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Diversity of opportunistic black fungi on babassu coconut shells, a rich source of esters and hydrocarbons

Mariana M. F. NASCIMENTO[a,b], Vania A. VICENTE[a], Juliana V. M. BITTENCOURT[c], Jane Mary L. GELINSKI[a,d], Francesc X. PRENAFETA-BOLDÚ[e], Maycoll ROMERO-GÜIZA[e], Gheniffer FORNARI[a], Renata R. GOMES[a], Germana D. SANTOS[a], A. H. G. GERRITS VAN DEN ENDE[b], Conceição D. M. P. S. DE AZEVEDO[f], G. Sybren DE HOOG[a,b,g,*]

[a]Microbiology, Parasitology and Pathology Graduate Programme, Federal University of Paraná, Curitiba Department of Basic Pathology, Federal University of Paraná, Curitiba 81540-970, Brazil
[b]Westerdijk Fungal Biodiversity Institute, P.O. Box 85167, 3508 AD, Utrecht, The Netherlands
[c]Technological Federal University of Paraná, Ponta Grossa 84016210, Brazil
[d]University of West of Santa Catarina – UNOESC, Videira 89900-000, Santa Catarina, Brazil
[e]IRTA, GIRO Joint Research Unit IRTA-UPC, Torre Marimon, 08140 Caldes de Montbui, Barcelona, Spain
[f]Department of Medicine, Federal University of Maranhão, São Luís 65020070, Brazil
[g]Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, P.O. Box 94248, 1090 GE, Amsterdam, The Netherlands

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ABSTRACT

The present study assessed the diversity of black yeast-like fungi present on babassu coconut shells, a substrate rich in lipids and several volatile organic compounds (VOCs) including aromatic hydrocarbons. Using different isolation methods, one-hundred-six isolates were obtained and were identified by ITS sequencing as members of the genera *Exophiala*, *Cladophialophora*, *Veronaea*, and *Rhinocladiella*. Two novel species were discovered. Eight strains were selected for assessing their ability to grow on toluene and phenyl acetate as the sole carbon and energy source. All strains tested were able to assimilate phenyl acetate, while two out of eight were able to use toluene. VOCs profiling in babassu samples was also investigated by GC-ToF MS, revealing that a complex mixture of VOCs was emitted, which included alkylbenzenes such as toluene. Assimilation of alkylbenzenes by the black yeasts might therefore be the result of evolutionary adaptation to symbiotic interactions with higher plants. The potential relationship between lipid/aromatic hydrocarbon metabolism and pathogenicity is also discussed.

* *Corresponding author*. Westerdijk Fungal Biodiversity Institute, P.O. Box 85167, 3508 AD, Utrecht, The Netherlands.
E-mail address: s.hoog@westerdijkinstitute.nl (G.S. De Hoog).

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Introduction

Babassu (*Orbignya phalerata* Mart., *Arecaceae*) is a native palm tree in the northern and northwestern regions of Brazil, especially in Maranhão, Piaui, Tocantins and Goias States (Teixeira 2008). Particularly in Maranhão state, babassu exploration for application in food, soap and skin products has great economic importance for local development and as a complementary activity to rural people's subsistence (Souza *et al.* 2011). On the other hand, the Maranhão province is known as one of the main endemic areas of chromoblastomycosis, a chronic, mutilating skin disease caused by black fungi (Azevedo and Gomes *et al.* 2015; Queiroz-Telles 2015). Several authors (Marques *et al.* 2006; Vicente *et al.* 2012, 2014) reported on a possible connection between the presence of black fungi on babassu shells and the prevalence of chromoblastomycosis in Maranhão, suggesting that chromoblastomycosis in this province might be an occupational disease.

