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Fungal endophyte diversity in the leaves of the medicinal plant *Myracrodruon urundeuva* in a Brazilian dry tropical forest and their capacity to produce L-asparaginase

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ABSTRACT

We studied the fungal endophyte community of the leaves of *Myracrodruon urundeuva* in Brazil along with their potential to produce L-asparaginase. In total, 187 endophytes were isolated. The colonization rate of leaf fragments from caatinga (10.41 %) was lower than those from *brejo de altitude* (39.58 %). Sequences of ITS rDNA from all endophytes revealed relationships with Ascomycota (Botryosphaeriales, Chaetothyriales, Diaporthales, Eurotiales, Glomerellales, Hypocreales, and Pleosporales) and Basidiomycota (Polyporales). The most frequent endophytes were members of the genus *Diaporthe*. *Talaromyces* was an indicator genus for caatinga while *Phyllosticta*, *Diaporthe*, and *Colletotrichum* were for *brejo de altitude*. The composition of endophytic fungi in caatinga and *brejo de altitude* differed significantly, its richness and diversity (H' = 3.41) being significantly higher in *brejo de altitude*. *Diaporthe* sp. URM 7793 was the best producer of L-asparaginase (2.41 U/g), followed by *Diaporthe* sp. URM 7779 (2.00 U/g), *Talaromyces* sp. URM 7785 (1.91 U/g), and *Diaporthe* sp. URM 7792 (1.47 U/g). The composition of endophytic fungi assemblages is strongly influenced by the type of ecosystem. In its natural habitat, *M. urundeuva* harbors an important diversity of endophytes, which could be used to produce L-asparaginase.

Keywords: Brazilian ecosystems, *Diaporthe*, endophytes, enzymes, fungal richness

Introduction

Endophytic fungi coexist with their hosts without causing damage to them, contributing to adequate conditions for survival under environmental stresses, such as high temperatures and nutrient scarcity in arid environments (Khidir *et al.* 2010). The relationship between endophytes and their respective plant hosts may increase in complexity if the hosts are medicinal plants (Zhao *et al.* 2011; Venieraki *et al.* 2017). The first such compound was "Taxol" a substance produced by the endophyte *Taxomyces andreanae* isolated from the medicinal plant *Taxus brevifolia* (Stierle *et al.* 1993), which brought with it great benefits to the study of anticancer substances. From this early research began the investigation of other plants regarding the diversity of endophytes and their capacity to produce several substances of biotechnological interest (Wang & Dai 2011; Chandra 2012).

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Medicinal plants are promising sources for the development of natural medicines, promoting an increase in the use of these plants worldwide (Chen *et al.* 2016). However, native medicinal plants are endangered because of their indiscriminate use as well as anthropogenic pressure on their natural habitat (Chen *et al.* 2016; Hassler 2018). *Myracrodruon urundeuva* (Anacardiaceae), which has a geographical distribution from Mexico to South America, is one such plant (Monteiro *et al.* 2012). In Brazil, the presence of this species has been verified mainly in the tropical dry forest (Caatinga forest) and in high altitude areas that comprise fragments of the Atlantic Forest in the middle of a semiarid region. These ecosystems are called *brejos de altitude* (a montane rainforest). They harbour various fauna and flora species that are endangered in Brazil owing to constant danger from anthropic, economic, and environmental pressure (Lorenzi 2008; IUCN 2017). *Myracrodruon urundeuva* presents great versatility—its branches and leaves have innumerable medicinal properties, mainly anti-inflammatory, antiulcerogenic, and cicatrizant activities—and some scientific research has investigated these potentialities (Mahmoud *et al.* 2011).

The diversity of endophytic fungi from some plants in the Caatinga forest, mainly Cactaceae species, has been reported (Bezerra *et al.* 2012; 2013; 2017a; b; Freire *et al.* 2015; Santos *et al.* 2015b). These studies have revealed a remarkable mycodiversity associated with the plants living in dry environments, contradicting some studies that have shown that the largest diversity of endophytes is mainly associated with plants in humid forests (Arnold *et al.* 2001; Banerjee 2011). Besides the great importance of the caatinga and *brejo de altitude* ecosystems in the preservation of biodiversity, several disturbances are present in these areas, mainly deforestation for agriculture and cattle breeding, causing pressure on the environment and affecting the balance in these ecosystems (Castelletti 2003; Porto *et al.* 2004).

To encourage the preservation of this diversity, some studies have shown the biotechnological capacity of endophytes from Brazilian dry areas to produce biomolecules of industrial interest, such as antibacterial agents and enzymes (Bezerra *et al.* 2012; 2015; Pires *et al.* 2015; Santos *et al.* 2015b). The percentage of endophytes producing biologically active compounds is approximately 80 %, which has prompted several studies to verify the capacity of these microorganisms to produce various metabolites (Liu *et al.* 2016; Venieraki *et al.* 2017).

