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Antibacterial potential of endophytic fungi of the Amazon medicinal plant mulateiro *Calycophyllum spruceanum* (Benth.) Hook. f. ex K. Schum.

Aline Barreto dos Santos Coradin[1], Gleison Rafael Queiroz Mendonça[1], Leila Priscila Peters[2] and Clarice Maia Carvalho[1,3*]

[1]Programa de Pós-graduação em Ciência, Inovação e Tecnologia para a Amazônia, Universidade Federal do Acre, Rodovia BR-364, Km 04, Distrito Industrial, 69920-900, Rio Branco, Acre, Brasil. [2]Centro de Ciências da Saúde e do Desporto, Universidade Federal do Acre, Rio Branco, Acre, Brasil. [3]Centro de Ciências Biológicas e da Natureza, Universidade Federal do Acre, Rio Branco, Acre, Brasil. *Author for correspondence. E-mail: clarice.carvalho@ufac.br

ABSTRACT. *Calycophyllum spruceanum*, known as mulateiro, is an Amazonian medicinal plant traditionally used to treat skin infections, scars, and gastrointestinal and uterine conditions. Some of its therapeutic properties are attributed to bioactive compounds produced in association with endophytic fungi. This study aimed to evaluate the antibacterial potential of endophytic fungi isolated from *C. spruceanum*. Leaves and stems underwent surface sterilization and were cultured on four types of media: PDA, PDA supplemented with plant extract, SDA, and SDA supplemented with plant extract, incubated at 18 °C and 28 °C. The isolated fungi were classified into morphospecies using classical taxonomy. Crude extracts of fungal metabolites were tested for antibacterial activity against Gram-positive and Gram-negative bacteria through the disk diffusion assay. In total, 650 fungal isolates were obtained, 486 filamentous fungi and 164 yeasts, grouped into 248 morphospecies. The most frequently isolated genus was *Phomopsis* (30.9%), followed by *Colletotrichum* (16.7%) and *Guignardia* (5.1%). A higher number of fungi were recovered from leaves (60.3%) compared to stems (39.7%). Among the isolates, 23 demonstrated antibacterial activity against *Escherichia coli*, and one isolate exhibited inhibitory activity against *Klebsiella pneumoniae*, both Gram-negative bacteria of clinical relevance. This is the first report describing the endophytic fungal community associated with *C. spruceanum*, highlighting its potential as a source of novel antibacterial compounds, particularly effective against Gram-negative pathogens. These findings contribute to the bioprospecting of Amazonian biodiversity and open perspectives for the development of natural products with pharmaceutical applications.

Keywords: endophytic fungi; Amazon biodiversity; Antibacterial activity; Natural products; *Phomopsis*; *Colletotrichum*.

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Introduction

The Amazon Forest has about 50% of the planet's biodiversity, and almost 70% of the forest area is located in the Brazilian Amazon. Recognized as an essential biological center of global diversity, Brazil is considered one of the most biodiverse countries on the planet, with about one-third of the world's rainforests (Pylro et al., 2014). However, few studies have been conducted with plants of this biome.

Regarding the microorganisms of this biome, the knowledge and use of this biodiversity is even rarer. Among the species of microorganisms that can be studied are endophytic microorganisms. They are fungi and bacteria that live inside plants and have aroused scientific interest, being an important source of bioactive products (Hardoim et al., 2015; Gouda et al., 2016). They are capable of producing enzymes (Ahmed et al., 2016; Krishnapura & Belur, 2016; Toghueo et al., 2017), antitumor (Kathiravan & Raman, 2010; Pandi et al., 2010; Somjaipeng et al., 2016; Kasaei et al., 2017) and antimicrobial compounds (Vieira et al., 2014; Adewale et al., 2015; Jin et al., 2017; Monteiro et al., 2017).

An example of the strong interaction between plants and endophytes is the antitumor taxol, a metabolite produced by the endophytic fungus *Taxomyces andreanae*, isolated from *Taxus brevifolia*. Both the plant and fungus produce the same active substance used in breast and uterine cancer treatments (Kathiravan & Raman, 2010; Somjaipeng et al., 2016; Kasaei et al., 2017).

