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Characterization of cultivable endophytic fungi from the medicinal plant *Senna reticulata* (Willd.) H. S. Irwin & Barneby

Caracterização de fungos endofíticos cultiváveis da planta medicinal Senna reticulata (Willd.) H. S. Irwin & Barneby

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ABSTRACT

The objective of this work was to characterize the endophytic fungi community of the medicinal plant *S. reticulata*. Vegetable tissue samples from *S. reticulata* plants, stems and leaves, were collected, washed and disinfected by immersion in 70% etanol, 2% sodium hypochlorite, 70% alcohol, and washing in sterile distilled water. The samples were fragmented and inoculated in PDA and Sabouraud culture media, with and without plant extract. After inoculation, the plates were incubated at 18 ºC or 28 ºC for 30 days. The isolated fungi were characterized in terms of their macromorphology and micromorphology. A total of 309 *S. reticulata* endophytic fungi, 178 stem fungi and 131 leaf fungi, were isolated. A greater number of isolates was obtained in plant I, totaling 194. Sabouraud medium with plant extract at 18 ºC, were the best conditions for isolation of endophytic fungi of *S. reticulata*. 27 morphospecies were identified, distributed in ten genera, *Phomopsis* (32.4%), *Penicillium* (13%), *Aspergillus* (5.8%), *Acremonium* (2.6%), *Colletotrichum* (1.6%), *Curvularia* (1.6%), *Alternaria* (0.3%), *Trichoderma* (0.3%), *Cladosporium* (0.3%) and *Xylaria* (0.3%). *Senna reticulata* has a diverse community of endophytic fungi, with *Phomopsis* as dominant. This is the first report of endophytic fungi from the medicinal plant *Senna reticulata*.

Keywords: Pasture killer; Medicinal plants; *Phomopsis*.

RESUMO

O objetivo deste trabalho foi caracterizar a comunidade de fungos endofíticos da planta medicinal *S. reticulata*. Amostras de caule e folhas de *S. reticulata* foram coletadas, lavadas e desinfetadas por imersão em etanol 70%, hipoclorito de sódio 2%, álcool 70% e lavagem em água destilada estéril. As amostras foram fragmentadas e inoculadas em meios de cultura BDA e Sabouraud, com e sem extrato vegetal. Após a inoculação, as placas foram incubadas a 18 ºC ou 28 ºC por 30 dias. Os fungos isolados foram caracterizados quanto à macromorfologia e micromorfologia. Um total de 309 fungos endofíticos de S. reticulata, 178 fungos do caule e 131 fungos foliares foram isolados. Um maior número de isolados foi obtido na planta I, totalizando 194. O meio Sabouraud com extrato da planta a 18 oC, foram as melhores condições para o isolamento dos fungos endofíticos de *S. reticulata*. Foram identificadas 27 morfoespécies, distribuídas em dez gêneros, *Phomopsis* (32,4%), *Penicillium* (13%), *Aspergillus* (5,8%), *Acremonium* (2,6%), *Colletotrichum* (1,6%), *Curvularia* (1,6%), *Alternaria* (0,3%), Trichoderma (0,3%), Cladosporium (0,3%) e Xylaria (0,3%). Senna reticulata possui uma comunidade diversificada de fungos endofíticos, com *Phomopsis* como dominante. Este é o primeiro relato de fungos endofíticos da planta medicinal *Senna reticulata.*

Palavras-chaves: Mata-pasto; Plantas medicinais; *Phomopsis*

1. INTRODUCTION

Senna reticulata (Willd.) H. S. Irwin & Barneby is a pioneer Amazonian species, found in abundance in lowland regions, river beds and other environments that are subject to flooding (PAROLIN, 2005). However, the species also grows in upland regions, colonizing sites above 25 m from the river level, where it occurs more frequently in highly sedimented soils (PAROLIN et al., 2002). It belongs to the Leguminosae family and has leaves composed of 8 - 14 pairs of leaves that have an oval shape. The flowers are large, yellow and pollinated by insects. The fruits are long and dry. They have many branches scattered irregularly (PAROLIN, 2005).

