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ARTIGO ORIGINAL

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Selection of endophytic fungi from the amazon plant Araçá-boi (Eugenia stipitata Mc Vaugh) producers of l-asparaginase

Seleção de fungos endofíticos de Araçá-boi (Eugenia stipitata Mc Vaugh) produtores de l-asparaginase

Bruno Jhosef Freires de Souza[1], Leandro Cavalcante Santos[2], Laura Nadyne da Silva Silvestre[3], Camila Ferreira Martins Freire[4], Adriana Valente de Oliveira[5], André Leonam Lopes Isquierdo[6], Geyse Souza Santos[7], Clarice Maia Carvalho[8].

RESUMO

Araça-boi (Eugenia stipitata), espécie vegetal nativa da região amazônica, com potencial nutricional e medicinal, porém, são poucos os trabalhos científicos desenvolvidos com a espécie, e nenhum com o potencial de seus fungos endofíticos. A enzima L-asparaginase catalisa a reação de quebra do aminoácido L-asparagina, que tem sido utilizado como quimioterápico para o tratamento do câncer. Em virtude do exposto, este trabalho teve como objetivo selecionar fungos endofíticos de Eugenia stipitata com potencial para produzir a enzima L-asparaginase. Fungos endofíticos foram isolados de folhas e caules de três indivíduos coletados em Rio Branco, Acre, Brasil. Os tecidos foram submetidos à desinfecção superficial e os fragmentos foram inoculados em meio Potato Dextrose Agar. Os fungos emergentes foram purificados e agrupados em morfoespécies, sendo identificados por meio de análise micromorfológica. Para avaliar a produção da enzima L-asparaginase, um representante de cada morfoespécie foi inoculado em meio BDA e incubado a 28 °C por 7 dias, com verificação da produção de L-asparaginase em Czapex Dox. Foram isolados 79 fungos endofíticos, 35 (44,3%) da folha e 44 (55,7%) do caule, agrupados em 33 morfoespécies de acordo com suas características macromorfológicas. Os gêneros identificados foram Aspergillus, Curvularia, Guignardia, Paecilomyces, Penicillium, Phomopsis e Xylaria, com maior frequência para o gênero Phomopsis 12,7%, seguido de Guignardia 8,9%. Dos 33 fungos endofíticos analisados, 24 produziram L-asparaginase, que pertencem aos gêneros Paecilomyces (6), Phomopsis (3), Aspergillus (2), Curvularia (1), Penicillium (1) e Xylaria (1). Os fungos endofíticos de Eugenia stipitata têm potencial para produzir L-asparaginase.

Palavras-chave: Antitumoral; Phomopsis; Guignardia; Paecilomyces.

ABSTRACT

Araça-boi (Eugenia stipitata), a plant species native from Amazon region, with nutritional and medicinal potential, however, there are few scientific works developed with the species, and none with the potential of its endophytic fungi. The enzyme L-asparaginase catalyzes the reaction of breaking the amino acid L-asparagine, which has been used as a chemotherapeutic agent for the treatment of cancer. Due to the above, this work aimed to select endophytic fungi from Eugenia stipitata with potential to produce the enzyme L-asparaginase. Endophytic fungi were isolated from leaves and stems of three individuals collected in Rio Branco, Acre, Brazil. The tissues underwent superficial disinfection and fragments were inoculated in Potato Dextrose Agar medium. The emerging fungi were purified and grouped into morphospecies, and identified through micromorphological analysis. To evaluate the production of the enzyme L-asparaginase, a representative of each morphospecies was inoculated in PDA medium and incubated at 28 °C for 7 days, with verification of production of L-asparaginase in Czapex Dox. A total of 79 endophytic fungi were isolated, 35 (44.3%) of the leaf and 44 (55.7%) of the stem, grouped into 33 morphospecies according to their macromorphological characteristics. The genera identified were Aspergillus, Curvularia, Guignardia, Paecilomyces, Penicillium, Phomopsis and Xylaria, with the highest frequency for the genus Phomopsis 12.7%, followed by Guignardia 8.9%. Of the 33 endophytic fungi analyzed, 24 produced L-asparaginase, which belong to the genera Paecilomyces (6), Phomopsis (3), Aspergillus (2), Curvularia (1), Penicillium (1) and Xylaria (1). The endophytic fungi from Eugenia stipitata have the potential to produce L-asparaginase.