An Amazonian medicinal plant of interest for studies of biological activity is *Calycophyllum spruceanum* (Benth.) Hook. f. ex K. Schum., of the Rubiaceae family, popularly known as mulateiro, pau-mulato and

slippery by the Brazilian riverside population (Record & Hess, 1943; Araújo et al., 2016; Baldin et al., 2016). Native to the Amazon region, it is often found in Brazil, Colombia, Ecuador, Peru and Bolivia (Rizzini, 1978; Ugarte-Guerra & Domínguez-Torrejón, 2010).

Different tissues of *C. spruceanum* are used in folk medicine to treat skin, oral mucosa and eye infections (Costa et al., 2011), topical treatment of cellulite, diabetes, parasite control (Revilla, 2001), skin blemishes, prevention of aging (Figueiredo-Filho et al., 2016), control of hair loss, wrinkles, skin blemishes and scars (Santos et al., 2016).

Metabolites produced by *C. spruceanum* have been little studied. The presence of tannins and phenols has been reported to be responsible for the plant's action in delaying cellular aging and antioxidant photoprotection (Santos et al., 2016; Vargas et al., 2016) and secoiridoids with activity against the trypomastigote forms of *Trypanosoma cruzi* (Zuleta et al., 2003).

This is the first study on the antibacterial activity of endophytic fungi of the Amazonian medicinal plant *Calycophyllum spruceanum*.

Material and methods

Isolation of endophytic fungi

Stem and leaf samples of three individuals of *C. spruceanum* were collected at the Brazilian Agricultural Research Corporation (EMBRAPA-AC) campus (10° 02′ 2.143″ S and 67° 41′ 28.174″ W) between October 2014 and April 2015 (Figure 1). Plant samples were washed and disinfected with 70% alcohol for 1 minute, 2.5% sodium hypochlorite for 2 minutes, 70% alcohol for 30 seconds, and twice in sterile distilled water (Lima et al., 2011).

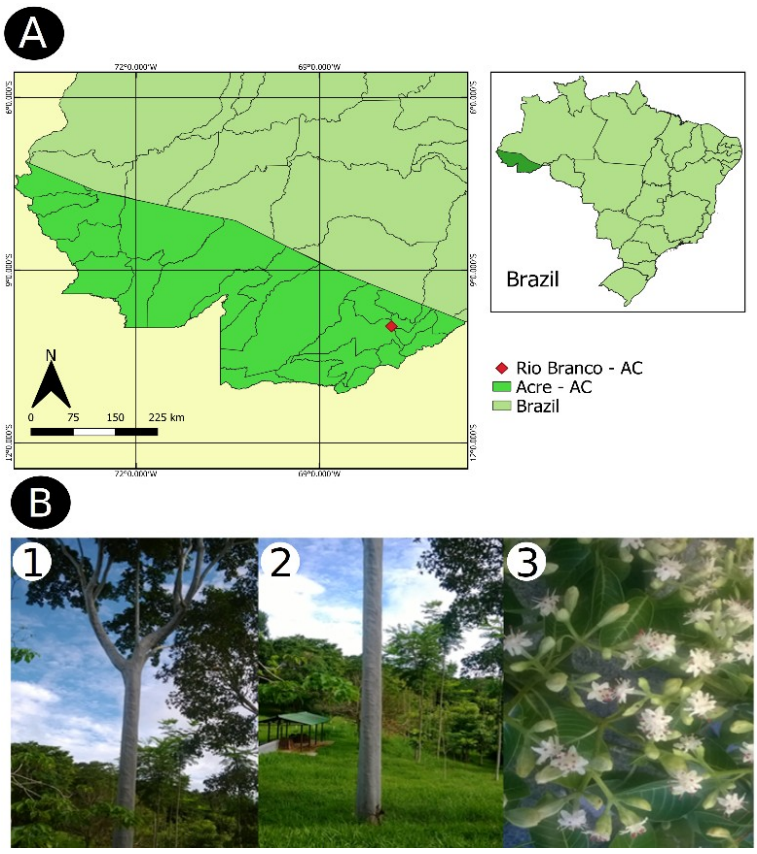

Figure 1. Study area. A. Location of plant material collection; B.1. *Calycophyllum spruceanum* plant; B.2. Stem; B.3. *C. spruceanum* flowering.

