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Antibacterial activity of endophytic fungi isolated from *Croton lechleri* (Euphorbiaceae)

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***Croton lechleri* is a native species from the Amazon and used with relative frequency in folk medicine in Brazil and other countries. Diversity and antibacterial activity of endophytic fungi associated with this plant were studied here. Samples of leaves and stems were used and 575 endophytic fungi were isolated (307 from leaves and 268 from stems), comprising 284 morphotypes distributed in 13 genera and unknown. The most frequently isolated genera were *Phomopsis* (30.78%), *Penicillium* (21.57%) and *Pestalotiopsis* (16.70%). Diversity and richness of species were higher in leaf tissues. Fifty-five fungi presented antibacterial activity. The fungi with the highest activity were *Phomopsis* (6.34%), *Penicillium* (3.17%), and those unknown (5.28%). *Penicillium* sp. 9 showed the highest antibacterial activity against *Enterococcus faecalis* and *Phomopsis* sp. 8 and *Phomopsis* sp. 9 against *Streptococcus pneumoniae* and *Staphylococcus aureus*. *Curvularia* sp. 1 and a fungus that could not be identified (Unknown sp. 9), showed the highest antibacterial activity against *Klebsiella pneumoniae* and *Escherichia coli*, respectively. Only two fungi (*Penicillium* sp. 9 and *Curvularia* sp. 1) inhibited the five tested bacteria. Endophytic fungi of *C. lechleri* harbor a great diversity of endophytic fungi, which have the potential for producing antibacterial compounds.**

Key words: Dragon's blood, antibacterial agent, endophytic fungi, microbial interaction.

INTRODUCTION

Croton lechleri, a tree that grows in Mexico, Venezuela, Ecuador, Peru, and Brazil, popularly known as dragon's blood (Gupta et al., 2008), is used by local communities to cure respiratory infections, diarrhea, gastric ulcers,

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herpes, and skin infections (Carlson, 2002).

C. lechleri latex has curative effects (Pieters et al., 1995), being antitumor (Rossi et al., 2003; Alonso et al., 2012; Montopoli et al., 2012), antioxidant (Lopes et al., 2004; Marino et al., 2008; Rossi et al., 2011), antibacterial (Bussmann et al., 2010; Rossi et al., 2011), antidiarrheal (Cottreau et al., 2012), and antimutagenic (Lopes et al., 2004; Fão et al., 2012; Rossi et al., 2013). The traditional use of plants with medicinal properties has promoted studies on the diversity and bioprospection of endophytic fungi (Strobel et al., 2004).

The presence of endophytic microorganisms in medicinal plants has been observed in many species (Hilarino et al., 2011; Premalatha and Kalra, 2013; Bezerra et al., 2015). These organisms are often involved in complex relationships between the synthesis, degradation, and accumulation of secondary metabolites of biotechnological interest (Müller et al., 2016). In many cases, there is an important symbiotic interaction with host plant, involving the production of compounds that can reduce herbivory in plant tissues, confer resistance to plant pathogens, and produce growth regulators to increase plant development (Kumar and Verma, 2017).

Endophytic microorganisms inhabit the interior of plants for at least one period of its life cycle and may colonize inter- and intracellular spaces of plants (Azevedo et al., 2000). These organisms do not harm plants but exhibit an asymptomatic relationship (Hardoim et al., 2015).

The use of endophytic microorganisms as a source of bioactive compounds or secondary metabolites is an interesting strategy since these microorganisms inhabit the interior of plants without causing any apparent symptom of the disease and growing in this environment involves continuous metabolic interaction between endophyte and host (Finkel et al., 2017).

Although several studies on biological activities and chemical composition of *C. lechleri* can be found in the literature, none of them is related to endophytic community and biological activity of its metabolites. Thus, endophytic fungi from leaves and stems of *C. lechleri* and their in vitro biotechnological potential for controlling pathogenic bacteria were assessed in this study.

MATERIALS AND METHODS

Collection of plants and isolation of endophytic fungi

Samples of leaves and stems from three individuals of *C. lechleri* were collected at the Federal University of Acre (9°57′26.2″ S and 67°52′29.1″ W) between September 2014 and February 2015.

