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Research Paper

Endophytic fungi from *Myrcia guianensis* at the Brazilian Amazon: Distribution and bioactivity

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Abstract

Beneficial interactions between plants and microorganisms have been investigated under different ecological, physiological, biochemical, and genetic aspects. However, the systematic exploration of biomolecules with potential for biotechnological products from this interaction still is relatively scarce. Therefore, this study aimed the evaluation of the diversity and antimicrobial activity of the endophytic fungi obtained from roots, stems and leafs of *Myrcia guianensis* (Myrtaceae) from the Brazilian Amazon. 156 endophytic fungi were isolated and above 80% were identified by morphological examination as belonging to the genera *Pestalotiopsis*, *Phomopsis*, *Aspergillus*, *Xylaria*, *Nectria*, *Penicillium* and *Fusarium*. Fermented broth of those fungi were assayed for antimicrobial activity and four inhibited the growth of *Staphylococcus aureus, Enterococcus faecalis, Candida albicans* and *Penicillium avellaneum*. As the strain named MgRe2.2.3B (*Nectria haematococca*) had shown the most promising results against those pathogenic strains, its fermented broth was fractioned and only its two low polar fractions demonstrated to be active. Both fractions exhibited a minimum bactericidal concentration of 50 $\mu g.mL^{-1}$ against *S. aureus* and a minimum fungicidal concentration of 100 $\mu g.mL^{-1}$ against *P. avellaneum*. These results demonstrate the diversity of fungal genera in *M. guianensis* and the potential of these endophytic fungi for the production of new antibiotics.

Key words: secondary metabolites, fungus/plant interaction, antibiosis, Amazonian endophytic fungi.

Introduction

In the last two decades, the increasing number of scientific studies involving endophytic fungi has allowed the development of their concept. One of the most accepted concepts is that endophytic fungi are those that live asymptomatically in the apoplastic spaces or within the plant cells, at least during a significant part of their life cycle (Petrini, 1991). A more recent definition considers endophytes to be any cultured or uncultured organism that inhabits the interior of plant tissues and organs without

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causing damage to their host. These can be the type I, which includes those microorganisms that do not produce external structures in the plant, or type II, those that produce external structures in the plant (Miller *et.al*, 2010).

The great interest in endophytic microorganisms comes from the perception that they occupy the same ecological niches of plant pathogens and therefore have great potential for use in biological control. The strict relationship between endophytes and their hosts makes them natural candidates for use as agents of disease and insect control (Hallmann *et al.*, 1997; Miller *et.al*, 2010). Over the last years, other evidences have justified this interest emphasizing that endophytic fungi can be ecologically important to their host, sometimes giving them support, and other times being the protagonists in fundamental processes of plant survival (Artursson *et al.*, 2006; Yue *et al.*, 2000).

The diversity of endophytic fungi that colonizes different parts of the plants has promoted a wide variety of questions about the potential of these microorganisms as a consequence of the various types of environments, such as desert, tropical and arctic, in which these host species are present (Rosa *et al.*, 2009; Wali *et al.*, 2008). The biotechnological potential of endophytic fungi is emphasized by the amount of scientific investigations in this area, showing that these microorganisms can produce a very large number of compounds, many of which have biological activities of interest, such as phytohormones (Vidal and Jaber 2009), antimalarial, antiviral (Isaka *et al.*, 2007; Lehtonen *et al.*, 2006; Suryanarayanan *et al.*, 2009), antioxidant, antileishmanial (Cubilla-Rios *et al.*, 2008; Khidir *et al.*, 2010; Schulz and Boyle, 2005), cytotoxic (Bashyal *et al.*, 2005; Davis *et al.*, 2008), and antimicrobial (Aly *et al.*, 2008; Schulz *et al.*, 2002; Zhou *et al.*, 2008) compounds.

Thus, this type of research contributes to the elucidation of new fungal compounds that have potential applications in the pharmaceutical industry. Furthermore, due to the increase in poor soil and most adverse environment, Amazonian plants can host fungi that may also contribute to the development of agribusiness sectors, through the discovery of new compounds for development of cultivars and to combat of pests, such as bacteria and pathogenic fungi.