Plant samples were fragmented (5 mm) and inoculated into two types of culture media (Potato Dextrose Agar – PDA and Sabouraud Dextrose Agar – SDA). The effect of supplementing the culture medium with plant extract (10% v/v) was also evaluated. For producing the plant tissue extract, 100 g of fresh tissue were ground in 500 mL distilled water, filtered on filter paper, and 500 mL of an infusion of 200 g of potato were added to prepare PDA + extract medium or 500 mL distilled water for SDA + extract (Lima et al., 2011). The inoculated plates were incubated at 18 °C and 28 °C for up to 30 days. The fungi were purified in PDA culture medium,

organized into morphospecies according to the macromorphological characteristics of the colony and stored using mineral oil and distilled water methods (Buell & Weston, 1947; Castellani, 1963). Macro and micromorphological analyses, using the blade cultivation technique, were performed for identification, and then compared with the specific literature (Barnett; Hunter, 1999)

Molecular characterization

The two endophytic fungi with the best antibacterial activity were submitted to genomic deoxyribonucleic acid (DNA) extraction using the Quick-DNA Fungal/Bacterial miniprep kit (Zymo Research) following the manufacturer's instruction. The rDNA amplification of the ITS region was done in a 50 µL reaction mixture, which included 2 µL DNA template (1–20 ng), 0.4 µM of primers ITS1 (5′- TCCGTAGGTGAACCTGCGG -3′) and ITS4 (5′- TCCTCCGCTTATTGATATGC -3′), 1.5 mM $MgCl_2$, 0.2 µM dNTPs, 5 µL of Taq buffer, and 1.25 U Taq DNA polymerase (Qiagen). The PCR amplification was performed on a cycler PCR machine (Bio-Rad) with the initial denaturation at 95°C for 2 min, followed by 35 cycles of amplification (95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min) and an extension step (72 °C for 7 min) (White, 1990). The PCR products around 600 to 700 pb were purified using the QIAquick PCR Purification Kit (Qiagen) and quantified in 2% agarose gel. The purified PCR products were sent for sequencing.Of the fungi with the best antibacterial activity only 1 was successfully amplified using primers ITS1 and ITS4.

The obtained sequences were analyzed and edited in the Bioedit 7.0.9.1 (Hall, 1999) aligned with the region sequences (ITS1-5.8S-ITS4) using the ClustalW program, later deposited in the databases of GenBank, and calculating the species similarity index with the BLAST. After identifying the species of interest, a phylogenetic tree was built by the Neighbor-Joining method (Jukes & Cantor, 1969) using the software MEGA 7.0 (Arantes et al., 2016).

Antibacterial assay

One fungus of each morphospecies was inoculated in PDA culture medium and incubated at 28 °C for 14 days, being cultivated in Potato Dextrose broth (PD) for 14 days at 28 °C without agitation. 2 mL of medium containing fungal metabolites was extracted by liquid-liquid partition with ethyl acetate and solubilized in 300 µL of dimethylsulfoxide (DMSO) (Lima et al., 2011).

Antibacterial activity of fungal extracts was done by disk diffusion method against the pathogenic bacteria *Staphylococcus aureus* (ATCC 12598), *Streptococcus pneumoniae* (ATCC 11733), *Escherichia coli* (ATCC 10536), and *Klebsiella pneumoniae* (ATCC 700603) (NCCLS, 2003).

Pathogenic bacteria were cultivated at 37 °C for 4 to 6 hours, and their turbidity was adjusted to 0.5 on the McFarland scale. Bacteria were then inoculated in Petri dishes containing Muller-Hinton agar, deposited on paper discs, then 20 µL of fungal extracts, and incubated at 37 °C for 24 hours. Fungal extracts that did not allow bacterial growth around the disk were considered to have antibacterial activity. Tests were performed in triplicate, and the produced inhibition halos were measured in millimeters (NCCLS, 2003).

Data analysis

The infection index (FI) was calculated from the relationship between the number of fragments that emerged from endophytic fungi by the total number of fragments used in the experiment.