Due to its great competitive character, *S. reticulata* is popularly known in the Amazon for pasture killer (PAROLIN, 2003). It can grow up to 4 m in the first months after establishment in the soil, at 12 m as an adult. It is usually found measuring between 4 and 8 m in height, with a diameter at chest height (DBH) of 5.2 cm (PAROLIN, 1998). Characteristically, red ants build nests on *S. reticulata* and attack all intruders very aggressively, especially in the Costa do Catalão, where caterpillars can reduce the leaf area of the seedlings by 20% (PAROLIN, 2001). The plant species is rich in secondary compounds, and it is used to treat intestinal infections, fungal diseases (PAROLIN, 2001), antirheumatic and dermatitis (PAROLIN, 2001; LIZCANO, 2010).

Study of the chemical constituents of *S. reticulata*, there are six anthraquinones, 1,8-dihydroxy-3-methylanthroquinone (chrysofanol), 1,8-dihydroxy-3-methyl-6-metaxyanthraquinone (fisciona), 3-carbinol-1, 8-dihydroxyanthraquinone, 1,3,8-trihydroxyanthraquinone, 1,6,8-trihydroxy-3-metaxyanthraquinone (lunatin), 1,6,8-trihydroxy-3-methylanthraquinone (emodine), β-sitosterol and stigmasterol steroids, α and β amirine triterpenes and the campferol flavonoid and the chrysofanol biantrone (SANTOS; SILVA, 2008).The great distribution, abundance and ethnobotany, aroused interest in studies on the biological activities of this plant. The leaf of *S. reticulata* has antioxidant activities (LIZCANO et al., 2010; MATULEVICH-PELÁEZ et al., 2017) and reduced blood glucose rates in diabetic rats (CRISTANCHO et al., 2009). The root can be used to treat malaria (VIGNERON et al., 2005), and hydroethanolic extracts of the bark and leaf showed antifungal activity against the fungi *Microsporum canis*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* (OLIVEIRA, 2009).

Plants live in constant association with microorganisms, which can be beneficial or harmful. A portion of these microorganisms, mainly bacteria and fungi, inhabit the interior of these plants, called endophytes. These organisms internally colonize plant tissues at some point in the life cycle, without causing apparent damage (DE SILVA et al., 2019). This association can confer several advantages to the plant, such as the decrease in herbivory, the increase of tolerance to abiotic stresses and the control of pathogenic microorganisms (ZHANG et al., 2006).

In addition to research on biological activity in plants, endophytic microorganisms from plant tissues are interest to the scientific community, especially because of the potential in agriculture and industry, in the production of antibiotics and other secondary metabolites of pharmacological interest, such as the anti-tumor taxol (STIERLE et al., 1993), and also as pest inhibiting agents (AHMAD et al., 2020) and pathogens (PETERS et al., 2020). Thus, microorganisms and plants can be studied in the search for new biologically active substances.

Despite this relevance, no study on the endophytic fungi community of *Senna reticulata* was found in the literature. Thus, the objective of this work was to describe the community of cultivable endophytic fungi of the medicinal plant of *Senna reticulata*.

2. MATERIAL AND METHODS

Plant material

Leaf and stem samples from two Senna reticulata plants were collected at the Zoobotanic Park of the Federal University of Acre located in the city of Rio Branco, Acre, Brazil (Figure 1).