To this day, only a small number of studies on this theme, focusing on species belonging to the Myrtaceae family, was published, although this family has numerous species throughout Brazil, many of which are present in the Brazilian Amazon (Landrum and Kawasaki, 1997). The choice of the host plant species is important for the isolation of endophytes of interest from the standpoint of the antimicrobial activity. In this sense, the family Myrtaceae is noted for producing a variety of compounds, some with proven antimicrobial activity (Cruz, 1982). Hence, the aim of this study was to isolate and evaluate the antimicrobial activity of the endophytic fungi obtained from the roots, stems and leafs of *Myrcia guianensis*, a common plant species of the Brazilian Amazon, in order to contribute to the understanding of endophyte communities and their potential as new antibiotic sources.

Materials and Methods

Plant collection and isolation of the endophytic fungi

Three specimens of *M. guianensis* were collected in April 2009, at 35 m distant from each other, in an Amazonian savanna area, located in the São Pedro community, which belongs to the city of Santarém (Pará State), in the following coordinates: latitude 02°32'08.9" S and longitude 54°54'23.9" W, at an elevation of 19 meters relative to sea level. Their identifications were realized at INPA (National Institute of Amazon Research) Herbarium, and a voucher specimen was deposited under the registration number 181913.

Samples of roots, stems and leaves of the specimens were washed with autoclaved distilled water, and stored in sterile plastic bags at 6 °C. For the isolation process, the samples were washed with detergent under tap water, fragmented into 10x12 cm pieces, and subjected to a sequence of submersions in different solutions in the following order and time: (i) for the leaves, 70% alcohol for 1 min; sodium hypochlorite 3% for 2.5 min, 70% alcohol for 30 seconds, and sterile distilled water for 2 min; (ii) for the roots and stems, 70% alcohol for 1 min; sodium hypochlorite 4% for 3 min, 70% alcohol for 30 seconds, and sterile distilled water for 2 min (Souza *et al.*, 2004). In this specific situation, the water was plated and incubated at 26 °C as a control of sterilization procedure. Since the roots and stems fragments were divided into cortex and bark for subsequent inoculation, the endophytes were isolated from five parts of the plants: leaves (L), stem cortex (S), stem bark (Sb), root cortex (R) and root bark (Rb).

The plant material was cut into pieces of approximately 5x5 mm and inoculated in Petri dishes (2 plates with 9 fragments for each tissue) containing PDA medium added with chloramphenicol 50 $\mu g.mL^{-1}$. A total of 270 fragments were inoculated (45 fragments from each part of the plant of the three specimens, in duplicate) and incubated at 18 °C for 8 days, following by 4 days, at 26 °C. According to the cultivable endophytes that had been raised, they were transferred to test tubes containing inclined PDA medium. In order to identify the morphological characteristics of each isolate, they were inoculated individually into Petri dishes and analyzed.

The isolates were stored in duplicate into mineral oil for the anamorphic fungi (Castellani, 1939); into 2 mL microtubes containing 20% glycerol; and in Petri dishes containing PDA medium, in duplicate, for sterile mycelia and Ascomycetes (telemorphic fungi). All isolated endophytic fungi were deposited into the Collection of the Laboratory of the School of Health Sciences, State University of Amazonas (ESA/UEA).

Diversity analysis

For the analyze of the diversity, the isolates were grouped according to their macro and micromorphological characteristics. They were identified by their macroscopic vegetative characteristics, which were color, texture, topography, diffuse pigmentation, color, and topography of the back of the colony, and well as by their microscopic reproductive structures, using the microculture technique and comparing the obtained results with taxonomic keys (Barnett and Hunter, 1972; Hanlin, 1996).

The colonization rates (CR) of the fungi isolated were achieved by the ratio between the total number of isolates and the number of fragments within the sample, and the relative frequency (RF) was based on the ratio of the total number of isolates of a group and the total number of isolates. The microhabitats occupied by the isolated fungi (Azevedo, 1999) were also evaluated. For the statistical analysis of colonization rates, the Tukey test with $p \geq 0.05$ was used.