Keywords: Antitumor; Phomopsis; Guignardia; Paecilomyces.

1 Bacharel em Engenharia Agronômica, Universidade Federal do Acre, Rio Branco, Acre, Brasil;
E-mail: Jhosef.bj21@gmail.com
http://orcid.org/0000-0002-0575-3314

2 Mestre em Ciência, Inovação e Tecnologia para a Amazônia, Universidade Federal do Acre, Rio Branco, Acre, Brasil;
http://orcid.org/0000-0002-0575-3314

3 Mestre em Ciência, Inovação e Tecnologia para a Amazônia, Universidade Federal do Acre, Rio Branco, Acre, Brasil.
https://orcid.org/0009-0002-1104-8352

4 Mestre em Ciência, Inovação e Tecnologia para a Amazônia, Universidade Federal do Acre, Rio Branco, Acre, Brasil.
http://orcid.org/0000-0002-8815-4252

5 Mestre em Ciência, Inovação e Tecnologia para a Amazônia, Universidade Federal do Acre, Rio Branco, Acre, Brasil.
http://orcid.org/0000-0002-8463-0610

6 Mestre em Ciências da Saúde na Amazônia Ocidental, Universidade Federal do Acre, Rio Branco, Acre, Brasil.
https://orcid.org/0009-0002-4578-8893

7 Doutora em Biodiversidade e Biotecnologia da Amazônia Legal, Universidade Federal do Acre, Rio Branco, Acre, Brasil.
http://orcid.org/0000-0003-2886-2959

8 Docente no Centro de Ciências

1. INTRODUCTION

Eugenia stipitata McVaugh belongs to the Myrtaceae family, being popularly known as araçá-boi, pé-de-yogurte, araçá-açu (FALCÃO et al., 2000), it is a native plant of the Brazilian Western Amazon, and can be found in other countries, such as Peru, Bolivia, Ecuador and Colombia (SACRAMENTO et al., 2008).

Eugenia stipitata produces a yellow-colored fruit, thin skin, juicy pulp, little fibrous and extremely acidic, however it has a pleasant flavor and aroma with high nutritional value, and can be used industrially for the production of ice cream, creams, juices and sweets (ANDRADE, 1997).

The fruit of E. stipitata has favorable economic potential, due to the low costs for cultivation and maintenance, without the need for high agricultural technology for production, in addition to being accepted by the taste of consumers (ANDRADE et al., 2021).

The fruit of E. stipitata may also contain a high content of phenolic compounds, carbohydrates, proteins and vitamins C and E, being considered therefore a potent antioxidant that strengthens the immune system (FIGUEROA-MÉNDEZ; RIVAS-ARANCIBIA, 2015) It is a fruit that has a low content of lipids and ash, but has a lot of moisture (SOUZA et al., 2018).

Eugenia stipitata is rich in bioactive compounds, which reduce oral, gastrointestinal, urogenital and intestinal inflammations in humans, in addition, it can be used in the treatment of diarrhea and bleeding (SOUZA et al., 2018).

In addition to the plant, the endophytic fungi of this plant species can also be studied. Several classes of molecules can be produced by endophytic fungi with clinical interest, such as antimicrobial (SPECIAN et al., 2014), antitumor (CREMASCO et al., 2009), antibiotic, antifungal, cytotoxic, antiviral, immunosuppressive, antiparasitic, among others (SCHULZ, BOYLE, 2005; ZHANG et al., 2006; DEMAIN et al., 2009; SILVA, 2010; ALY et al., 2010).