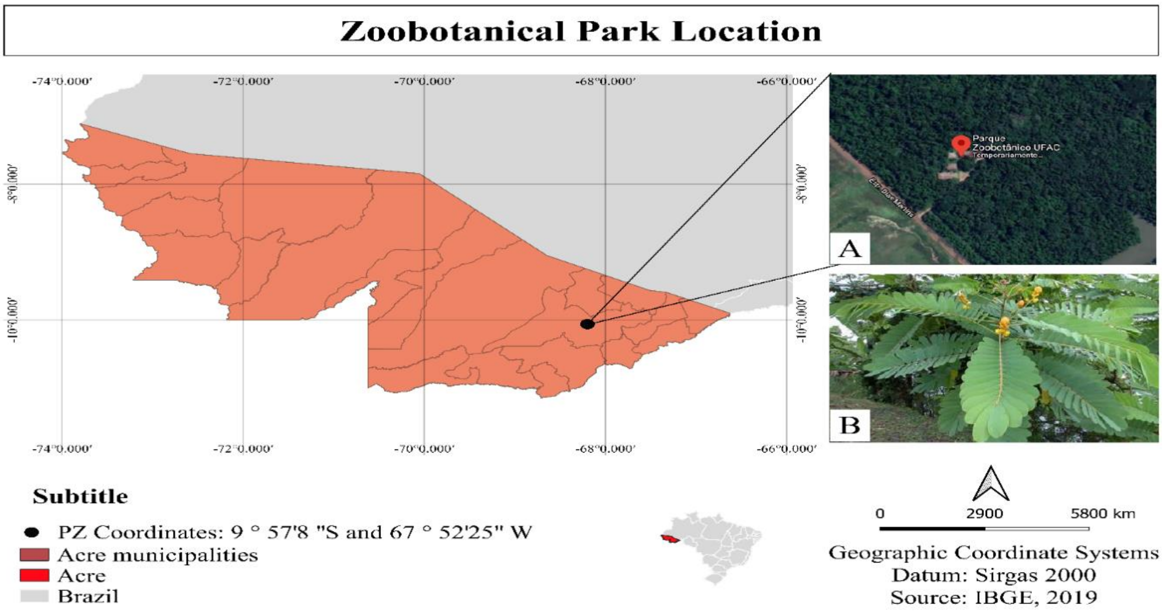

Figure 1. Location map of the *S. reticulata* collection point. A) Satellite image of the Zoobotanical Park. B) Image of the *S. reticulata* plant.

Isolation of endophytic fungi

Senna reticulata leaf and stem were washed with a sponge and detergent in running water to remove solid residues and epiphytic microorganisms. After washing, the material underwent superficial disinfection by immersion in 70% ethanol (1 min), 2% sodium hypochlorite (4 min), 70% alcohol (30 sec) and washing in sterile distilled water (1 min) three times. The disinfected plant material was cut into fragments of 5 mm in diameter and inoculated into a Petri dish containing Potato-Dextrose-Agar-PDA, PDA + 10% plant extract, Sabouraud-SDA, SDA + plant extract a 10%, all supplemented with chloramphenicol antibiotics (100 µg mL^{-1}), to inhibit bacterial growth, and incubated at 18 °C or 28 °C for 30 days. To prepare the PDA + extract medium, 500 mL of 200g of potato infusion was added to 500 mL of plant extract, and for the preparation of the SDA+extract medium 500 mL of water, and the reagents used in the preparation of each culture medium were solubilized (ARAÚJO et al., 2010).

The fungal colonies with distinct characteristics according to macroscopic observations (color, texture and growth characteristics in culture medium) were purified using the streak technique by depletion in Petri dishes containing the PDA culture medium, and incubated for 48 h. After the purity of the colonies was confirmed, the isolates were inoculated into tubes containing PDA medium (ARAÚJO et al., 2010), and the fungi were preserved in distilled water (CASTELLANI, 1963) and mineral oil (BUELL; WESTON, 1947).

Morphological characterization

For the macromorphological characterization, the fungi were grouped into morphospecies according to the characteristics of the colony, such as color, texture and pigment production. One representative of each morphospecies was used for micromorphological identification. For this, microculture was carried out, the fungi were inoculated in 1 cm^2 cubes of PDA and covered with coverslips, inside Petri dishes. The plates were incubated at room temperature for 7 days for mycelial growth and the coverslips stained with lactophenol blue for visualization of reproductive structures under an optical microscope (LACAZ et al., 1998; BARNETT; HUNTER, 1999).

Data analysis

The relative frequency of isolated endophytic fungi was calculated by the number of fungi per tissue, culture medium, temperature or genus divided by the total number of isolated fungi, multiplied by one hundred, in the Excel program. GraphPad Prism 5.0 was used to make figures and QGIS was used for the map. Principal Component Analysis (PCA) was performed using the software Statistica 2.0 to evaluate the influence of culture media on the isolation of fungal endophytes.