The frequency of colonization (FC) of the fungal genera was calculated using the formula FC = NCOL/Nt × 100, where NCOL is the number of segments colonized by each fungus and Nt is the total number of fragments. The relative frequency of isolation (RF) was calculated as the number of isolates of a species divided by the total number of isolates, expressed as a percentage.

The number of dominant species and the Simpson's and Shannon indices were calculated to analyze the diversity of the endophytic community of *C. spruceanum*. The formula for calculating the Simpson's diversity index = 1 − ∑ (pi) 2. Shannon-Wiener diversity (H′) = −∑ pi ln pi, where pi is the proportion of frequency of colonization of species in a sample. Species equivalence (E) was calculated using the formula E = H/ln S, where S is the number of species in the sample.

Principal Component Analysis (PCA) was conducted using Statistica 2.0 software to assess the impact of different culture media on the isolation of fungal endophytes.

The antibacterial activity data were examined through statistical analysis with SPSS statistical package version 22.0. Mean and standard deviation were analyzed and treatments compared to drug control using paired Student's t-test. The significance level in T-test is assumed at p-value < 0.05 (5%).

Results

Diversity of endophytic fungi from *Calycophyllum spruceanum*

Endophytic fungi were isolated from different *C. spruceanum* tissues (stem and leaf) under different growing conditions (PDA, PDA + extract, SDA and SDA + extract). A total of 650 endophytic fungi were obtained from 480 samples of *C. spruceanum*, with 92.1% IF, being isolated 486 filamentous fungi and 164 yeasts, which were analyzed only quantitatively since they were not included in the identification and antibacterial activity steps.

The distribution of endophytes varied across plant tissues. Among the 486 isolated endophytic fungi, leaf presented higher colonization, with 293 fungi (60.3%), than stem, with 193 fungi (39.7%) (Table 1).

Table 1. Number and relative frequency percentages (RF%) of fungal endophyte isolated from *Calycophyllum spruceanum* according to the plant tissue, culture medium and temperature.

Genus	Plant Tissue		Culture Medium						Temperature ºC		Total	RF (%)
	Leaf	Stem	PDA	PDA + leaf	PDA + stem	SDA	SDA + leaf	SDA + stem	18	28		
Phomopsis	98	52	49	17	15	36	20	14	70	80	150	30.9
Colletotrichum	69	12	21	16	6	27	8	2	40	41	81	16.7
Guignardia	24	1	3	9	-	2	10	1	19	6	25	5.1
Fusarium	5	15	12	1	2	4	-	1	15	5	20	4.1
Xylaria	3	16	8	1	3	3	-	4	16	3	19	3.9
Pestalotiopsis	5	11	7	-	2	3	-	4	10	6	16	3.3
Aspergillus	3	10	5	1	1	5	-	1	9	4	13	2.7
Penicillium	5	8	3	-	1	7	1	1	6	7	13	2.7
Curvularia	8	2	4	2	-	1	1	2	3	7	10	2.1
Beauveria	4	1	1	-	1	2	1	-	2	3	5	1.0
Cladosporium	2	3	1	-	2	-	1	1	5	-	5	1.0
Pyricularia	1	1	-	-	-	-	1	1	2	-	2	0.4
Trichoderma	1	1	-	1	-	1	-	-	-	2	2	0.4
Acremonium	-	1	-	-	-	1	-	-	-	1	1	0.2
Epicoccum	1	-	-	1	-	-	-	-	-	1	1	0.2
Unknown	64	59	38	15	14	32	17	7	70	53	123	25.3
Total	293	193	152	64	47	124	60	39	267	219	486	100.00
RF (%)	60.3	39.7	31.3	13.2	9.7	25.5	12.4	8.3	54.9	45.1		

RF = relative frequency; PDA = Potato Dextrose Agar; SDA = Sabouraud Dextrose Agar.

PDA culture medium provided the largest amount of isolates, with 152 (31.3%), followed by SDA medium, with 124 (25.5%). Among the media with extract, a more significant number of isolates were found in leaf extract media, with 124 (25.5%), when compared to the media with stem extract, with 86 (17.7%).

Temperature with the highest amount of isolated fungi was 18 °C in both analyzed tissues, with 267 isolates (54.9%), while 28 °C had 219 fungi (45.1%).