Endophytic fungi are microorganisms that live inside plant tissues, in symbiotic association and can spend all or part of their life cycle colonizing healthy plant tissues (BEZERRA et al., 2013; SONG et al., 2017).

Colonization can play an important role in the survival of the host plant, such as improved nutrient absorption (MALINOWSKI et al., 1999), increased resistance against certain pathogens (PIMENTEL et al., 2006), development and production of growth-

promoting metabolites, such as gibberellins (CHOI et al., 2005; RIM et al., 2005) and auxins (DAI et al., 2008), in addition to producing secondary metabolites for pharmaceutical use (SPECIAN et al., 2014).

Among the metabolites produced by endophytic microorganisms, the enzyme L-asparaginase can be highlighted, which has been used as a chemotherapeutic agent for the treatment of cancer in humans, such as acute lymphoblastic leukemia and lymphosarcoma (AVRAMIS; TIWARI, 2006), Hodgkin's lymphoma, acute myelomonocytic leukemia, chronic lymphocytic leukemia, reticulosarcoma and melanosarcoma (LOPES et al., 2017).

L-asparasiginase is an enzyme that catalyzes the breakdown reaction of the amino acid L-asparagine, which results in aspartic acid and ammonia (NARTA et al., 2007). In some leukemic cell lines, L-asparaginase causes these cells to be unable to produce enough L-asparagine, so they are dependent on extracellular source for this amino acid, and L-asparaginase degradation blocks protein synthesis, leading to with this, cell death by apoptosis (KRISHINA; NIBHA, 2012)

L-asparaginase from filamentous fungi has the advantage of being of eukaryotic origin, closer to human cells, different from those from bacteria, thus minimizing hypersensitivity reactions (AVRAMIS; TIWARI, 2006; SHRIVASTAVA et al., 2016).

According to the Brazilian Ministry of Health, L-asparaginase can be considered the standard method for the treatment of acute lymphocytic leukemia, being sold commercially under the names Elspar® by Merk, originated from Escherichia coli, and Erwinase by Speywood®, however, when used in the long term, they can trigger allergic reactions and anaphylaxis (VERMA et al., 2007).

Due to the great clinical interest, the need to know the biodiversity of endophytic fungi of Amazonian species, the search for new sources of L-asparaginase, and because there is no work on the endophytic fungi of E. stipitata, this study aimed to identify endophytic fungi from araçá-boi (Eugenia stipitata) producing L-asparaginase.

2. MATERIAL AND METHODS

Collection of botanical material and isolation of endophytic fungi

Leaf and stem were collected from three specimens of Eugenia stipitata in the city of Rio Branco, Acre, Brazil in September 2018 for the isolation of endophytic fungi (Figure 1).

After collection, the material was washed in running water and neutral detergent to remove excess epiphytes, organic matter and solid residues.