The collected botanical material was processed and samples with no disease signs were selected and washed to eliminate the excess epiphytic. Samples were separated for preparing culture media containing plant tissue, stem, or leaf extracts, and samples for isolating endophytic fungi.

Samples were disinfected with 70 % alcohol for 1 min, 2.5% sodium hypochlorite for 2 min, 70% alcohol for 30 s, and washed in sterile distilled water twice (Pereira et al., 1993).

Tissues were fragmented into 5 mm diameter samples and inoculated in potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA) culture media, with and without 10% plant tissue extract. Plant extract was produced by using 100 g of fresh leaves or stems in 500 mL distilled water and filtered on filter paper. An infusion of 200 g of potato was added to prepare PDA+extract medium or 500 mL distilled water to prepare SDA+extract medium, being solubilized the reagents (Lima et al., 2011). All media received chloramphenicol (100 µg mL^{-1}) in order to inhibit bacterial growth. The inoculated samples were incubated at 18 and 28°C and observed daily for 30 days (Azevedo et al., 2010).

Fungi were purified in PDA culture medium, classified into morphotypes according to their macromorphological characteristics, and stored using mineral oil and distilled water techniques (Azevedo et al., 2010). For identification, macro and micromorphological analyses were performed and compared with specific literature (Barnett and Hunter, 1998).

Fungus cultivation and metabolite extraction

Endophytic fungi were cultured in potato dextrose broth (PD) by inoculating ten blocks of pure culture agar (5 mm^2) under active growth and with 14 days in 125 mL Erlenmeyer flasks containing 20 mL of medium. Flasks were incubated for 14 days at 28°C without shaking. Subsequently, 2 mL of broth was extracted with an equal volume of ethyl acetate by liquid-liquid partition and the extract in ethyl acetate was collected and evaporated. The crude extract was dissolved in 300 µL dimethylsulfoxide (DMSO) for antibacterial bioassay (NCCLS, 2003).

Antibacterial activity determination

The antibacterial activity of fungal extracts was determined by the agar diffusion method against the pathogenic bacteria *Staphylococcus aureus* (ATCC 12598), *Streptococcus pneumoniae* (ATCC 11733), *Enterococcus faecalis* (ATCC 4083), *Escherichia coli* (ATCC 10536), and *Klebsiella pneumoniae* (ATCC 700603) (NCCLS, 2003). Pathogenic bacteria were cultured at 37°C for 4 to 6 h in Luria-Bertani medium and their turbidity was adjusted to 0.5 McFarland scale. Bacteria were then inoculated into Petri dishes containing Muller-Hinton agar (MH), deposited on these paper disks (5 mm diameter) and then 20 µL of endophytic extract, and incubated at 37°C for 24 h. The endophytic extract that did not allow bacterial growth around the disc was considered as having antibacterial activity, and the inhibition halos produced were measured in millimeters. All determinations were performed in triplicate (NCCLS, 2003).

Statistical analysis of data

The infection index (FI) was calculated from the relationship between the number of fragments from which the endophytic fungi emerged and the total number of fragments used in the experiment (Azevedo et al., 2010).

The relative frequency of isolation (RF) was calculated as the number of isolates of a species divided by the total number of isolates, being expressed as a percentage (Bezerra et al., 2015). For the diversity analysis of the endophytic community of *C. lechleri*, the Simpson and Shannon indices were used to calculate the number of dominant species (Bezerra et al., 2015).

The formula for calculating the Simpson diversity index is $1 - \Sigma (pi)^2$. Shannon-Wiener diversity $(H') = -\Sigma\ pi \ln pi$, where pi is the

Table 1. Number and relative frequency percentages of endophytic fungi isolated from *C. lechleri* according to plant tissue, culture medium and temperature.