The 486 endophytic fungi were grouped into 248 morphospecies after analyzing their macroscopic characteristics, distributed in 16 genera and a group called unknown because it does not produce reproductive structure for identification.

The following genera were observed as dominant in *C. spruceanum*: *Phomopsis*, *Colletotrichum*, *Guignardia*, *Fusarium*, *Xylaria*, *Pestalotiopsis*, *Aspergillus*, *Penicillium*, *Curvularia*, and *Cladosporium*, while *Pyricularia*, *Trichoderma*, *Acremonium*, and *Epicoccum* were observed as accidental (Figure 2).

Some genera have specificity for tissue, culture medium, or temperature, showing the importance of using different nutritional and environmental sources for the isolation of microorganisms (Figure 3). *Epicoccum* was isolated only on leaf and *Acremonium* only on the stem. *Pyricularia* was isolated only on PDA + extract, *Acremonium* only in SDA, *Epicoccum* only in PDA + leaf extract, and *Pestalotiopsis* was not isolated in medium with leaf extract. *Cladosporium* and *Pyricularia* were isolated only at 18 °C and *Trichoderma*, *Acremonium* and *Epicoccum* only at 28°C.

Phomopsis was isolated on leaves and stem, all media, and at both temperatures, with 150 isolates (30.9%), behaving as a generalist and dominant in *C. spruceanum*. *Colletotrichum* obtained 81 fungi (16.7%) from the total of isolates, the second most frequent, being observed in both tissues in all culture media and temperatures.

The endophytic community associated with *C. spruceanum* was abundant and diverse. Among the 480 plant fragments, 650 isolates were obtained. All three individuals were colonized by endophytic fungi. The different tissues analyzed present differences in composition, richness, and diversity.

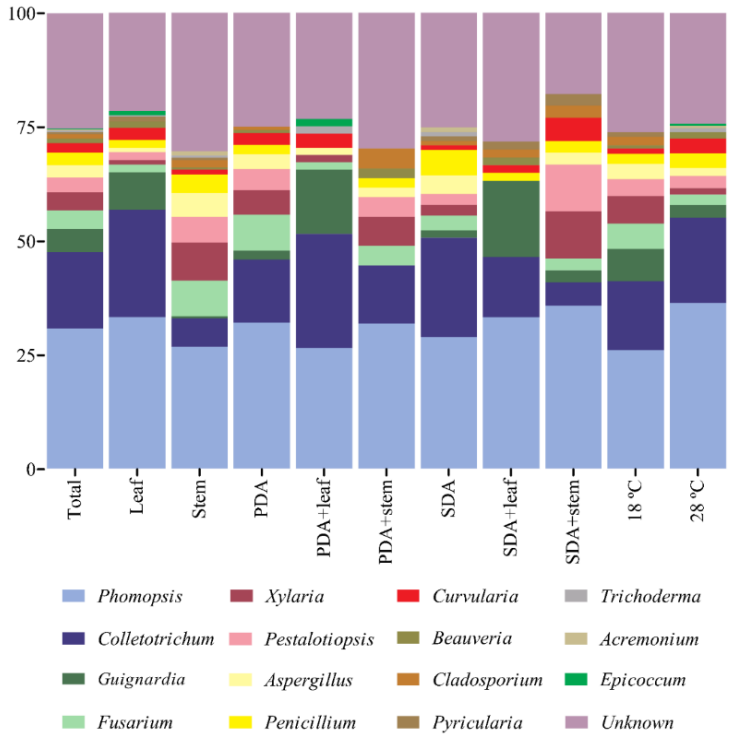

Figure 2. Frequency distribution of endophytic fungi from *Calycophyllum spruceanum* according to plant tissues, culture media, and growth temperatures.