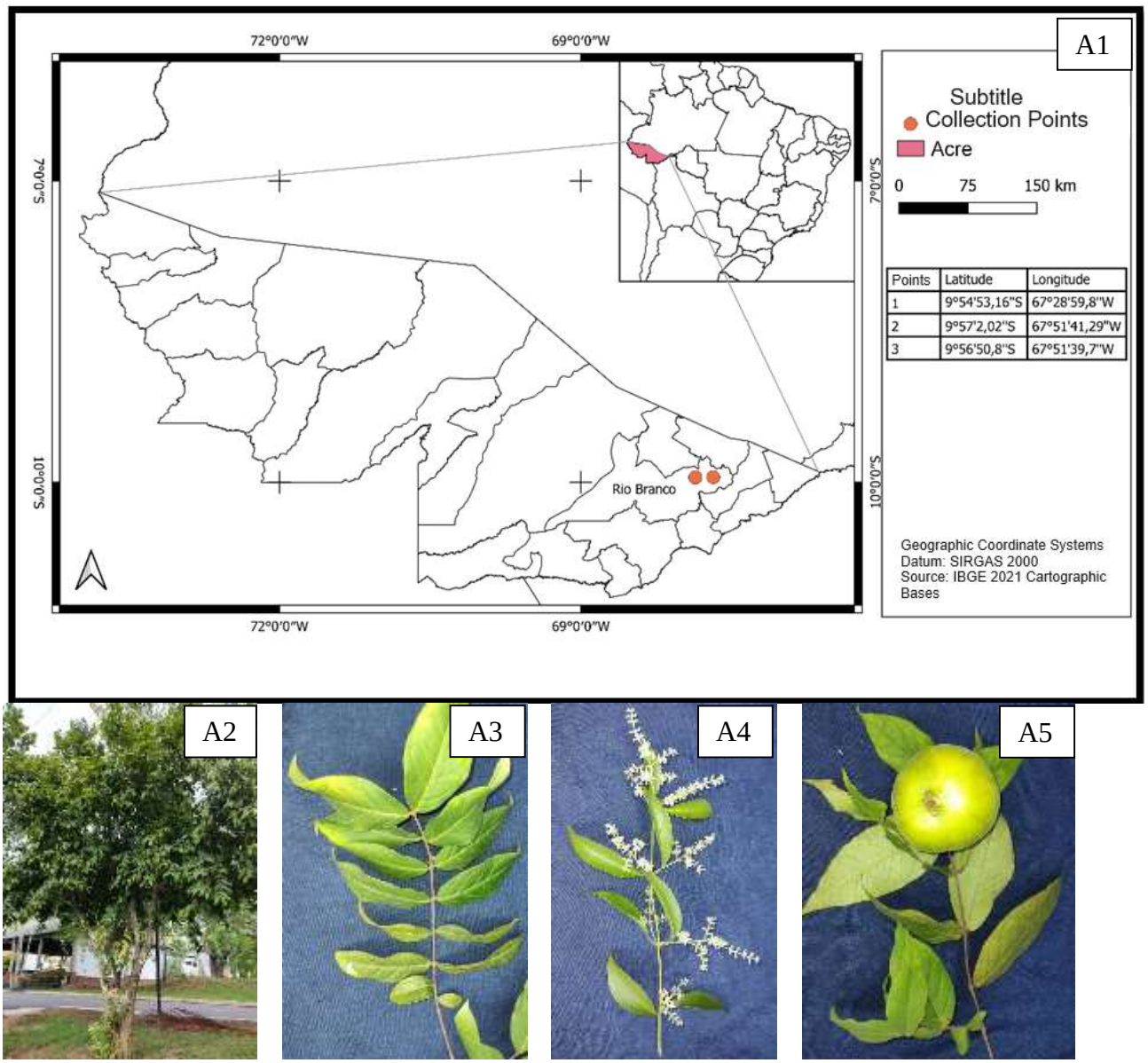

Figure 1. Leaf and stem collected from *Eugenia stipitata*. A 1. Collection locations. A2. Plant. A3. Sheet. A4. Inflorescence. A5. fruit.

In an aseptic chamber, the leaf and stem underwent a superficial disinfection process, by immersion in 70% alcohol for 1 min, 3% hypochlorite for 3 min, 70% alcohol for 1 min and washing in sterilized distilled water twice, where it was 50 µL of the last washing water was removed and inoculated in the Potato-Dextrose-Agar (PDA) medium for asepsis control (PEREIRA et al., 1993).

After disinfection, leaf discs and stem fragments measuring 5 mm in diameter were removed and inoculated into Petri dishes containing PDA medium supplemented with the antibiotic chloramphenicol (100 mg/L) and incubated at 28 ºC for up to 30 days. The fungal colonies were purified in PDA medium using the stripping technique by depletion, and then maintained in a tube containing inclined PDA medium. The isolated fungi were stored using mineral oil and distilled water techniques (ARAÚJO et al., 2002).