Genus	Plant tissue		Culture medium						Temperature (°C)		T[a]	RF (%)
	Leaf	Stem	PDA	PDA+ leaf	PDA+stem	SDA	SDA+leaf	SDA+stem	18	28		
Phomopsis	63	114	59	25	43	36	4	10	82	95	177	30.78
Penicillium	82	42	27	-	18	17	45	17	52	72	124	21.57
Pestalotiopsis	69	27	32	20	-	24	20	-	52	44	96	16.70
Colletotrichum	16	7	-	1	6	15	-	1	8	15	23	4.00
Aspergillus	12	8	10	-	8	-	2	-	18	2	20	3.48
Fusarium	14	5	6	6	-	7	-	-	8	11	19	3.30
Xylaria	3	14	-	-	9	8	-	-	4	13	17	2.96
Guignardia	2	12	4	2	-	8	-	-	6	8	14	2.43
Curvularia	4	7	-	-	1	4	-	6	3	8	11	1.91
Nigrospora	2	4	-	-	4	2	-	-	-	6	6	1.04
Chaetomium	1	-	-	1	-	-	-	-	1	-	1	0.17
Paecilomyces	-	1	-	-	-	-	-	1	-	1	1	0.17
Rhizopus	1	-	1	-	-	-	-	-	-	1	1	0.17
Unknown	38	27	11	13	15	16	5	5	33	32	65	11.30
T[a]	307	268	150	68	104	137	76	40	267	308	**575**	
RF (%)	53.39	46.61	26.09	11.83	18.09	23.83	13.22	6.96	46.43	53.57		

a= total identified in the sample.

proportion of species colonization frequency in a sample. Species equivalence (E) was calculated by using the following formula: E = H / ln S, where S is the number of species in the sample (Bezerra et al., 2015).

RESULTS

A total of 575 fungi (307 from leaves and 268 from stems) were isolated from 160 fragments of *C. lechleri* and grouped into 284 fungal morphotypes, distributed in 13 genera and unknown. The infection index (FI) for *C. lechleri* was 93%, being 96% for leaves and 90% for stems. Colonization and frequency of endophytic fungi were higher in leaves (53.39%) than in stems (46.61%) of *C. lechleri* (Table 1).

The most frequent genera were *Phomopsis* (30.78%), *Penicillium* (21.57%), and *Pestalotiopsis* (16.70%) isolated from leaf and stem. Three endophytic genera (*Rhizopus*, *Paecilomyces*, and *Chaetomium*) had a lower frequency and were isolated only once (0.17 %) (Table 1). Two genera were isolated only from leaf (*Chaetomium* and *Rhizopus*) and one exclusively from the stem (*Paecilomyces*).

Endophytic fungi richness was higher in *C. lechleri* leaves. Among the 575 analyzed fungi, only 65 (11.30%) were not identified. The medium with the highest amount of isolated endophytic fungi was PDA+plant tissue extract (29.91%), followed by PDA (26.09%) (Figure 1).

The temperature was also a factor that influenced the isolation of endophytic fungi from *C. lechleri*. In this sense, 308 fungi (53.57%) were isolated at 28°C and only 267 (46.43%) at 18°C, having as a specialist at 18°C the genus *Chaetomium* and at 28°C the genus *Paecilomyces* and *Rhizopus*.

Endophytic community diversity isolated from different tissues of *C. lechleri* was compared using the α-diversity indices (Table 2). Simpson and Shannon-Wiener endophytic fungi diversity were higher in leaves. Species richness was also higher in leaves. Little difference was observed regarding species uniformity among the studied tissues.

The antibacterial activity of ethyl acetate extract from each of the 284 fungal morphotypes was analyzed (Table 3). Only two samples inhibited all the tested bacteria, that is, the extract of *Penicillium* sp. 9 and *Curvularia* sp. 1. The extracts of *Phomopsis* sp. 3, 4, and 10 and *Pestalotiopsis* sp. 2 had antibacterial action only against gram-positive bacteria, *S. aureus*, and *S. pneumoniae*. Only the extract of *Penicillium* sp. 6 showed activity for the gram-negative bacteria *E. coli* and *K. pneumoniae*.