Fungal metabolites production

For the fungal metabolic production, 20% of the isolates from each morphological group were selected. These strains were inoculated in triplicate into 50 mL Erlenmeyer flasks containing 10 mL of potato dextrose liquid medium (PD) added with 0.2% of yeast extract under sterile conditions (Souza *et al.*, 2004). The flasks were incubated into a shaker during 8 to 11 days, according to the growth of each group, at 26 °C and 120 rpm. As negative control, flasks containing only culture media were incubated under the same conditions. After the cultivation period, the crude fermentation broth was separated from the mycelium by vacuum filtration and sterilized by filtration through a 22 μm Millipore membrane. The crude fermentation broth was stored at 4 °C.

The fungal strain that had presented the most promising results in the antimicrobial activity tests was grown for a preparative scale, in order to obtain a sufficient amount of the crude fermentation broth that would ensure the assessment of the bioactive compound. At this stage, 75 Erlenmeyer flasks (capacity 250 mL) containing 100 mL of PD liquid medium were used. The methodology to obtain the metabolic medium was the same described above.

Fungal metabolites extraction and fractioning

The mycelium was extracted three times with ethanol, and after filtration the ethanolic mixture was concentrated under vacuum, resulting in the mycelial extract (ME). The crude fermentation broth was partitioned with ethyl acetate, and the organic extract was concentrated under reduced pressure, resulting in the fermentation broth extract (FBE). The dried extracts (ME and FBE) were weighed and stored at 4 °C. After the assessment of antimicrobial activity, the extract that showed the most promising results was fractionated in an open column with normal phase silica (70-230 mesh) in a gradient mode. The mobile phases were: dichloromethane:ethyl acetate 1:1 (FA1), ethyl acetate 100% (FA2); ethyl acetate:acetone 1:1 (FA3), acetone 100% (FA4), 100% methanol (FA5) and methanol:water 8:2 (FA6).

Preliminary characterization of fungal metabolites

Chemical prospecting in ME and FBE extracts were performed to observe the presence of four groups of compounds already found as having antimicrobial activity, which can be present in the secondary metabolism of endophytic fungi (Yu *et al.*, 2010). Phenols and tannis (reaction with $FeCl_3$), alkaloids (Hager, Mayer and Dragendorff reagents) and quinones (reaction with NH_4OH) were evaluated according to the method proposed by Matos (2009).

Qualitative analysis of the antimicrobial activity

The evaluation of the antimicrobial activity of the crude fermentation broth was performed by the agar diffusion method (Souza *et al.*, 2004). The fungal metabolites were tested against standard strains obtained from the Collection of Amazon Bacteria (CBAM) and from the Collection of Amazon Fungi (CFAM) of the Oswaldo Cruz Foundation, Manaus, Amazonas (FIOCRUZ-AM). The strains used were *Staphylococcus aureus* (CBAM-026), *Bacillus cereus* (CBAM-289), *Enterococcus faecalis* (CBAM-309) *Pseudomonas aeuriginosa* (CBAM-024) and the fungal strains of *Candida albicans* (CFAM-1132) and *Penicillium avellaneum.* The fungus *P. avellaneum* is an species which has been used by many researchers as a target of anticancer and antifungal compounds (Floss *et al.*, 2004; Hanka and Barnett 1974; Kittakoop *et al.*, 2009).

Cell suspensions were prepared using the same media the microorganisms were inoculated in: Müller-Hinton for bacteria and Sabouraud for the fungi strains. For the preparation of the inoculum suspension, a concentration of 10^6 cfu for bacteria, and 10^3 cfu for fungi (fungal spore suspension) were standardized. An amount of 100 μL from the cell suspension was applied and spread using a Drigalski spatula through a Petri dish (20x150 mm) containing specific solid medium: brain heart infusion (BHI) for bacteria and Sabouraud for fungi. After that, 26 wells with 5 mm in diameter were placed on the culture medium of each plate and filled with 100 μL of the crude fermentation broth. All plates were incubated under aerobic conditions for 24 and 72 h, at 28 °C for fungi and 37 °C for bacteria. After that, the inhibition zones were measured. As positive control, antibiotics were used: amoxicillin for bacteria, and ketoconazole for fungi, both at 2.0 $mg.mL^{-1}$. As the negative control, only the crude fermentation broth was used.