Morphological Characterization

For morphological characterization, a macroscopic analysis of the characteristics of the fungal colonies (color, texture, pigmentation and color of the back of the colony) was done, and fungi with similar characteristics were organized into morphospecies. A microscopic analysis of the reproductive structures, resistance structures or hyphae morphology was also done, using the microculture technique (SHIRLING; GOOTTLIEB, 1966), and compared to the reproductive structures with specific literature (BARNETT; HUNTER, 1998).

Qualitative evaluation of L-asparaginase production

For the qualitative evaluation of L-asparaginase-producing fungi, a representative of each morphospecies was used to analyze the degradation of the amino acid L-asparagine (GULATI et al., 1997), using Czapex Dox medium (2g glucose, 10g L-asparagine, KH2PO4 1.52g, MgSO4.7H2O 0.54g, Cu(NO3).3H2O 0.001g, ZnSO4.7H2O 0.001g, FeSO4.7H2O 0.001g, agar 15g to 1L of medium, 0.009% bromothymol blue) (THEANTANA et al., 2009).

Three fungal plugs measuring 5 mm in diameter were inoculated into plates containing Czapex Dox medium and incubated at 28 °C for 7 days, with a positive result for enzyme production, the formation of a purple zone halo due to the release of ammonia by the hydrolysis of asparagine and alkalinization of the medium (THEANTANA et al., 2009).

Statistical analysis

Descriptive analysis of isolation frequency in absolute values and percentage was performed. The factors analyzed were identified genera, numbers of morphospecies and number of species as a function of the isolation tissue (leaf and stem). All analyzes were performed using the Graphpad Prism 6 statistical program.

3. RESULTS

Isolation of endophytic fungi

79 fungal isolates were obtained from the three specimens of Eugenia stipitata, 35 (44.3%) from leaf and 44 (55.70%) from stem, which were grouped into 33 morphospecies according to their macromorphological characteristics.

The genera Aspergillus, Curvularia, Guignardia, Paecilomyces, Penicillium, Phomopsis and Xylaria were identified. Guignardia and Penicillium showed specificity for leaf, while Curvularia showed specificity for stem (Figure 2).

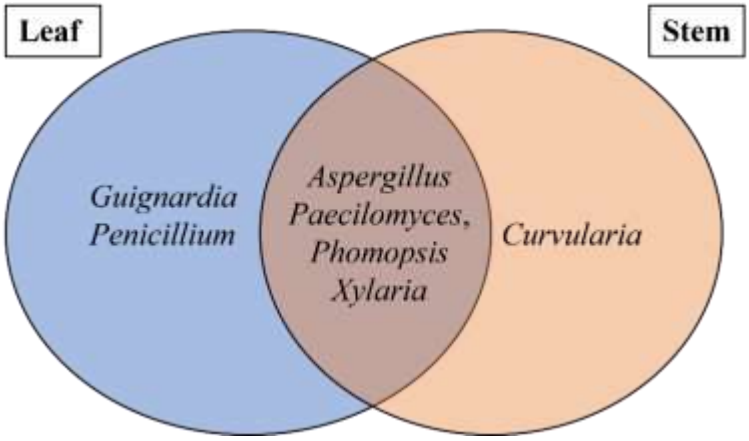

Figure 2. Venn diagram showing the distribution of the identified genera in the leaf and stem of the *Eugenia stipitata* plant.

The frequency of genera found on leaves and stems demonstrates that the most frequent endophytic fungi identified on leaves are Guignardia and Aspergillus, while on stems, the most frequent genera were Phomopsis and Paecilomyces (Table 1, Figure 3).

Table 1. Endophytic fungi isolated from Eugenia stipitata according to plant tissue.