3. RESULTS

A total of 309 endophytic fungi of *Senna reticulata*, 178 of stem and 131 of leaf were isolated (Table 1). A greater number of fungi was isolated from individual I, collected in January, 194 isolates, and the tissue stem with the largest number (110). From individual II, 115 fungi were isolated, and the stem had a greater number of isolates than leaf, with 68 fungi (Figure 1).

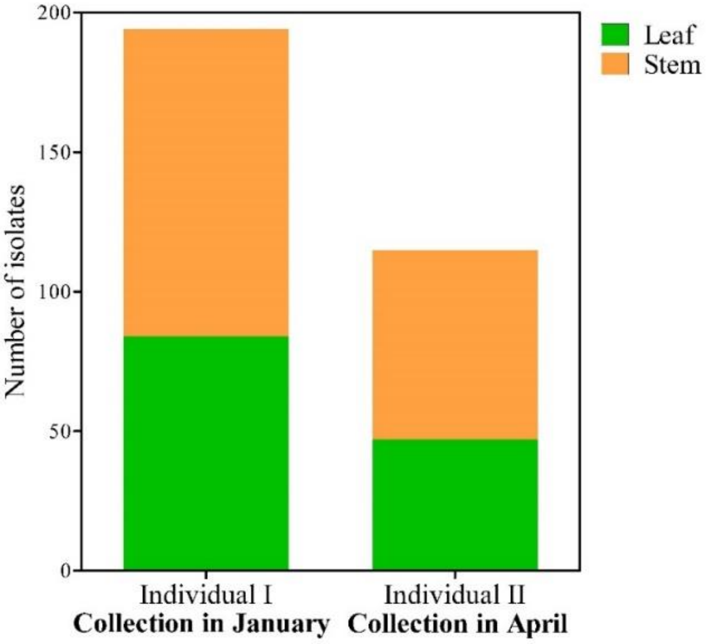

Figure 1. Number of isolates of *S. reticulata* endophytic fungi per individual and plant tissue.

The greatest fungal growth was with the addition of the plant extract to conventionally used media, and the SDA + extract medium presented the highest amount of isolates, with 98 fungi. About temperature, 18 °C had the best result for isolation of S. reticulata endophytic fungi with 169 isolates (Table 1).

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

Genus	Plant Tissue		Culture Medium				Temperature		T	RF (%)
	Leaf	Stem	PDA	PDA + extract	SDA	SDA + extract	18 ºC	28 ºC		
Phomopsis	35	65	15	23	13	49	52	48	100	32.4
Penicillium	20	20	12	5	13	10	23	17	40	13.0
Aspergillus	8	10	5	3	5	5	-	18	18	5.8
Acremonium	3	5	-	3	3	2	5	3	8	2.6
Colletotrichum	3	2	-	3	-	2	-	5	5	1.6
Curvularia	-	5	-	-	5	-	2	3	5	1.6
Alternaria	-	1	-	-	-	1	1	-	1	0.3
Trichoderma	1	-	-	-	-	1	1	-	1	0.3
Cladosporium	-	1	-	1	-	-	1	-	1	0.3
Xylaria	1	-	-	1	-	-	1	-	1	0.3
Mycelia sterilia	60	69	48	19	34	28	83	46	**129**	41.8
Total	**131**	**178**	**80**	**58**	**73**	**98**	169	140	**309**	100
RF (%)	42.4	57.6	25.9	18.8	23.6	31.7	54.7	45.3		

The isolated fungi were classified into 133 morpho-species, and 27 identified morpho-species distributed in ten genera, *Phomopsis* (32.4%), *Penicillium* (13%), *Aspergillus* (5.8%), *Acremonium* (2.6%), *Colletotrichum* (1.6%), *Curvularia* (1.6%), *Alternaria* (0.3%), *Trichoderma* (0.3%), *Cladosporium* (0.3%) and *Xylaria* (0.3%). The other morphospecies were not identified because they do not have reproductive structures, considered sterile mycelium (41.8%)