Antimicrobial activities of extracts and fractions

Extracts (ME and FBE) and fractions (FA1-FA6) were tested against the same pathogenic strains as previ-

ously described. On the case of positive results, new assays were done in order to determine the Minimum Inhibitory Concentration (MIC), in accordance with the predetermination of the National Committee for Clinical Laboratory Standards (NCCLS), and using the broth microdilution method, according to Cos and coworkers (2006). For these tests, the extracts and fractions were dissolved to 2.0 $mg.mL^{-1}$ in DMSO:water (1:9), and 100 µL of the solution were applied in ELISA plates in the serial dilution mode. In each well, 20 µL of the pathogenic organism suspensions were inoculated. All tests were performed in triplicate. The plates were incubated at 32 °C and after 24 and 48 h the inhibition zone was measured in mm.

Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) tests were also performed. Aliquots of the positive results observed for MIC tests were inoculated into Petri dishes containing appropriated culture media (Cos *et al.*, 2006). The plates were incubated at 37 °C for 24-48 h. The MBC or MFC was considered the lowest concentration of the fraction in which was observed no cell growth on the surface of the inoculated culture medium.

Identification of the most promising strain

The identification of the most promising strain was confirmed by sequencing of the fungus ITS-1 and ITS-2 rDNA and compared with sequences from the GenBank. The strain was stirred for 6 days (120 rpm) on potato-dextrose (PD) medium at room temperature. From the mycelium separated by filtration, the genomic DNA was extracted by the CTAB method (White *et al.*, 1990) as adaptation of Souza and coworkers (2008).

Results

Isolation of endophytic fungi

From the 270 inoculated fragments of *M. guianensis*, 156 endophytic fungi were isolated. Of these, 53 were isolated from the specimen 1, 54 from the specimen 2 and 49 from the specimen 3 (Table 1). Considering the parts of the plants, the larger numbers of isolates were obtained from stem barks, 50 (32.1%) and leaves, 47 (30.1%). Root cortices provided the lowest number, with five fungi, totaling 3.2% of the isolates (Table 1).

Endophytic fungi diversity

All isolated fungi were assembled in 14 morphogroups, seven of known and seven of unknown genera. The genera identified and their relative frequencies were: *Pestalotiopsis* (33.3%), *Phomopsis* (25.0%), *Aspergillus* (11.5%), *Xylaria* (5.1%), *Penicillium* (2.5%), *Nectria* (1.9%), *Fusarium* (0.6%) and *Guignardia* (0.6%). The relative frequencies of unidentified groups were: Unidentified group 5 (5.1%), Unidentified group 6 (6.4%), Unidentified group 7 (2.5%), Unidentified group 8 (1.9%), Unidentified group 10 (1.9%) and Unidentified group 11 (1.2%) (Table 2).

For strain MgRe2 3.3 that showed the best results in the antimicrobial assays it was obtained DNA sequences, with 539 base pairs, which were compared with the NCBI database. The pairwise comparisons revealed trustfully identity with 99% of *Nectria haematococca*. This result was confronted with the morphological analyzes, and confirmed using specialized bibliography (Grafenhan *et al.*, 2011; Hanlin, 1996).

Antimicrobial activity

From the crude fermentation broth of the 46 endophytic fungi selected for the tests against the pathogenic strains, three were positive against *S. aureus*, and *E. faecalis*, one against *C. albicans* and and two against *P. avellaneum*. None of the isolated fungi was active against *P. aeruginosa B. cereus* (Table 3). 42 did not show any activity against the pathogens tested.