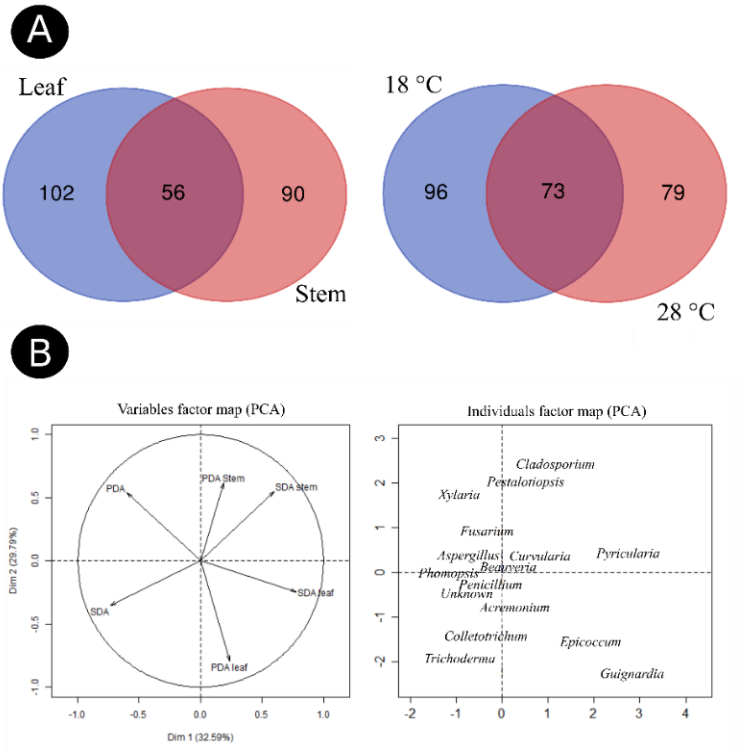

Figure 3. A. Venn diagram for plant tissue and temperature for fungal endophyte isolation from *Calycophyllum spruceanum*. B. Factorial maps for variables and individuals according to culture medium for isolation endophytic fungi genres from *Calycophyllum spruceanum*.

The diversity present between the identified genera in relation to the total isolated fungi is confirmed by the Shannon and Simpson's index and by the low uniformity index. Thus, the diversity analysis of *C. spruceanum* presented values of 5.3 for Shannon, 0.99 for Simpson's, and 0.86 for Evenness (Table 2).

On plant tissue, a higher abundance (293) and richness (158) of species was observed in leaf than stem. About diversity, the Simpson's index had no difference between tissues, but the Shannon index was higher on the stem than leaf, as well as Evenness.

The PDA medium presented higher richness (152), abundance (116), and diversity by the Shannon (4.67) and Simpson's (0.99) indices than the other culture media. The highest abundance and richness were observed at 18°C.

Table 2. Diversity indices of endophytic fungi from *Calycophyllum spruceanum* according to the plant tissue, culture medium and temperature.

	Abundance	Species Richness	Shannon's index (H')	Simpson's diversity (1-*D*)	Evenness
Tissue Type					
Leaf	293	158	4.82	0.99	0.85
Stem	193	146	4.88	0.99	0.93
Culture Medium					
PDA	152	116	4.67	0.99	0.93
PDA + Leaf	64	50	3.77	0.97	0.91
PDA + Stem	47	39	3.60	0.97	0.94
SDA	124	92	4.37	0.98	0.91
SDA + Leaf	60	47	3.73	0.97	0.91
SDA + Stem	39	33	3.45	0.97	0.94
Temperature					
18° C	267	169	5.00	0.99	0.89
28° C	219	152	4.85	0.99	0.90
Total	486	248	5.3	0.99	0.86

Antibacterial activity of metabolite extracts from endophytic fungi isolated from *Calycophyllum spruceanum*

Of the 243 metabolite extracts of endophytic fungi from *C. spruceanum*, none showed activity against the gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pneumoniae*. However, activity was observed against gram-negative bacteria, one with activity against *Klebsiella pneumoniae*, *Xylaria* genus, and 22 extracts produced inhibition halos against *Escherichia coli* (Table 3). The fungus of the genus *Phomopsis* showed the highest zone of inhibition against *E. coli*, and based on comparative analyses of the ITS rDNA gene, isolates 2.3131 and 2.3162 is phylogenetically related to *Phomopsis* sp. (Figure 4) with GenBank accession number OQ540869 and OQ540870, respectively.

Table 3. *In vitro* antibacterial activity of metabolite extracts of endophytic fungi from *Calycophyllum spruceanum*.