Genus	Vegetal Tissue		Total	RF (%)
	leaf	stem		
Phomopsis sp.	2	8	10	12.7
Guignardia sp.	7	-	7	8.9
Paecilomyces sp.	2	5	7	8.9
Xylaria sp.	1	2	3	3.8
Aspergillus sp.	3	-	3	3.8

Penicillium sp.	1	-	1	1.3
Curvularia sp.	-	1	1	1.3
NI sp.1	3	5	8	10.1
NI sp.2	2	4	6	7.6
NI sp.3	2	**3**	5	6.3
NI sp.4	1	3	4	5.1
NI sp.5	1	2	3	3.8
NI sp.6	1	2	3	3.8
NI sp.7	1	2	3	3.8
NI sp.8	1	2	3	3.8
NI sp.9	1	1	2	2.5
NI sp.10	1	1	2	2.5
NI sp.11	1	1	2	2.5
NI sp.12	1	1	2	2.5
NI sp.13	1	1	2	2.5
NI sp.14	1	1	2	2.5
Total	**34**	**45**	**79**	**100.0**

NI= not identified.

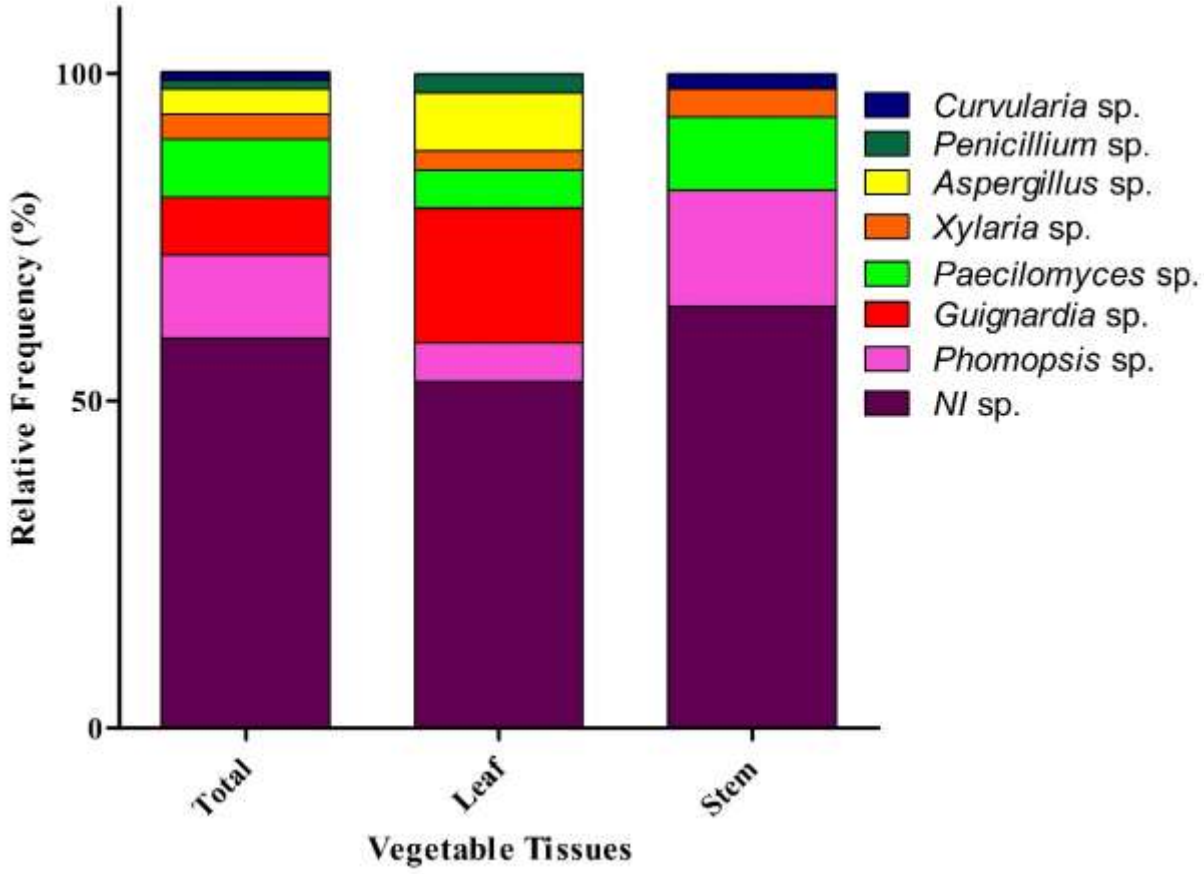

Figure 3. Relative frequency of endophytic fungi isolated from *Eugenia stipitata* according to plant tissue.
NI= not identified.