Nectria haematococca: Extracts, fractions, MIC and MBC

Antibiosis sensitivity tests performed with the mycelia - ME (0.76 g) - and with the fermentation broth extracts - FBE (3.58 g) - of this strain confirmed the presence of bioactive substances with inhibitory effect against *S.*

Table 1 - Total of fungi isolated and colonization rates in each tissue of *Myrcia guianensis.*

Plant parts[a]	Specimen 1		Specimen 2		Specimen 3		Averages	
	n° of isolates	CR	n° of isolates	CR	n° of isolates	CR	n° of isolates	CR
L	15	0.166	19	0.211	13	0.144	15.66	0.173[a]
R	_	_	3	0.033	2	0.022	1.66	0.018
Rb	10	0.111	9	0.100	11	0.122	10.00	0.111[b]
S	11	0.122	7	0.077	6	0.066	8.00	0.088[b]
Sb	17	0.188	16	0.177	17	0.188	16.66	0.184[a]
Total	53	0.196	54	0.200	49	0.181	52.00	0.192[a]

[a]For the isolation it was used a total of 90 fragments from each specimen of *M. guianensis*. L – Leaves; R – Root Cortex; Rb – Root Bark; S – Stem Cortex; Sb – Stem Bark; CR – colonization rate. Averages with a same letter indicate no significant difference according to the Tukey test ($p < 0.05$).

Table 2 - Morphological groups and relative frequencies of endophytic fungi isolated from *Myrcia guianensis.*

Morphogroup	Specimen 1				Specimen 2				Specimen 3				Total	RF (%)
	L	R	S	T	L	R	S	T	L	R	S	T		
Pestalotiopsis	6	4	11	21	1	2	5	8	8	6	9	23	52	33.3
Phomopsis	3	6	7	16	5	4	6	15	2	2	4	8	39	25.0
Aspergillus	2	1	2	5	2	1	3	6	–	1	6	7	18	11.5
Xylaria	1	–	1	2	1	–	3	4	1	1	–	2	8	5.1
Unknown 6	1	–	2	3	1	–	1	2	2	–	1	3	8	5.1
Unknown 7	–	–	–	–	4	–	4	8	1	–	1	2	10	6.4
Unknown 8	–	–	–	–	1	1	2	4	–	–	–	–	4	2.5
Unknown 9	1	–	–	1	1	–	1	2	–	–	–	–	3	1.9
Penicillium	–	–	1	1	–	–	2	2	–	–	1	1	4	2.5
Unknown 10	2	–	–	2	–	1	–	1	–	–	–	–	3	1.9
Unknown 11	–	–	–	–	–	1	–	1	–	1	–	1	2	1.2
Nectria	–	–	1	1	–	–	1	1	–	–	1	1	3	1.9
Fusarium	–	1	–	1	–	–	–	–	–	–	–	–	1	0.6
Guignardia	–	–	–	–	–	–	–	–	1	–	–	1	1	0.6
Total	16	12	24	53	16	10	28	54	15	11	24	49	156	53.3

L – leaves; R – root; S – stem; T – total of each specimen.

Table 3 - Antibiosis results of the crude fermentation broth from endophytic fungi isolated from of *M. guianensis* which presented some activity against the pathogenic strains.

Endophytic fungi	Genera	Tested microorganisms					
Strains		Pa	Sa	Ef	Bc	Ca	Pv
MgF2.1.2	*Pestalotiopsis*	–	++	++	–	–	–
MgF1.2.1	*Phomopsis*	–	++	++	–	–	–
MgR2.1.1	Unknown 10	–	–	–	–	++	++
MgRe2.2.3B	*Nectria*	–	++	++	–	–	+++
Negative control		–	–	–	–	–	–
amoxicilin		+++	++	+++	+++	NT	NT
ketoconazole		NT	NT	NT	NT	+++	+++

Pa - *Pseudomonas auriginosa*; Sa - *Staphylococcus aureus*; Ef - *Enterococcus faecalis*; Bc - *Bacillus cereus*; Ca - *Candida albicans*; Pv - *Penicillium avellaneum*; NT – Not tested.
+ Inhibition zone between 10 and 15 mm; + + Inhibition zone between 15 and 30 mm; + + + Inhibition zone between 30 and 45 mm.

aureus, *E. faecalis* and *P. avellaneum* only in the FBE. Negative results were observed for ME (Table 4). The fractions FA1 (0.35 g), FA2 (0.52 g), FA3 (0.11 g), FA4 (0.09 g), FA5 (1.32 g) and FA6 (0.19 g) obtained from the chromatographic fractioning of FBE were tested against the same pathogenic strains. FA1 and FA2 showed positive result against the tested strains (Table 4).