Pathogenic Bacteria	Endophyte	Zone Inhibition (mm ± SD)
Escherichia coli		
	Phomopsis (2.3162)	26 ± 2.2
	Phomopsis (2.3131)	24 ± 2.3
	Phomopsis (2.2408)	20 ± 1.9*
	Phomopsis (2.3268)	20 ± 1.3*
	Phomopsis (2.3495)	19 ± 1.3*
	Phomopsis (2.2444)	19 ± 1.2*
	Phomopsis (2.2455)	19 ± 1.5*
	Phomopsis (2.3275)	17 ± 3.2*
	Phomopsis (2.3365)	19 ± 0.9*
	Xylaria (2.2802)	10 ± 0.5*
	Xylaria (2.3061)	10 ± 1.8*
	Xylaria (2.3167)	10 ± 0.9*
	Beauveria (2.3119)	11 ± 1.3*
	Cladosporium (2.3241)	11 ± 1.2*
	Fusarium (2.1599)	10 ± 0.5*
	Guignardia (2.3245)	10 ± 0.2*
	Penicillium (2.2426)	10 ± 0.4*
	Unknown (2.2481)	10 ± 1.3*
	Unknown (2.2556)	10 ± 1.3*
	Unknown (2.2743)	11 ± 2.4*
	Unknown (2.2761)	10 ± 2.2*
	Unknown (2.3098)	11 ± 1.6*
Control	Chloramphenicol	26 ± 0
Klebsiella pneumoniae		
	Xylaria (2.2800)	10 ± 0.8*
Control	Chloramphenicol	13.7 ± 0.5

*Significant differences ($p < 0.05$) using Student's t test; Antibacterial activity: low (halo 8-12 mm), medium (halo 13-19 mm) and high (halo >19 mm).

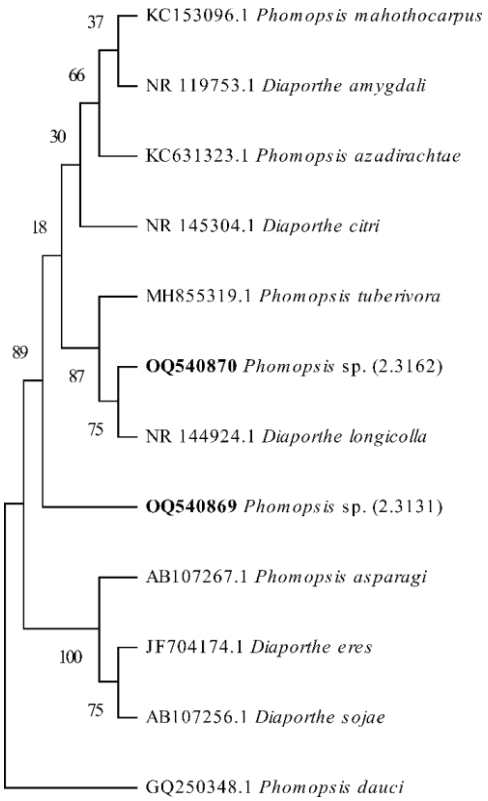

Figure 4. Neighbor-joining tree from ITS sequences showing the relationship between *Phomopsis* sp. (2.3131) and *Phomopsis* sp. (2.3162) of the present study and other closely related *Phomopsis* and *Diaporthe* species retrieved from the GenBank. Bootstrap values (1000 replicates) are shown on the branches. Bar = 2 nucleotide substitutions per 100 nucleotides.

Discussion

A total of 650 endophytic fungi were isolated from *C. spruceanum*, 486 filamentous fungi and 164 yeasts. This contradicts the claim that yeasts constitute a scarce group of the leaf and stem endophytic microorganism community (Wang & Guo, 2007) This difference can be explained by the endophytic microbial composition being influenced by plant species, geographic distribution, plant age, annual precipitation, available nutrients, humidity, among other factors (Silva et al., 2006).

The leaf of *C. spruceanum* presented higher colonization, with 60.3%, than the stem, with 39.7%. Leaves may be good habitats or more likely to be colonized by endophytic fungi. Similar findings have been described, with leaves cited as the plant tissue with the largest endophytic population, as it is one of the most conducive paths for infections (Nascimento et al., 2015; Szilagyi-Zecchin et al., 2016).