Some endophytes have been studied for their capacity to produce the enzyme L-asparaginase (Santos *et al.* 2015a). This enzyme is widely used in the treatment of cancer; however, when produced by bacteria, it shows strong side effects in the patients (Loureiro *et al.* 2012; Lopes *et al.* 2017). Conversely, L-asparaginase produced by fungi has fewer side effects and, thus, has been investigated in several fungal species, mainly endophytes (Thirunavukkarasu *et al.* 2011; Santos *et al.* 2015a). Besides its pharmacological uses, L-asparaginase is also employed to decrease the formation of the carcinogenic substance acrylamide during the production of carbohydrate-rich, thermally treated foods (Pedreschi *et al.* 2008; 2011; Kumar *et al.* 2014).

Studies verifying L-asparaginase production by endophytes have garnered great interest because of the capacity of these microorganisms to produce the enzyme under laboratory as well as industrial conditions (Chow & Ting 2015; Santos *et al.* 2015a). To the best of our knowledge, this is the first study to report the diversity and biotechnological potential of endophytes from the medicinal plant *M. urundeuva* growing in the caatinga and *brejo de altitude* ecosystems. We aimed to investigate the diversity of endophytic fungi in the leaves of the medicinal plant *M. urundeuva* growing in two Brazilian ecosystems and investigate their capacity to produce the enzyme L-asparaginase.

Materials and methods

Study area

Samples of *M. urundeuva* Allemão (Anacardiaceae) were collected from two areas of the Brazilian tropical dry forest (Caatinga forest) in north-eastern Brazil, the municipalities of Serra Talhada (7°57'21.38"S 38°17'43.92"W) and Triunfo (7°49'32.99"S 38°6'54.21"O), Pernambuco state, during the dry season (July 2016) after one of the longest periods of drought in the last 50 years. These areas include different phytophysiognomies, which are influenced by the semiarid climate-type BSh, according to the classification of Köppen (1948), with an average annual temperature of 35 °C and an average annual rainfall of 380–800 mm, concentrated within a short period of two to four months followed by a long dry period lasting up to eight months.

The Serra Talhada area is a typical caatinga forest wherein plant species mainly belonging to the families Fabaceae, Euphorbiaceae, and Cactaceae are present (Santos *et al.* 2014). The Triunfo area, which is the other area, is called *brejo de altitude* and is an isolated fragment of humid forest (Atlantic Forest) present in the caatinga ecosystem. These fragments are islands of Atlantic Forest isolated on plateaus and mountain ranges varying from 600 to 1200 m in altitude surrounded by caatinga lowlands (Medeiros *et al.* 2017).

Plant collection and isolation of endophytic fungi

Leaves of the medicinal plant *M. urundeuva* were randomly collected from eight trees (length, 3-m tall) from each studied area, totalling 16 trees. The leaves were packed in paper bags and processed in the laboratory within 48 h. To isolate endophytic fungi, the plant material was processed as suggested by Bezerra *et al.* (2013; 2015). Leaf

fragments were placed on potato-dextrose-agar medium (PDA) supplemented with chloramphenicol (100 mg/l) and tetracycline (50 mg/l) to restrict bacterial growth. The Petri dishes were incubated at 28 °C ± 2 °C for up to 30 d. Fungal endophyte growth was observed every day, and any colony found was isolated, purified and maintained on PDA for later identification. To verify the efficacy of surface sterilization, 1 ml of water from the last rinses were inoculated onto Petri dishes containing the same medium and incubated under the same conditions.

Plant collections were registered to the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) under number 61879-1 and authentication code 89369941. Representative cultures of the isolated endophytes are deposited in the URM culture collection (Micoteca URM Profa. Maria Auxiliadora Cavalcanti ISO 9001:2015) at Universidade Federal de Pernambuco, Recife, Brazil.

DNA extraction, PCR, and sequencing

For genomic DNA extraction, all the endophytic fungi isolated were cultured on PDA, and the Wizard® Genomic DNA Purification Kit (Promega, USA) was used to perform the DNA extraction according to the manufacturer's instructions. The primers set ITS5 and ITS4 (White *et al.* 1990) was used to amplify part of the ITS region (first and second internal transcribed spacer regions and intervening 5.8S nrDNA). PCR, sequencing, and sequence analyses were performed as described by Bezerra *et al.* (2017a; b).

Phylogenetic analyses

Searches using the BLASTn tool in the GenBank database at NCBI were used to verify the phylogenetic relationships derived from all ITS rDNA sequences. Subsequently, selected sequences deposited in GenBank were aligned with the sequences obtained in this study to verify their relationships based on Maximum Likelihood (ML) and Bayesian Inference (BI) analyses. The online MAFFT interface (Katoh & Standley 2013) was used to perform the alignment, and MEGA v. 7.0 (Kumar *et al.* 2016) was used for sequences adjustments. ML analysis was performed using MEGA v. 7.0 with 1000 bootstrap replicates, and gaps were treated as missing data. BI analysis was performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) launched from Topali 2.5 (Milne *et al.* 2004) using two runs over 1×10^6 generations with a burn value of 25 %. The nucleotide substitution model was estimated using the online tool Findmodel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html), which suggested a Jukes–Cantor (JC) model. The tree obtained was printed with TreeView v. 1.6.6 (Page 1996). Newly generated ITS sequences were deposited in GenBank (MG870390–MG870542).