Among the tested extracts, 55 (19.37%) presented antibacterial activity against at least one of the five tested bacteria. *S. aureus* presented lower resistance to the tested extracts, presenting a sensitivity to 33 samples (11.62%), while *E. faecalis* presented the highest resistance, with sensitivity only to six samples (2.11%). Nineteen (6.70%), 16 (5.63%), and 11 (3.87%) endophytic extracts were active against *E. coli*, *S. pneumoniae*, and *K. pneumoniae*, respectively. Only extracts from *Curvularia* sp. 1 (2.1152) and *Penicillium*

Table 2. Diversity indices of endophytic fungi from C. lechleri according to plant tissue, culture medium, and temperature.

Diversity index	Abundance	Species richness	Shannon-Wiener diversity	Simpson diversity	Species evenness
Tissue type					
Leaf	307	157	4.64	0.99	0.81
Stem	268	128	4.40	0.98	0.79
Culture medium					
PDA	150	77	3.86	0.97	0.77
PDA+Leaf	68	37	3.13	0.93	0.74
PDA+Stem	104	57	3.69	0.96	0.80
SDA	137	63	3.75	0.97	0.76
SDA+Leaf	76	20	2.60	0.91	0.60
SDA+Stem	40	30	3.24	0.95	0.75
Temperature (°C)					
18	308	131	4.40	0.98	0.77
28	267	155	4.67	0.99	0.84
Total sample	575	284	5.21	0.99	0.82

Table 3. Antimicrobial activity of endophytic fungi isolated from *C. lechleri* against pathogenic bacteria species.

Endophytic fungi	Isolate	Antagonistic activity against*				
		Efa	Spn	Sau	Eco	Kpn
Phomopsis sp. 1	2.1157	-	-	-	10.5±0.5	-
Phomopsis sp. 2	2.1183	-	-	8.7±1.1	-	-
Phomopsis sp. 3	2.1187	-	11.5±0.9	15.4±1.1	-	-
Phomopsis sp. 4	2.1188	-	11.2±0.6	10.9±0.9	-	-
Phomopsis sp. 5	2.1198	-	-	-	8.6±0.7	-
Phomopsis sp. 6	2.1231	-	-	-	8.7±1.3	-
Phomopsis sp. 7	2.1269	-	-	-	6.4±0.5	-
Phomopsis sp. 8	2.1264	-	18.4±0.5	-	-	-
Phomopsis sp. 9	2.1367	-	10.9±1.1	16.3±0.6	7.0±0.8	-
Phomopsis sp. 10	2.1430	-	7.5±0.8	11.2±0.8	-	-
Phomopsis sp. 11	2.1473	8.3±0.4	-	-	-	-
Phomopsis sp. 12	2.2507	-	-	8.3±0.2	-	-
Phomopsis sp. 13	2.2610	-	-	8.1±0.8	-	-
Phomopsis sp. 14	2.2653	-	6.9±0.4	-	-	-
Phomopsis sp. 15	2.2717	-	9.7±0.6	-	-	-
Phomopsis sp. 16	2.2719	-	8.9±0.3	-	-	-
Phomopsis sp. 17	2.2697	-	8.4±0.5	-	-	-
Phomopsis sp. 18	2.3021	-	-	10.7±0.0	-	-
Penicillium sp. 1	2.1339	-	-	7.8±0.5	-	-
Penicillium sp. 2	2.1351	-	-	12.2±0.1	11.9±0.2	12.1±0.4
Penicillium sp. 3	2.1391	-	8.4±0.3	12.9±0.6	-	12.4±0.5
Penicillium sp. 4	2.1429	-	-	9.0±0.3	-	12.8±0.6
Penicillium sp. 5	2.1478	-	-	6.5±0.8	-	16.2±0.6
Penicillium sp. 6	2.1636	-	-	-	11.3±0.2	7.6±0.5
Penicillium sp. 7	2.2635	-	-	13.9±0.9	-	-

Table 3. Cont'd.