Preliminary characterization of fungal metabolites

Qualitative analysis of the compounds present in the ME and FBE showed the presence of phenols in both extracts. The presence of tannins was found only in the ME, and the presence of quinones was observed only in the FBE. It was not observed the presence of alkaloids in any of the extracts.

Discussion

Isolation of endophytic fungi

The results showed that *M. guianensis* is a good source of endophytic fungi, since only one type of culture medium was used for the isolation process, and this unique method allowed the acquirement of a considerable number of endophytes (totaling 57.7% of the inoculated fragments). No microorganism had appeared from the last washing water, so the surface disinfection method was considered efficient.

The three specimens of *M. guianensis* presented similar relative distribution of isolated fungi among all parts of the plant. While someone could expect the higher numbers of isolates in the leaves and stem barks (Table 1), a smaller

Table 4 - Antibiosis activities of the extracts and fractions from the fermentation broth of the fungus *Nectria haematococca* cultivated in preparative scale.

Tested strains	Extracts		Fractions			Control			FA1		FA2	
	ME	FBE	FA1	FA2	FA3-6	CON	AMO	KET	MIC ($\mu g.mL^{-1}$)	MBC ($\mu g.mL^{-1}$)	MIC ($\mu g.mL^{-1}$)	MBC ($\mu g.mL^{-1}$)
S. aureus	–	23.3 ± 1.1	12.3 ± 0.5	12.3 ± 0.5	–	–	28.1 ± 0.28	NT	≥ 25	≥ 50	≥ 25	≥ 50
E. faecalis	–	24.3 ± 2.0	11.3 ± 0.5	11.6 ± 0.5	–	–	46.0 ± 1.00	NT	≥ 50	–	≥ 50	–
P. avellaneum	–	22.3 ± 2.0	23.3 ± 1.1	21.3 ± 0.5	–	–	NT	43.3 ± 0.6	≥ 12.5	≥ 100	≥ 12.5	≥ 100

ME – Ethanolic extract from the mycelium; FBE – ethyl acetate extract from the fermentation broth; FA1 – fraction from FBE obtained in dichloromethane:ethyl acetate (1:1); FA2 – fraction from FBE obtained in ethyl acetate 100%; FA3 – fraction from FBE obtained in ethyl acetate: acetone (1:1); FA4 – fraction from FBE obtained in acetone 100%; FA5 – fraction from FBE obtained in methanol 100%; FA6 – fraction from FBE obtained in methanol:water (8:2); AMO – Amoxicilin 2.0 $mg.mL^{-1}$; KET – ketoconazole 2.0 $mg.mL^{-1}$; CON – negative control DMSO: water (1:9); MIC – minimum inhibitory concentration; MBC – minimum bactericidal concentration; NT – not tested.

amount of fungi in the roots was somewhat unexpected, since as this tissue is in contact with the ground, which is a source of numerous microorganisms. However, several works have presented the leaves and stems as main parts where endophytes are found (Gazis and Chaverri, 2010; Suryanarayanan *et al.*, 2009), which may be related to how the endophyte penetrates the plant (by its aerial interactions), and also to the favorable conditions of certain tissues for the fungus protection (Ahlholm, 2002; Brand and Gow, 2009). On the other hand, the similarity among the results for *M. guianensis* specimens suggests that the isolation process is expressing the real number of cultivable endophytic fungi present in the healthy tissues. Besides, almost every isolated genus presented a relative frequency greater than 1, except *Guignardia*, known as an orange's pathogen (Spósito *et al.*, 2011) and *Fusarium*, the agent that causes the addlement of the roots (Yu *et al.*, 2004), both cases recognized as a systemic colonization (Table 2).

