activity being farm work[30].

Within this context, the objective of the present study was to determine the presence of etiological agents of chromoblastomycosis on the shell of babassu coconuts and on other natural sources.

Materials and methods

Study area: The study was carried out in the Fortaleza Village, Municipality of Pinheiro, Maranhão, Brazil, located 2°26'30" latitude South and 45°08'15" longitude West at an altitude of 60 m above sea level (Fig. 1). The climate is of the tropical humid type, with a mean annual rainfall of 1,800 mm. The soil is hydromorphic, with a pH of about 5.4, and is covered with an alluvial fluvial-marine field vegetation, where mangroves and babassu palm trees grow. The relative humidity in this region is about 80% and the mean annual temperature ranges from 25.4 to 27.5°C. The economy of the region is based on the culture of rice, corn, mandioca and extraction of the fruit of the babassu palm tree (*O. phalerata*).

This area was chosen because of the characteristics described above and because some patients with chromoblastomycosis who registered at our outpatient clinic of infectious disease of Federal University of Maranhão, Brazil, were natives of the region.

Sample collection: Sixty-eight samples of decomposing coconut shell, stem and leaves of the babassu palm, wood and bark of decomposing trees, cover vegetation, animal feces, thorns of the bactris palm (*Bactris acanthocarpa*), and soil were collected in April and September 2002. The material was collected close to the dwellings as well as at the work place of the patients, with the selection criterion being the observation of darkened areas which are suspect for the presence of dematiaceous fungi (Fig. 2a, 2b, 2c).

The material was collected using gloves and previously sterilized instruments (spoon, scissors, gardening shovel), stored in sterile plastic bags, and processed in the laboratory of the Nucleus of Tropical Disease of the Federal University of Maranhão, 2 to 30 days after collection.

Culture: Samples were cultured according to the standard technique described by Iwatsu *et al.*[1]

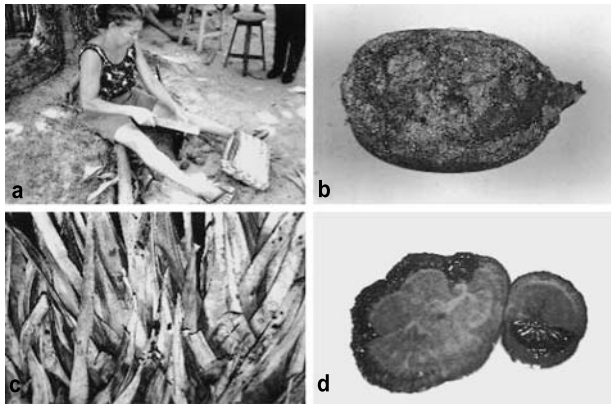

Fig. 2. (a) Manual extraction of babassu seeds. (b) Decomposing babassu coconut. (c) Colonized plant matter. (d) Colony isolated on Sabouraud dextrose agar.

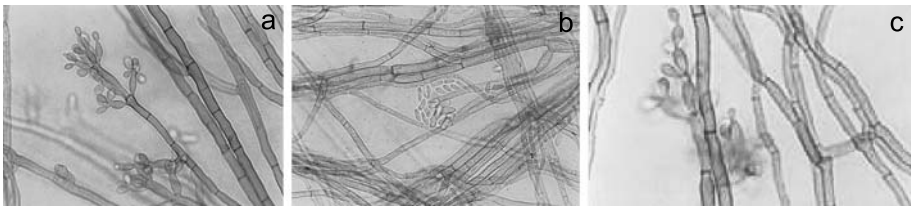

Fig. 3. Micromorphological characteristics of the isolates. (a) *Fonsecaea pedrosoi* showing septate conidiophores, erect with simpodial arrangement - magnification: 1.000 x (b) *Exophiala* sp. showing annellides producing oval annelloconidia. (c) *Cladophialophora* showing brownish septate hyphae with long chains of spindle-shaped conidia in the shape of a flattened lemon. Magnification: 1000 x.