Black yeast-like fungi are phylogenetically diverse, but the recurrent human opportunists nearly all belong to a single order, the Chaetothyriales. Many species display polymorphic life cycles (de Hoog & McGinnis 1987). The natural habitats of most species are unknown; despite their probably opportunistic nature, many have been reported from human-dominated environments and from humans only. Potential agents of the disease probably inhabit adverse (micro)-niches in decaying plant material (Vicente *et al.* 2008, 2012; Guerra *et al.* 2013), hydrocarbon-polluted sites (Prenafeta-Boldú *et al.* 2001a; Isola *et al.* 2013), railway sleepers (Döğen *et al.* 2013) or bathing facilities (Sudhadham *et al.* 2008). Some species, primarily in the genera *Exophiala* and *Cladophialophora*, are able to assimilate toxic volatile aromatics, to an extent that this might be beneficial for bioremediation of industrial pollution (Prenafeta-Boldú *et al.* 2001b; Zhao *et al.* 2010). However, the same species have been associated repeatedly with infectious disease (de Hoog *et al.* 2004; Najafzadeh *et al.* 2010, Najafzadeh *et al.* 2011; Azevedo *et al.* 2015). An ecological connection between aromatic hydrocarbon assimilation and human pathogenicity has been presumed (Prenafeta-Boldú *et al.* 2006). Elucidation of ecological and evolutionary parameters is necessary to understand those factors of habitat choice that seem to enhance opportunism on the human host (Vicente *et al.* 2008; Döğen *et al.* 2013; Gümral *et al.* 2014).

Babassu coconuts are rich in lipids, terpenes and other hydrocarbons (Garcia *et al.* 1995) and are a commodity for industrial purposes (Teixeira 2008; Santos *et al.* 2013). The shells provide a rather special environment recalcitrant to microbial decomposition. However, abundant presence of black fungi has been observed. The hypothesis of babassu shells possibly transmitting disease by these fungi is the subject of the present paper. We describe the coconut shell as an environment rich in volatile organic compounds (VOCs), possibly enhancing enrichment of agents of chromoblastomycosis.

Material and methods

Sampling area and isolation methods

Six samples of babassu coconut were collected in the municipality of São Luis, in Maranhão state, Brazil, on January 2014. The epicarp and mesocarp of the coconut were selected for fungal isolation. Three methods focused on black fungi were applied:

(i) **Direct plating**. Of each sample, approximately 1 g was added to 5 mL saline solution (8.5 g L^{-1}). After homogenization, 100 μL was plated directly onto Mycosel agar (Difco, Detroit, MI, U.S.A.) and Dichloran Rose Bengal Chloramphenicol Agar (DRBC, Himedia, Mumbai, India). Samples were incubated at 28 °C for 2 weeks.

(ii) **Selective enrichment on volatile aromatics** was adapted from Prenafeta-Boldú *et al.* (2001a) and Isola *et al.* (2013). Czapeck agar without glucose (sodium nitrate 2.0 g L^{-1}, dipotassium phosphate 1.0 g L^{-1}, magnesium sulphate 0.5 g L^{-1}, potassium chloride 0.5 g L^{-1}, and ferrous sulphate 0.01 g L^{-1}, chloramphenicol 0.1 g L^{-1}) was used for enrichment in 6 mL liquid culture medium in 15 mL flasks, inoculated with 100 μL of the suspension solution. Flasks were incubated at 25 °C for three weeks under controlled atmosphere rich in volatile aromatic hydrocarbon (toluene) which was supplied as a sole carbon and energy source. Hundred μL of the enriched culture was plated on Mycosel Agar (Difco) and incubated at 28 °C for two weeks and black colonies were transferred to Malt Extract Agar (MEA, malt extract 20 g L^{-1}, glucose 10 g L^{-1}, peptone 1 g L^{-1}, agar 15 g L^{-1}).

(iii) **Oil flotation** (Vicente *et al.* 2008). Approximately 20 g from each sample were incubated at room temperature for 30 min in 100 mL sterile saline solution containing 200 U penicillin, 200 μg L^{-1} streptomycin, 200 μg L^{-1} chloramphenicol and 500 μg L^{-1} cycloheximide. Subsequently 20 mL sterile mineral oil were added, followed by vigorous 5 min shaking. The flasks were left to settle for 20 min. The oil-water interphase was carefully collected, inoculated onto Mycosel agar (Difco) and incubated for 4 weeks at 28 °C.

Physiology and morphology

Laccase activity was assayed for nine selected isolates (CRMP1196, CRMP1198, CRMP1216, CRMP1227, CRMP1262, CRMP1256, CMRP1258, CMRP1226, CMRP1259) according to Sun *et al.* (2012) using ABTS-agar medium containing 5 mM 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (Sigma Aldrich). Agar plugs (3 mm diam) obtained from the edge of growing mycelium of tested fungi were inoculated and incubated at 24 °C in the dark for 7 d. Positive reaction was indicated by the formation of a green halo around the colony.

Cardinal growth temperatures were determined on MEA plates incubated in the dark for 3 weeks at temperatures of