In a study performed by Pinto (2011), in the isolation of endophytic fungi from leaves and stems of *M. sellowiana*, a lower frequency of isolates (12.7%) was observed when compared to the findings presented here (53.3%), as the treatment leaves in the disinfection step was the same surface, and the medium used for isolation also (BDA), the discrepancy in results may be related to seasonality of these microorganisms in the host, considering that the collection of plant material for the isolation occurred at different times in the year 2009, during the rainy season and the other during the dry season (Pinto, 2011).

The statistical analyses of the averages of the fungi isolated in each tissue showed that there is no significant difference ($p \leq 0.05$) between the number of isolates from the stem barks (Sb) and from the leaves (L). The same was observed for the number of isolates from the root barks (Rb) and from the stem cortices (S). On the other hand, there is a notable difference between the averages of colonization rate (CR) in the roots cortices (0.018), and in the stem barks (0.184). Clearly, for the conditions applied, the amount of cultivable isolates in the stem barks is ten times greater than in the root cortices, showing the importance of the plant fragmentation for the fungal isolation. Since many fungal species are not cultivable, in order to analyze the efficiency of the endophyte isolation, it is necessary to remember that these numbers just express the frequency of cultivable endophytes under determined isolation conditions, such as superficial disinfection, culture medium and growth temperature.

Endophytic fungal diversity

It was found a greater diversity of genera in the specimen 2, than in 1 and 3. In contrast, specimens 1 and 3 presented more isolates of the genus *Pestalotiopsis* (Table 2). Some of the most frequent isolates of *M. guianensis*, as *Pestalotiopsis* and *Phomopsis* are known as endophytes of other plant species from tropical climate, and some have

demonstrated significant potential for the production of useful biotechnological compounds, such as isopestacin isolated from *P. microspora*. This compound showed antifungal activity against *Pythium ultimum*, an oomycete that causes the addlement of the roots in plants of agricultural importance (Strobel *et al.*, 2002).

Another example is the lactones extracted from the crude fermentation broth of *Phomopsis* sp., an endophytic fungus from *Azadirachta indica*. A recent work it was described the antifungal action of these lactones against the important plant pathogen, *Ophiostoma minus*, with a minimum inhibitory concentration (MIC) of 31.25 $\mu g.mL^{-1}$ (Wu *et al.*, 2008).

Regarding the diversity of endophytes, it is important to note that the identified groups at the genus level were also found in other studies concerning tropical plants from the Amazon region (Hanada *et al.*, 2010; Souza *et al.*, 2008). This is the case of the genera *Pestalotiopsis*, *Phomopsis*, *Aspergillus*, *Penicillium* and *Xylaria*, which demonstrates the versatility of these endophytic microorganisms in colonize diverse host species. In this sense, it is important to consider that frequencies under 1% indicate genera not common in the plant's tissues. In some cases, those indicate that these fungi may not be a natural endophyte, but an epiphyte or even a plant pathogen trying to colonize the plant tissue transitorily. In this work, it was possible to observe the presence of two plant's pathogen genera, *Fusarium* and *Guignardia* (Spósito *et al.*, 2011; Yu, *et al.*, 2004), both found at a frequency of 0.6%, indicating its momentarily colonization.

Plants of *Myrcia* genus appear as a promising source of endophytic microorganisms, which produce metabolites with proven antimicrobial activity. Pinto (2011) evaluated the antimicrobial activity of extracts from endophytic fungi isolated from *M. sellowiana*, and found 62 extracts active against *S. aureus*, 20 against *E. coli* and 11 against both pathogens (Pinto, 2011). In addition to that, the author found 35 endophytic fungi strains that produced metabolites which are active against the pathogenic fungus *Colletotrichum gloeosporioides*. These findings justify the investigation among this vegetal genus as a source of antimicrobial producing endophytic fungi and corroborate the results presented in this work.