Evaluation of L-asparaginase production

The 33 morphospecies of endophytic fungi isolated from Eugenia stipitata were evaluated for the production of L-asparaginase and 24 had a positive result for the production of L-asparaginase by the production of purple halo in the culture medium, while the negative results did not cause color changes. (Figure 4).

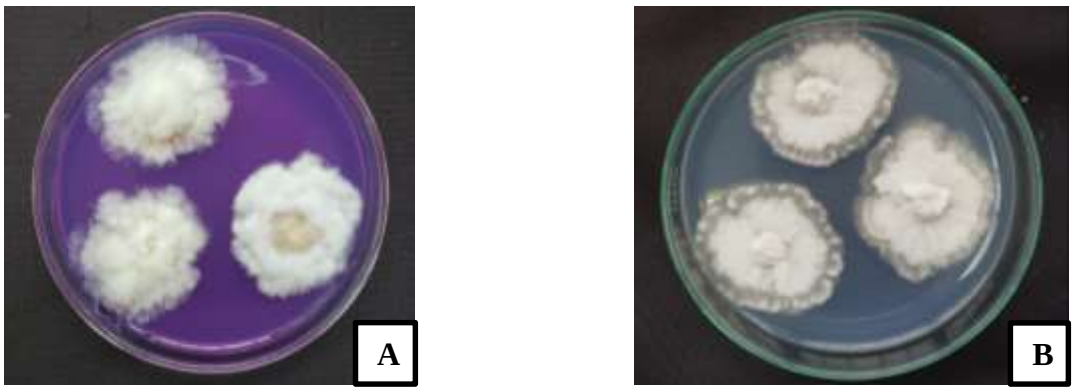

Figure 4. Qualitative evaluation of L-asparaginase-producing endophytic fungi of *Eugenia stipitata*. A. Positive for L-asparaginase production; B. Negative for L-asparaginase production.

According to Table 3, of the fungi analyzed for the production of L-asparaginase, fungi of the genera Paecilomyces (6), Phomopsis (3), Aspergillus (2), Curvularia (1), Penicillium (1) and Xylaria (1) had positive results.

Table 3. Qualitative analysis of L-asparaginase enzyme production by endophytic fungi isolated from Eugenia stipitata.

Fungus	Genus	L-asparaginase
2.6029	*Phomopsis* sp. 1	+
2.5991	*Phomopsis* sp. 2	+
2.5979	*Phomopsis* sp. 3	-
2.6020	*Phomopsis* sp. 4	-
2.5989	*Phomopsis* sp. 5	+
2.5962	*Guignardia* sp. 1	-
2.7966	*Paecilomyces* sp. 1	+
2.5976	*Paecilomyces* sp. 2	+
2.6007	*Paecilomyces* sp. 3	+
2.6021	*Paecilomyces* sp. 4	+

2..6037	*Paecilomyces* sp. 5	+
2.6000	*Paecilomyces* sp. 6	+
2.5975	*Xylaria* sp. 1	-
2.5988	*Xylaria* sp. 2	+
2.5970	*Xylaria* sp. 3	-
2.5971	*Aspergillus* sp. 1	+
2.6041	*Aspergillus* sp. 2	+
2.5969	*Penicillium* sp. 1	+
2.6004	*Curvularia* sp. 1	+
2.6035	NI sp. 1	+
2.5984	NI sp. 2	+
2.6023	NI sp. 3	+
2.5998	NI sp. 4	+
2.5981	NI sp. 5	-
2.5973	NI sp. 6	-
2.6009	NI sp. 7	-
2.5974	NI sp. 8	+
2.6015	NI sp. 9	-
2.6006	NI sp. 10	+
2.6003	NI sp. 11	+
2.6010	NI sp. 12	+
2.5963	NI sp. 13	+
2.6005	NI sp. 14	+
Total analyzed		**33**
Positive Total		**24**