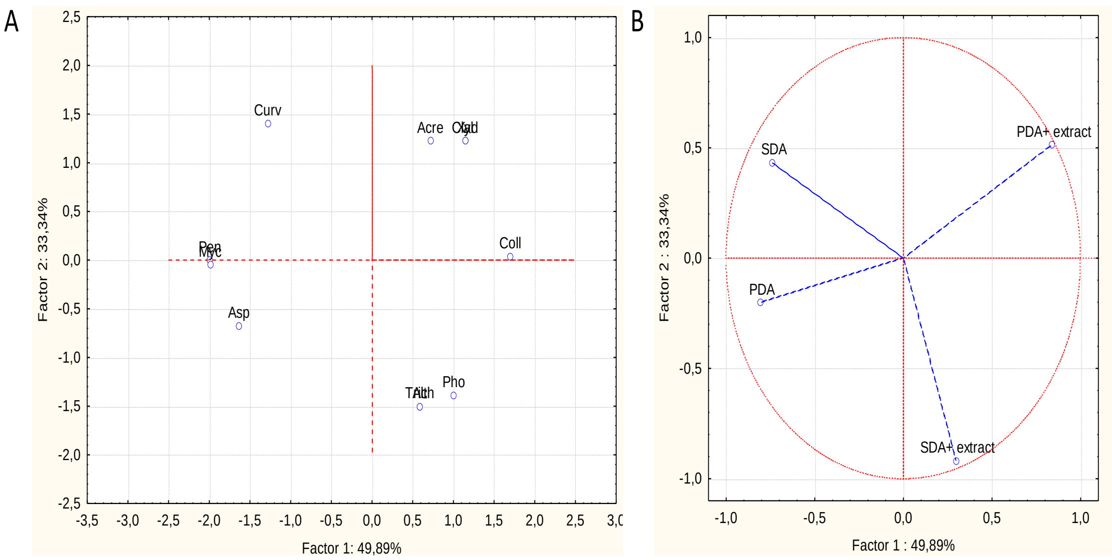

Figura 1. Principal component analysis (PCA) of media effect (PDA, PDA+extract, SDA and SDA+extract) on endophytic fungi isolation from *S. reticulata*. (A) Discriminations of endophytic fungi isolated on different media; (B) Loading plot of media for the first two principal components, PC1 and PC2.