Different culture media and temperatures were used to isolate endophytic fungi of *C. spruceanum*, as some microorganisms may have different nutritional requirements. Some fungi can grow in media with few inorganic nutrients, while others need complex organic nutrients (Lima et al., 2011).

Among the 248 morphospecies analyzed, 166 were identified at the genus level by traditional methods. Studies for taxonomic identification using more than one identification technique, such as molecular biology methods, have obtained better performance in the elucidation of emerging species or even in the identification of sterile mycelium cases (Passarini et al., 2013). In addition to morphological features, it is also important to use physiological features for identification (Nascimento et al., 2015).

Among the 486 fungi submitted to identification, 124 (25.5%) did not produce reproductive structure (unidentified), being classified as sterile mycelium. Similar results were obtained with *Cereus jamacaru*, with 30.3% of unidentified fungi (Bezerra et al., 2013).

The most commonly isolated endophytic fungal species in the tropics belong to the phylum Ascomycota, including anamorphs. The fungi of this phylum are cosmopolitan and can be isolated from all plant tissues of practically all terrestrial plants and transmitted horizontally, occurring in temperate and tropical environments (Hardoim et al., 2015). Basidiomycota and Zygomycota species are also isolated as endophytes, but represent a smaller number (Bezerra et al., 2013; Nascimento et al., 2015). Similar results were observed in *C. spruceanum*, in which the 16 identified genera belong to the phylum Ascomycota.

Species of the genus *Cladosporium*, *Colletotrichum*, *Fusarium*, *Guignardia*, *Pestalotiopsis*, *Phomopsis* and *Xylaria* are the most common in studies in Brazil, besides non-sporulating fungi or sterile mycelium, commonly reported in studies involving endophytic fungi (Nascimento et al., 2015).

According to the frequency, endophytic fungi can be classified as dominant when found more frequently in the analyzed tissues or accidental when they occur once or twice in the plant (Mahmoud et al., 2017). *Phomopsis* and *Colletotrichum* were the most frequent genera, isolated from all tissues, culture medium and temperature. The preference of an endophytic species for a host plant tissue is due to the chemical composition of that tissue. In fact, the different tissues of the host plant can be distinct microenvironments, allowing the development of a specific species of endophytic fungus (Malhadas et al., 2017).

Fungi of the genus *Colletotrichum* can develop from different life forms and act as endophytic, epiphytic, or phytopathogenic fungi (Sharma et al., 2017). *Phomopsis* and *Colletotrichum* are commonly isolated as endophytic in tropical plants and have been isolated from *Carapa guianensis* (Ferreira et al., 2015), *Myrcia guianensis* (Banhos et al., 2014), and *Mikania glomerata* (Polonio et al., 2015).

Twenty-four extracts of endophytic fungi of *C. spruceanum* showed antibacterial activity, such as fungi of the genera *Phomopsis* and *Xylaria*. *Phomopsis* is known to be a rich source of bioactive secondary metabolites of various structures, such as xanthones, diaryl ethers, cytochalasins, convolvulanic acid and mycotoxin fumonisin (Cai et al., 2017). In *C. spruceanum*, *Phomopsis* was the genus with the highest number of fungi (37.5%) with activity against *E. coli*. *Phomopsis* isolated from *Aspidosperma tomentosum* and *Spondias mombin* showed activity against bacteria, yeasts and filamentous fungi (Rao & Satish, 2015; Jouda et al., 2016).

Metabolites isolated from *Xylaria* are able to inhibit the growth of microorganisms, such as *Mycobacterium smegmatis* (Siebers-Wolff et al., 1993), bacteria and fungi, showing the potential of this genus as a producer of bioactive compounds (Macias-Rubalcava & Sanchez-Fernandez, 2017).

Conclusion

There is a high diversity of endophytic fungi associated with *Calycophyllum spruceanum*, a medicinal plant considered promising for further studies aimed at exploring endophytic communities in other tissues. Metabolites produced by these endophytic fungi demonstrated the ability to inhibit the *in vitro* growth of two clinically relevant Gram-negative pathogenic bacteria. This is the first report describing the endophytic fungal community associated with the Amazonian medicinal plant *Calycophyllum spruceanum*.

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