4. DISCUSSION

79 fungi were isolated from the three specimens of Eugenia stipitata, 35 from leaf (44.3%) and 44 from stem (55.7%), which were grouped into 33 morphospecies. The amount of endophytic microorganisms present inside the plants can vary according to the tissues of the analyzed plant (RIBEIRO; PAMPHILE, 2017).

Variations in the amounts of endophytic microorganisms in the plant may result from several factors such the conditions of the place where they are found or even the physiology of the plant (JIA et al., 2016). In the stems are the conducting vessels, xylem and phloem, with higher production of secondary metabolites, such as essential oils, which can serve as a substrate for microorganisms that inhabit the region with protective actions against these metabolites (OTERO et al., 2002).

Similar data were found in the isolation of endophytic fungi from Bacaba (Oenocarpus

bacaba), where the amount of microorganisms isolated from the stem was higher than those isolated from the leaf (13.5%) (DINIZ et al., 2020) and endophytic fungi from noni (Morinda citrifolia) in the Amazon region (ARAÚJO et al., 2021). However, in a study with endophytes of cat's claw (Uncaria tomentosa) in the Amazon, the amount of endophytic fungi isolated from leaves was higher than those isolated from stems (FERREIRA et al., 2021).

The genera Aspergillus, Curvularia, Guignardia, Paecilomyces, Penicillium, Phomopsis, Xylaria, and 14 morphospecies were not identified as endophytic fungi in Eugenia stipitata. These genera have also been isolated as endophytic fungi in other works in western Amazonia. Aspergillus, Penicillium, Phomopsis and Xylaria were also isolated as endophytes in Murumuru (Astrocaryum ulei) (DINIZ et al., 2021) and in noni (Morinda citrifolia) (ARAÚJO et al., 2021) and Curvularia, Guignardia, Penicillium, Phomopsis and Xylaria on cat's claw (Uncaria tomentosa) (FERREIRA et al., 2021).

Thirty-three morphospecies of endophytic fungi isolated from E. stipitata were used to analyze the production of L-asparaginase, and 24 had a positive result, Paecilomyces (6), Phomopsis (3), Aspergillus (2), Curvularia (1), Penicillium (1), Xylaria (1) and 10 unidentified morphospecies.

Fungi of these genera have also been reported in other studies producing L-asparaginase, such as Aspergillus (SARQUIS et al., 2004), Aspergillus, Guignardia and Penicillium (ALMEIDA, 2015), Paecilomyces (ROCHA, 2017), Xylaria and Phomopis (LOPES, 2016).

The enzyme L-asparaginase is used in the antineoplastic treatment in the therapy of acute lymphocytic leukemia, because the amino acid L-asparagine is an essential substrate for the tumor cell due to the high demand caused by the exacerbated cell proliferation that, consequently, generates a greater metabolic demand, mainly for protein synthesis (RYTTING, 2012). This enzyme is strategically used to eliminate the constant cell replication of tumor cells and prevent the supply of amino acids necessary for the synthesis of proteins that allow the cell to function and survive, consequently inducing cellular apoptosis (GUILLEME et al., 2013; KRALL et al., 2016).

5. CONCLUSION

Endophytic fungi isolated from leaves and stems from Eugenia stipitata have the potential to produce L-asparaginase, the most promising belonging to the genera Aspergillus, Curvularia, Paecilomyces, Penicillium, Phomopsis and Xylaria.

However, it is worth emphasizing the importance of future scientific studies to complement the data found in the present work, and work is still needed to quantify enzyme production.

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