Penicillium sp. 8	2.2698	11.3±0.5	-	13.1±0.2	-	-
Penicillium sp. 9	2.2710	10.5±0.4	11.4±0.4	12.7±0.5	11.2±0.3	9.2±0.4
Xylaria sp. 1	2.2609	-	-	9.3±0.4	-	-
Xylaria sp. 2	2.2630	-	-	-	6.8±0.0	-
Xylaria sp. 3	2.2976	-	-	-	-	8.4±0.2
Fusarium sp. 1	2.1431	8.6±0.3	11.4±0.2	-	-	-
Fusarium sp. 2	2.1388	-	-	-	-	13.2±0.3
Aspergillus sp. 1	2.1477	-	-	6.2±0.4	19.3±0.1	-
Aspergillus sp. 2	2.2875	9.9±0.2	-	7.9±0.3	11.9±0.3	12.7±0.6
Pestalotiopsis sp. 1	2.1114	-	-	8.0±0.3	-	-
Pestalotiopsis sp. 2	2.2705	-	11.2±0.2	7.2±0.6	-	-
Curvularia sp. 1	2.1152	10.1±0.0	8.5±0.4	10.8±0.7	9.7±0.2	19.0±0.1
Colletotrichum sp. 1	2.2707	-	-	7.9±0.3	-	-
Rhizopus sp. 1	2.2685	-	-	9.2±0.3	-	-
Paecilomyces sp. 1	2.1611	-	-	-	8.0±0.2	-
Unknown sp. 1	2.1132	-	-	12.5±0.2	-	-
Unknown sp. 2	2.1150	-	-	7.3±0.3	-	-
Unknown sp. 3	2.1207	-	13.1±0.2	-	10.2±0.4	-
Unknown sp. 4	2.1243	-	-	-	13.2±0.3	-
Unknown sp.5	2.1254	-	-	7.1±0.2	-	-
Unknown sp.6	2.1260	-	-	8.9±0.3	-	15.4±0.4
Unknown sp.7	2.1267	-	-	-	12.4±0.5	-
Unknown sp.8	2.1281	-	-	8.4±0.3	-	-
Unknown sp.9	2.1294	-	-	7.3±0.1	17.1±0.2	-
Unknown sp.10	2.1375	-	-	-	10.3±0.2	-
Unknown sp.11	2.1377	-	-	13.0±0.3	-	-
Unknown sp.12	2.2631	-	-	-	6.7±0.7	-
Unknown sp.13	2.2729	-	-	18.0±0.7	-	-
Unknown sp.14	2.2885	-	6.5±0.7	-	-	-
Unknown sp.15	2.3468	-	-	7.8±0.5	-	-
Chloramphenicol		13.3±0.4	19.0±0.8	19.3±0.5	26.0±0.0	13.7±0.5
Total		6	16	33	19	11

*Efa = *Enterococcus faecalis*; Spn = *Streptococcus pneumoniae*; Sau = *Staphylococcus aureus*; Eco = *Escherichia coli*; Kpn = *Klebsiella pneumoniae.*

sp. 9 (2.2710) presented antagonism for all the tested bacteria. *Penicillium* sp. 8 (2.2698) showed the highest activity against *E. faecalis*, while *Phomopsis* sp. 8 (2.1264) and *Phomopsis* sp. 9 (2.1367) presented the highest activity against *S. pneumoniae* and *S. aureus*, respectively. The fungi with the highest activity against gram-negative were *Curvularia* sp. 1 (2.1152) against *K. pneumoniae* and a fungus that could not be identified (Unknown sp. 9 - 2.1294) against *E. coli* (Figure 2).

DISCUSSION

A total of 575 endophytic fungi of *C. lechleri* were isolated, with colonization and frequency of endophytic fungi higher in leaves (53.39%) than in stems (46.61%) (Table 1). Studies with endophytic fungi have isolated most frequently fungi from stem than from leaves, unlike that observed in *C. lechleri* (Banhos et al., 2014; Bezerra et al., 2015). Other studies showed leaves with the highest colonization of endophytic fungi (Souza et al., 2004).