In this work it was also possible to verify the isolation of a fungi from the genus *Nectria*, a known producer of pigments such as anthraquinone and naphthoquinone (Barbier *et al.*, 1988; Barbier, *et al.*, 1990). The genus *Nectria* had not been previously described as endophytic fungi from Amazon hosts, or as a producer of antimicrobial compounds. Hence, the results obtained here demonstrate the limited knowledge of fungal species that comprise the Amazon ecosystem.

Antimicrobial activity

Even though only some of the isolated endophytes have been used in the analyses of the antimicrobial activity, which reduced the possibility of finding strains that produce bioactive compounds, and considering that they have been cultivated in only one culture medium, which cannot be suitable for gene expression that led to the production of some bioactive metabolites, it was gratifying that the strain MgRe2.2.3B, belonging to the genus *Nectria*, had shown activity against *S. aureus*, *E. faecalis* and *P. avellaneum*, a signal of its great potential as a source of antibacterial and antifungal compounds. It should be pointed out that the result of the antibiosis test of the crude fermented broth of this fungus against *P. avellaneum* was similar to the positive control ketoconazole. This fact supported the selection of this strain for subsequent assays, as well as for its molecular identification.

Extracts, fractions, MIC and MBC

The test results indicate that the active compounds are among the substances present in the fractions 1 and 2. A MIC of 25 $\mu g.mL^{-1}$ was obtained for FA1 and FA2 against *S. aureus* and of 12 $\mu g.mL^{-1}$ against *P. avellaneum*. Against *E. faecalis* the obtained MIC was 50 $\mu g.mL^{-1}$ for fraction 1 and 100 $\mu g.mL^{-1}$ for fraction 2 (Table 4). The minimum bactericidal concentration (MBC) results were found at 50 $\mu g.mL^{-1}$ for both FA1 and FA2 against *S. aureus*. The fractions did not present bactericidal capacity against *E. faecalis* in any of the tested concentrations. FA1 and FA2 showed fungicidal ability against *P. avellaneum*, but this capacity is only observed at a dose of 100 $\mu g.mL^{-1}$. Although the most promising results for the antimicrobial activity were obtained against the *P. avellaneum* strain, different results were observed during the fungicidal tests. These findings demonstrate the importance of such tests, since this evaluation confirms or denies the effective action of the investigated compound against pathogenic microorganisms.

Preliminary characterization of fungal metabolites

The production of phenols and quinones by endophytic fungi and antimicrobial activity of these compounds are fairly known. In a study carried out by Li and coworkers (2008), two new antimicrobial substances (pestalachloride A and B) were isolated, which are phenolic compounds produced by the endophytic fungus *Pestalotiopsis adusta* (Li *et al.*, 2008). In another work, it was observed the production of quinones by *Ampelomyces* sp., an endophytic fungus isolated from *Urospermum picroides*, with significant activity against the pathogenic bacteria *S. aureus*, *S. epidermidis* and *E. faecalis* (Aly *et al.*, 2008).

Antimicrobial activity of compounds produced by *Nectria haematococca* has not yet been described, but it is already known that this genus is a producer of a variety of

quinones, a class of compounds that could be identified in the *N. haematococca* extract using a qualitative assay. Such information expands the possibilities of research about this genus and its secondary metabolites, in this case resulting in a biotechnological perspective toward obtaining new antibiotics.

Final Remarks

The Amazonian plant *M. guianensis* proved to be a valuable source of endophytic fungi, both in terms of microorganisms number as in diversity, being detected at least 14 distinct morphogroups. It was possible to identify some known genera already described as producers of compounds with biotechnological interest. A fungus from the genus *Nectria,* which was not previously identified in the Amazon region, was also described in this work. The results of the antimicrobial tests demonstrate the production of bioactive compounds from the isolated microorganisms, both antifungal and antibacterial in some endophytic strains. The bioguided fractioning of an active extract from the fungus *Nectria haematococca* yielded positive results in the fractions of the crude fermentation broth which present quinones and phenols as chemical constituents.

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