and adapted by Vicente *et al.*[26]. Twenty grams of each sample was incubated in 100 m*l* 0.85% sterile saline containing 200 U/m*l* penicillin G, 200 μg/m*l* chloramphenicol, 200 μg/m*l* streptomycin and 500 μg/m*l* cycloheximide for 30 min at room temperature, and then 20 m*l* sterile mineral oil was added to each flask. The solution was homogenized by shaking for 5 min and left to stand for 20 min. One drop of the oil-water interface of the suspension was then seeded onto mycobiotic agar plates (Difco, Detroit, MI, USA) and the culture was incubated at 36°C for 4 weeks. After growth, dark colonies identified macroscopically were replated on mycobiotic agar medium (Fig. 3d).

The isolated strains were identified based on the macromorphology of the colonies after growth on Sabouraud dextrose agar (SDA) at 28, 36 and 40°C and based on the micromorphology of the conidia after growth on potato dextrose agar (PDA) as described by Riddell[32].

Results

The 68 samples collected from different natural substrates in the Fortaleza Village, Municipality of Pinheiro, Maranhão, are listed in Table 1.

The isolation technique used was found to be adequate for the recovery of saprobic isolates including the genera *Fonsecaea*, *Exophiala* and *Cladophialophora* (Table 2). All dematiaceous isolates grew on SDA at 28 and 36°C but not at 40°C.

Culture of the fungus *F. pedrosoi* on SDA yielded slow-growing colonies with a short black aerial mycelium and a black reverse. Micromorphological characteristics included brownish septate hyphae and *Cladophialophora* type of conidiogenesis. In the apex of the conidiophores, a serial arrangement of syimpodial conidiogenous cells bearing primary and secondary conidia was observed. (Fig. 3a).

The genus *Exophiala* was identified based on the development of olive to black colonies on SDA initially exhibiting yeast-like growth, which gradually developed a velvety texture, with a black reverse. Micromorphological analysis of PDA-grown cultures revealed an annellide type conidiophore and the production of oval annelloconidia (Fig. 3b).

Culture of *Cladophialophora* on SDA yielded dark, slow-growing colonies with a short aerial mycelium and a black reverse, which were found to be largely adhered to the culture medium. The presence of a brown pigment diffused within the medium was noted. Microscopical analysis revealed brownish septate hyphae and *Cladosporium* type sporulation characterized by chains of spindle-shaped conidia in the shape of a lemon (Fig. 3c).

The frequency of the different agents isolated from each natural substrate is shown in Table 2. Nine isolates of dematiaceous fungi were recovered from the different substrates analyzed, with the genus *Exophiala* being the most frequent (66.7%), followed by *Fonsecaea* (22.2%) and *Cladophialophora* (11.1%). Analysis of the different natural sources showed that most agents were recovered from decomposing plant matter (77.8%).

As shown in Table 2, the babassu palm tree was found to be an important plant source for the development of dematiaceous fungi, with 44.4% of the isolates being recovered from this plant. *Fonsecaea pedrosoi* was isolated from one sample of babassu coconut shell and decomposing plant matter each. *Exophiala* sp. was isolated from decomposing babassu shell, babassu stem, babassu leaves, decomposing wood, decomposing tree bark, and decomposing plant matter (one sample each), while *Cladophialophora* sp. was only isolated from decomposing wood (one sample).

Six (66.7%) of the nine isolates were obtained during collection in April, a period characterized by heavy rainfall in the region studied. The species isolated during this period included the genera *Fonsecaea* and *Cladophialophora*, while *Exophiala* was recovered both in April and September in equal proportions (33.3% each).