Similar to the results obtained in this study, *Phomopsis* and *Pestalotiopsis* were also one of the most frequent genera isolated as endophytic (Hilarino et al., 2011; Ferreira et al., 2015). Some fungal genera exhibited specificity in relation to the culture medium of isolation. *Rhizopus* was isolated only in PDA medium, *Chaetomium*

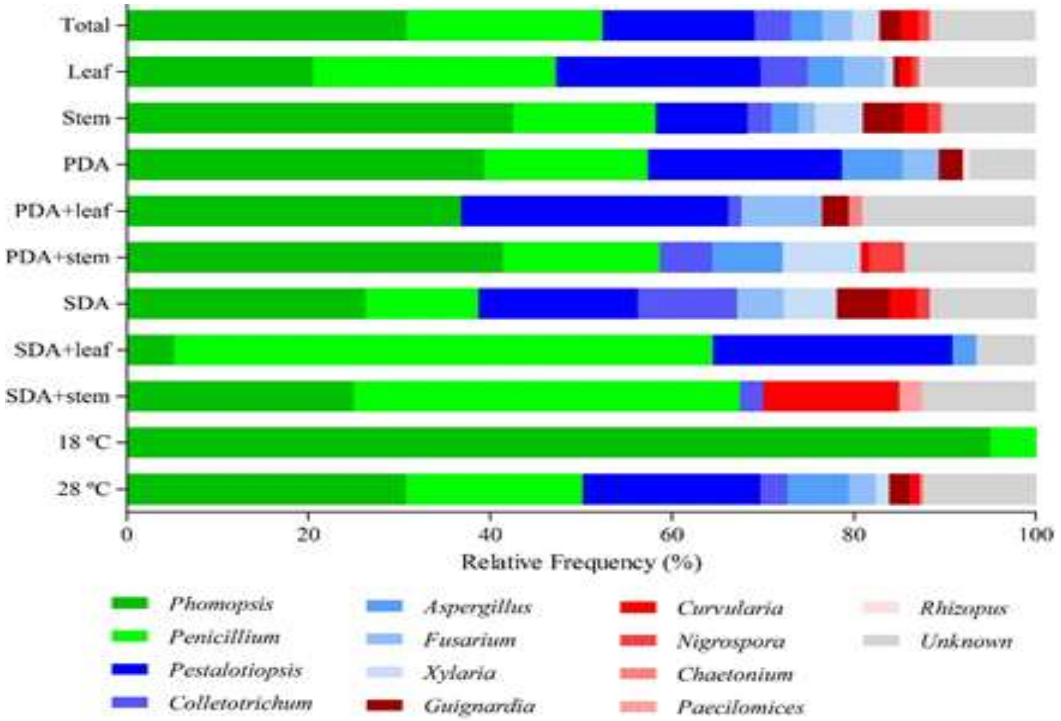

Figure 1. Fungal endophytic communities isolated from *C. lechleri* according to plant tissue, culture medium, and temperature.