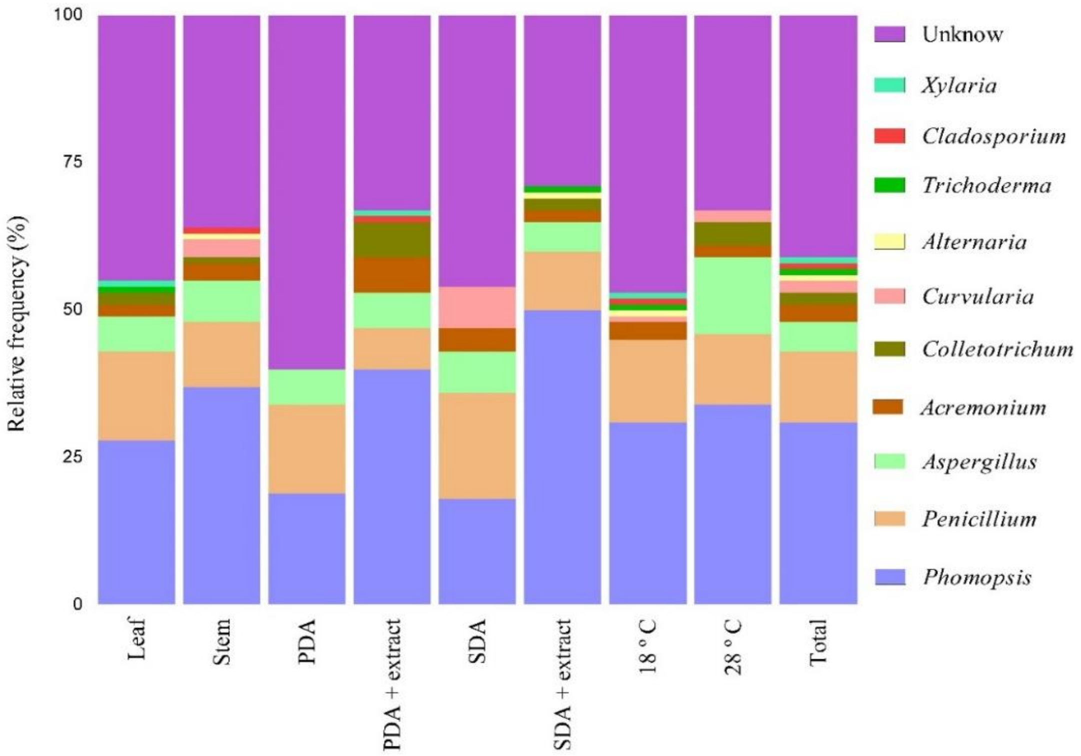

Figure 2. Relative frequency of genera of endophytic fungi of *S. reticulata* distributed vegetable tissue, culture medium and temperature.

The genera *Phomopsis*, *Penicillium* and *Aspergillus* grew in all culture media, however *Curvularia*, *Cladosporium*, *Xylaria*, *Alternaria* and *Trichoderma* were specific to a single culture medium (Figure 2A). Based on the PCA results, the *Phomopsis* genus was more abundant in the SDA + extract medium, while the *Penicillium* genus was more abundant in the non-extract medium (SDA and PDA) (Figure 1). The genera *Colletotrichum, Curvularia, Trichoderma, Cladosporium, Alternaria* and *Xylaria* were more abundant or only isolated in media with plant extract. The *Aspergillus* genus was more abundant in three media: PDA, SDA and SDA + extract. The *Acremonium* genus was more abundant in SDA and PDA + extract (Figure 1).

Phomopsis, Penicillium, Acremonium and *Curvularia* grew at 18 ºC and 28 ºC, the other fungi were specific for temperature (Figure 2B). *Trichoderma* and *Xylaria* were specific for leaf, and *Curvularia, Alternaria* and *Cladosporium* for stem, the other genera proved to be generalists (Figure 2C).