Table 1. Number of samples obtained from different substrates collected in the Fortaleza Village Municipality of Pinheiro, Maranhão, Brazil

Substrate	Number of samples	Percent %
Decomposing babassu coconut shell	18	26.5
Decomposing wood	7	10.3
Decomposing tree bark	5	7.4
Plant matter	8	11.8
Animal feces	2	3.0
Babassu stem	9	13.0
Thorn of the bactris palm (*Bactris acanthocarpa* Martius)	2	3.0
Babassu leaves	6	8.8
Soil	11	16.2
Total	68	100.0

Table 2. Frequency of dematiaceous fungi isolated from different natural substrates in the Fortaleza Village Municipality of Pinheiro, Maranhão, Brazil

Substrate	Isolated fungus			No. of isolates (%)
	Cladophialophora	*Exophiala*	*Fonsecaea*	
Decomposing babassu shell		1	1	2 (22.2)
Babassu leaves	−	1	−	1 (11.1)
Decomposing wood	1	1	−	2 (22.2)
Decomposing tree bark	−	1	−	1 (11.1)
Decomposing plant matter	−	1	1	2 (22.2)
Babassu stem	−	1	−	1 (11.1)
Total (%)	11.1	6 (66.7)	2 (22.2)	9 (100.0)

Discussion

In the present study, dematiaceous fungi (*F. pedrosoi*, *Cladophialophora* sp. and *Exophiala* sp.) were isolated for the first time from natural sources in an area of the State of Maranhão, a region regarded as an important focus of chromoblastomycosis in Brazil[6, 13].

In the "Baixada Maranhense", the activities of laborers range from farm work to the breaking of the babassu coconut and fishing[6, 29]. In addition, saprobic dematiaceous fungi found in this region such as *F. pedrosoi*, *Cladophialophora* sp. and *Exophiala* sp. have been shown to present a risk of infection to individuals living in these rural areas whose main activity is farm work. Exposure of this population to these fungi has been the main risk factor for contracting the disease[9, 31].

Although no studies exist relating the babassu coconut with the growth of dematiaceous fungi, Silva *et al.*[6] reported the cases of two patients with chromoblastomycosis in the gluteal region whose only professional activity was the breaking of this coconut. Notably, during this activity, the individuals sit on the shell of the coconut which consists of hard fibers that can cause microinjuries, thus permitting the penetration of these fungi (Fig. 2a).

One species among the dematiaceous fungi isolated was *F. pedrosoi*, the main causative agent of chromoblastomycosis in the Brazilian Amazon region[6, 8, 11]. The frequency of isolation of this fungus was higher than that reported by Iwatsu *et al.*[1], who only identified one isolate of this fungus among 117 samples obtained from nature. On the other hand, these authors identified 13 *P. verrucosa* isolates, a species not observed in the present study.

Exophiala was the most frequently isolated genus, in agreement with the study of Cristovao *et al.*[25], who isolated this fungus from the sandy soil of the beaches of the Negro river, Amazonas State, Brazil, a region with climatic conditions similar to those of the "Baixada Maranhense". Although *E. jeanselmei*, *E. spinifera* and *E. castellanii* have been described as agents of chromoblastomycosis, various species of this genus are more related to the etiology of pheohyphomycosis and black grain mycetoma[15, 21].

In the present study, the genus *Cladophialophora* was only isolated from decomposing wood. In the State of Paraná, this agent was recovered

from plant matter, tree stem and roots, soil and decomposing wood[26], while in the State of Falcon, Venezuela, *C. carrionii* was isolated from cactuses[24].

Decomposing plant matter, mainly wood, was the preferential habitat of the isolates obtained in the present study, similar to observations made by others[2, 22, 26]. The babassu palm tree (*Orbignya phalerata*) as well as the shell of its coconut were found to be an important plant source for the development of dematiaceous fungi such as *F. pedrosoi* and *Exophiala* sp. and probably an important risk factor for the contraction of chromoblastomycosis in the area studied.

The climatic conditions of the study area (a mean temperature of 25°C, annual rainfall of about 1,800 mm^3 and 80% relative humidity) seem to be adequate for the development of the fungi isolated in this study, in agreement with other reports[25].

Knowledge about the ecology of these fungi in nature can be useful for the differential diagnosis of fungal infections in a given geographic area where the disease occurs.

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