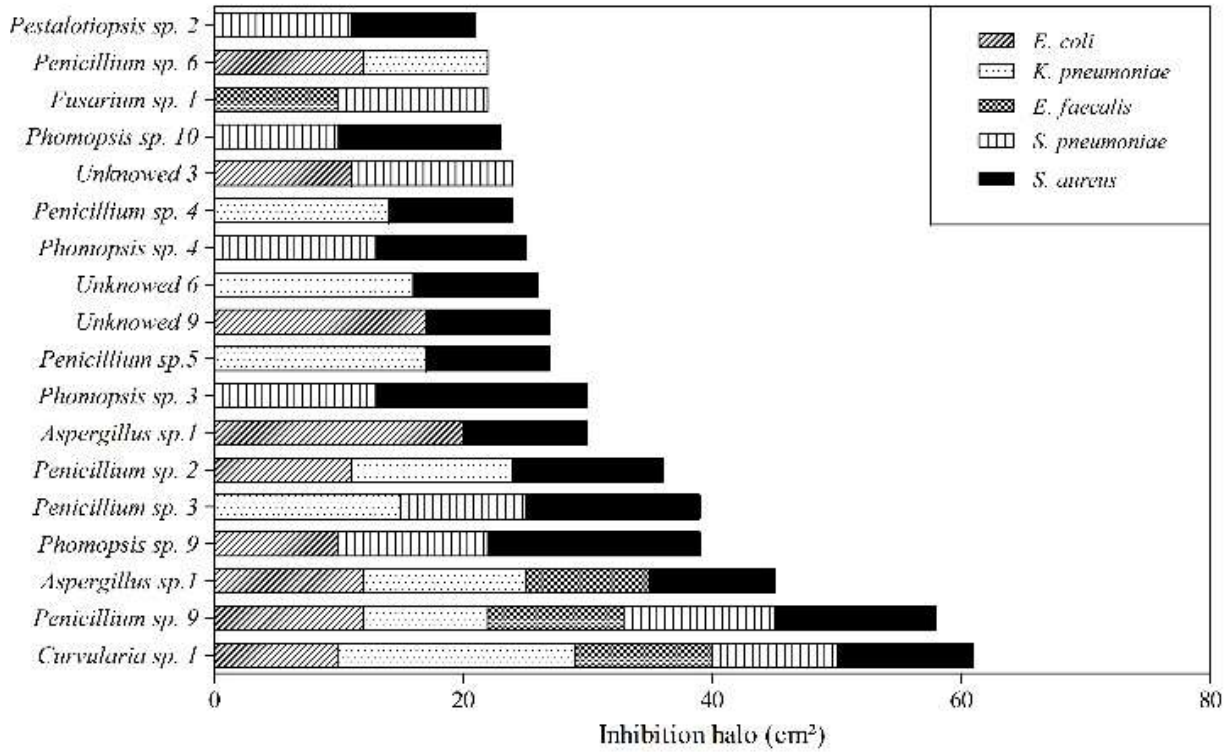

Figure 2. Antimicrobial activity (inhibition halo, in cm^2) against gram-positive and gram-negative bacteria presented by endophytic fungi isolated from *Croton lechleri*. Each value is expressed as the average of three independent experiments performed in triplicate.

only in PDA+extract, and *Paecilomyces* only in SDA+extract. Although PDA medium is the most used for isolating endophytic fungi, other culture media should be used to provide different nutritional sources. It is not common to use various culture media for isolating endophytic fungi (Pimentel et al., 2006), as well as the use of culture media with plant extracts is rarer still (Lima et al., 2011).

Two isolation temperatures were used to increase the number and diversity of endophytic fungi since it is possible to isolate fungi with slower growth at 18°C. However, few studies can be found in the literature using an isolation temperature of 18°C (Souza et al., 2004). In general, temperatures between 25 and 30°C are commonly used (Lima et al., 2011; Banhos et al., 2014; Bongiorno et al., 2016) or an ambient temperature of 28°C (Costa et al., 2012; Tayung et al., 2012), showing a large variation in this environmental factor (Table 1).

Endophytes have been reported as prolific producers of antimicrobial compounds. *Phomopsis*, *Penicillium* and *Xylaria* were the fungal genera that presented the highest antibacterial activity. Fungi of these genera are well known in the literature for their biological activities and several studies prove their potential as producers of biologically active secondary metabolites (Kobayashi et al., 2003; Prachya et al., 2007; Rukachaisirikul et al., 2008).

Fungi of the genus *Phomopsis* have often been isolated as endophytic and have demonstrated antibacterial activity (Kamei, 2008; Siqueira, 2008; Garcia et al., 2012; Deshmukh et al., 2015). Similar to that observed for the endophytic fungi *C. lechleri*, *Penicillium*, *Aspergillus*, and *Xylaria* stood out in secondary metabolite production, being among the genera frequently isolated as endophytic from tissues of several plants and the most frequently selected in bioprospecting studies (Elias, 2015).

In addition, other studies with endophytes have observed fungi of the genus *Penicillium* with antibacterial activity (Pastre et al., 2007; Borges, 2014; Bezerra et al., 2015). Fungi of the genus *Xylaria* are often isolated as endophytic from tropical plants and their metabolites have antibacterial activity, as observed in prospecting studies (Souza et al., 2004; Campos et al., 2015).

Endophytic fungi of the genus *Curvularia* have been observed in prospecting studies with antibacterial activity (Furtado et al., 2007; Bezerra et al., 2013; Nascimento et al., 2015).

Conclusion

C. lechleri hosts a rich community of endophytic fungi with antibacterial potential against bacteria pathogenic to human, especially against Gram positive. This study intends to contribute to the understanding of endophytic/plant interactions and open new perspectives on the biotechnological potential of endophytic microorganisms from Amazonian plants, which are practically unexplored in this field but have a great potential.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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