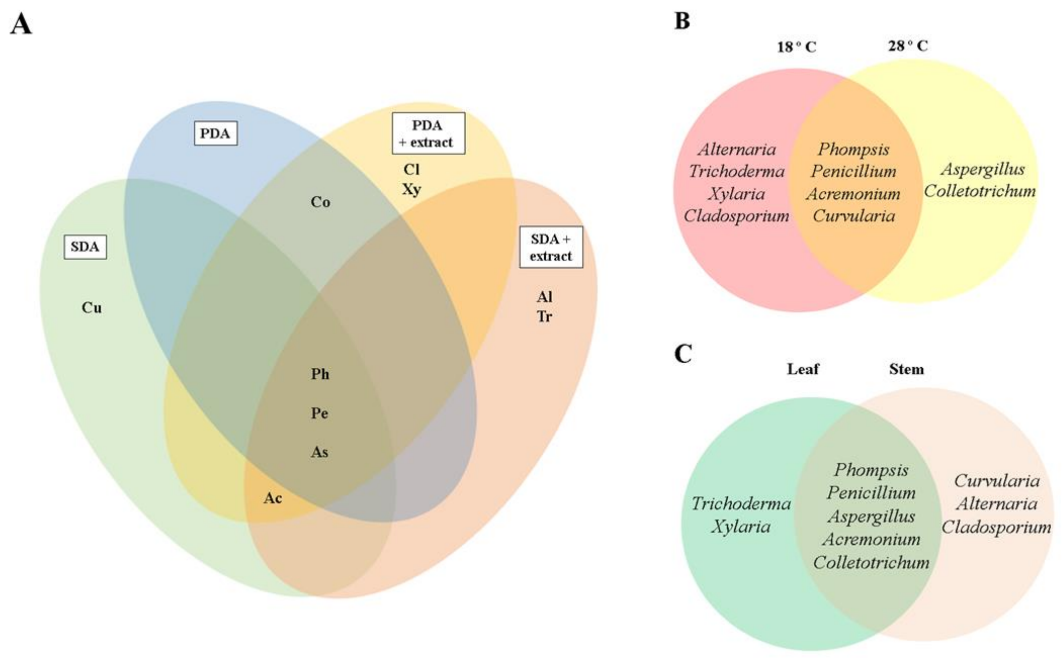

Figure 3. Venn diagram showing the effect of experimental conditions on the isolation of endophytic fungi isolated from *S. reticulata*. A) Genera isolated according to the culture medium used (Co: *Colletotrichum*; Ph: *Phomopsis*; Xy: *Xylaria*; Pe: *Penicillium*; As: *Aspergillus*; Ac: *Acremonium*; Fu: *Fusarium*; Tr: *Trichoderma*; Cl: *Cladosporium*; Cu: *Curvularia*; Al: *Alternaria*). B) Genera isolated according to temperature. C) Genera isolated according to the plant tissue.

4. DISCUSSION

Senna reticulata has been shown to harbor important genera of endophytic fungi. The isolation of the endophytic community of a plant can be affected by numerous variables, such as the time of collection of plant samples, as observed in this work, given the greater fungal recovery of individual I, collected in January in the amazon winter. In addition, in this study greater isolation of stem fungi was observed, which may be related to the presence of characteristic tissue substances such as - bisabolol, which has a protective function to the fungal community that colonizes it (SIEBER; DOWORTH, 1994; OTERO et al., 2002).

As for the experimental conditions, there were more fungi isolations in Sabouraud medium with the addition of the plant extract and at a temperature of 18 ºC. These data differ from most studies with endophyte isolation, which use only PDA medium and temperatures above 20 ºC as ideal conditions for isolation (LIANG et al., 2012; YADAV et al., 2014). This fact demonstrates that there is no single ideal condition for the isolation of endophytic microorganisms, and that it is necessary to use different nutritional sources, different temperatures, and even variation in the pH of the medium, to start fungal growth (DE FREITAS SIA et al., 2013).

Thus, fungi that may be in latency will receive the necessary stimulus to start growth, with greater knowledge from the endophytic community (CARNAUBA et al., 2007; SIA, 2012; DA SILVA et al., 2020). Other aspects that also act in the recovery of the endophytic community are: genetic quality, plant age, geographical distribution and environmental conditions (FAETH; FAGAN, 2002; OKI et al., 2009; HARDOIM et al., 2015).

Based on the morphological characteristics of the isolated fungi, it was possible to organize them into ten genera, *Phomopsis, Penicillium, Aspergillus, Acremonium, Colletotrichum, Curvularia, Alternaria, Trichoderma, Cladosporium* and *Xylaria*. *Phomopsis* was the genus with the highest frequency of isolation, totaling 32.4%. This genus is often isolated as an endophyte from Amazonian plants like *Carapa guianenses* and *Euterpe precatoria* (FERREIRA et al., 2015; PETERS et al., 2020). Metabolites of fungi of the genus *Phomopsis* have antimicrobial activity, inhibition of the growth of *Trypanosoma cruzi* amastigotes and antiviral action against the yellow fever virus (FERREIRA et al., 2015). It also has environmental applications, due to the degradation of N-heterocyclic indole xenobiotics and 4-hydroxybenzoic acid (CHEN et al., 2011; CHEN et al., 2013).

Penicillium and *Aspergillus* were isolated with a frequency of 13 and 5.8%, respectively. Both are similar genera in terms of ecological characteristics, as they are fungi with easy dissemination in the environment and can be recovered from plants, soil, water, air, among others (BENOIT et al., 2013; CRUZ et al., 2013). As endophytes, it has provided important biotechnological applications in different areas of knowledge. In agriculture, *Penicillium citrinum* and *Aspergillus terreus* provided induction of disease resistance, and growth promotion in sunflower (*Helianthus annuus* L.) (WAQAS et al., 2015). In health, P*enicillium* has shown antimicrobial, antimalarial and antituberculosis activity (INTARAUDOM et al., 2013; KOREJO et al., 2014). As for the genus *Aspergillus*, an important pharmaceutical product is produced from *A. terreus*, called lovastatin. This compound is highly required as it is a medication used to control cholesterol in humans (RAGHUNATH et al., 2012).

Acremonium was isolated with 2.6% frequency, and this is a cultivable endophytic fungus with biotechnological applications. This genus produces important enzymes, such as cellulases and hemicellulases in the degradation of lignocellulosic residues for the purpose of ethanol production (ALMEIDA et al., 2011). In agriculture, this endophyte has produced active compounds, such as gliotoxin, which acts in the biocontrol of *Pythium myriotylum*, a causal agent of soft rot in ginger, in addition to being efficient against the nematode *Meloidogyne incognita* (YAN et al., 2011; ANISHA; RADHAKRISHNAN, 2015). Antimicrobial activity against human pathogens was observed in *Acremonium* isolated from *Sesbania grandiflora* (L.) (POWTHONG et al., 2013).

Colletotrichum and *Curvularia* were isolated at the same frequency of 1.6% each. *Colletotrichum* is a genus of great importance in agriculture because it causes diseases in plants and economic damage, as is the case of anthracnose in papaya (RIBEIRO et al., 2016). However, strains isolated as endophytes of this genus have shown benefits to the host plant as promoting plant growth by transferring phosphorus to *Arabidopsis* shoots and participating in the production of indole-3-acetic acid (HIRUMA et al., 2016; NUMPONSAK et al., 2018). *Curvularia* can also become a phytopathogen, mainly in seeds and trigger economic losses. However, compounds isolated from endophytic strains have shown that fungi of the genus *Curvularia* have metabolites with antimicrobial, antioxidant and acetylcholinesterase inhibitor activities (SILVA et al., 2014; KAANICHE et al., 2019).

Alternaria, Trichoderma, Cladosporium and *Xylaria* were the four least frequent genera in the isolation of *S. reticulata*, with only 0.3% each. Both are commonly isolated as endophytes from plants such as *Euterpe precatoria, Avicennia officinalis, Hevea brasiliensis* and *H. guianensis* (CHAVERRI et al., 2011; RANGANATHAN; MAHALINGAM, 2019; PETERS et al., 2020). *Alternaria* has been the focus of research in the production of secondary metabolites due to its composition by steroids, terpenoids, pyranones, quinones and phenolics (LOU et al., 2013). Some metabolites are for medicinal purposes, with antifungal and antidiabetic activity, and others for agricultural purposes, with potential insecticide against *Spodoptera litura* (SINGH et al., 2012; CHAGAS et al., 2013; GOVINDAPPA et al., 2015).

Trichoderma is one of the most studied fungal genera in the literature due to its biotechnological potential for industrial, pharmaceutical, environmental and agricultural areas (SCHUSTER et al., 2010; MACHADO et al., 2012). Secondary metabolites of *Trichoderma* have promoted antifungal and anticancer activity (MING et al., 2012; SHENTU et al., 2014). In agriculture, the main focus is the biocontrol of pests and diseases, which has had an antagonistic effect for pepper moth (*Duponchelia fovealis*) and black rot in cocoa (*Phytophthora palmivora*) (HANADA et al., 2008; AMATUZZI et al., 2018).

Cladosporium have promoted sustainable ecological technology by mediating the biological synthesis of silver nanoparticles (AgNPS) with anti-diabetic and anti-alzheimer activity (POPLI et al., 2018). In addition, it has antitumor action due to the production of taxol and antimicrobial by the production of plumbago (ZHANG et al., 2009; VENKATESWARULU et al., 2018). *Xylaria* is known as a wood decomposing fungus (PHOTITA et al., 2001), but when isolated as an endophyte it produces important substances such as griseofulvin, diketopiperazines and cytochalasins with antifungal, antioxidant and anticholinesterase activities (CHAPLA et al., 2018).

Therefore, it was possible to observe the presence of fungi with several biotechnological applications, which are associated with the medicinal plant *S. reticulata*. In this way, future studies should focus on bioprospecting these strains for industrial, pharmaceutical or agricultural purposes.

5. CONCLUSIONS

There is a wide variety of endophytic fungi in *Senna reticulata*, these being more frequent in stem, more isolated in Sabouraud medium with plant extract at 18 ºC, and the genus *Phomopsis* is dominant. This is the first report from the endophytic fungi community of the medicinal plant *Senna reticulata*.

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