Colonization rate and absolute and relative frequencies

The endophytic colonization rate (TC) was obtained from the ratio between the number of colonized fragments (Nf) and the total number of fragments (Nt) from the plant tissue (TC = Nf/Nt × 100) (Araújo *et al.* 2002). The absolute frequency was calculated as the total number of endophytes isolated. For the relative frequency, the number of isolates of each genus was divided by the total number of isolates, and this value was multiplied by 100 (Larran *et al.* 2002).

Ecological and statistical analyses

We calculated the richness (S), Shannon-Wiener diversity index (H′) and Fisher's alpha of fungal endophytes for all samples. The richness was defined as the number of species in each sample and the Shannon-Wiener diversity index was calculated based on the equation $H' = -\Sigma(Pi \ln(Pi))$, where $Pi = ni/N$, ni = number of endophytic isolates of the species i and N = total number of endophytic isolates of all species (Shannon-Weaver 1949), and for statistical purposes, the values of H′ were converted into Exp (H′). Fisher's alpha was calculated based on the equation $S = \alpha^* \ln(1 + n/\alpha)$, where S is the number of taxa, n is the number of isolates, α is Fisher's alpha (Fisher *et al.* 1943). Species accumulation curves were determined, and total richness (observed) was compared with estimated richness using the first-order Chao index (Chao 1) to evaluate the sufficiency of the sampling effort.

Permutation multivariate analysis of variance (PERMANOVA), based on Bray-Curtis distance, was applied to test whether the endophytic fungi assemblages differed between ecosystems, and the variation in the composition of endophytic fungal community was visualized using non-metric multidimensional scaling (NMDS). These analyses were performed using relative abundance data.

Indicator species analysis (ISA) was performed to detect possible associations between endophytic fungal species and their respective environments. The indication values (IndVal) were calculated for each species and the significance was determined by the Monte Carlo test using 9999 permutations (Dufrêne & Legendre 1997). A species was considered an indicator for each area when it had $p < 0.05$ and IndVal ≥25 %.

All analyses were performed using R software v.3.4.3 (R Development Core Team 2017). PERMANOVA was conducted using the function adonis, NMDS was performed with metaMDS function and the richness (observed and estimated) and diversity of endophytic fungi in each sample was calculated using the "vegan" package (Oksanen *et al.* 2017). ISA was performed using "indicspecies" with 9999 permutations (Cáceres & Legendre 2009). The differences in richness and diversity of endophyte fungi between areas were analyzed using one-way analysis of variance (ANOVA) implemented in the *agricolae* package (Mendiburu 2017).

Screening of endophytic fungi for L-asparaginase production

For L-asparaginase production, 20 endophytes belonging to nine genera (7 isolates from caatinga and 13 from *brejo de altitude*) were randomly selected. L-asparaginase was produced and modified as per the studies by Loureiro *et al*. (2012) and Silva *et al*. (2018), respectively. Briefly, the liquid medium Czapek Dox's (Saxena & Sinha 1981), as modified by Gulati *et al*. (1997), was used in the pre-fermentation step; it contained (in g/L): glucose (14.0), L-asparagine (10.0), KH2PO4 (1.52), KCl (0.52), MgSO4•7H2O (0.52), CuNO3•3H2O (0.01), ZnSO4•7H2O (0.01), FeSO4•7H2O (0.01), and $(NH_4)_2SO_4$ (2.0). The pH of this medium was adjusted to 6.2, and 50 mL in Erlenmeyer flasks was inoculated with five discs (5 mm) of fungus mycelium and incubated with shaking at 120 rpm for 96 h at 30 °C. The cultures were filtered, and the mycelium was inoculated in fermentative medium, as described above, in the absence of $(NH_4)_2SO_4$. The glucose concentration was adjusted to 2 g/L, and the culture was reincubated under the same conditions.

L-asparaginase activity

L-asparaginase activity was determined according to Drainas *et al*. (1977) and modified as follows: 0.1 g of mycelium from each culture obtained during the fermentation step was macerated in 1.5 mL Tris-HCl buffer (20 mM, pH 8.6) and vortexed. To the samples, 0.2 mL (100 mM) of stock L-asparagine solution and 0.2 mL (1 M) of stock hydroxylamine solution (pH 7.0) were added, and they were incubated at 37 °C with shaking at 150 rpm. After 30 min, to stop the reaction, 0.5 mL ferric chloride reagent [10 % (w/v) FeCl3 plus 5 % (w/v) trichloroacetic acid in 0.66 mol·L HCl] was added to all samples and blanks (Tris-HCl and mycelium). The reaction mixture was centrifuged at 6000 rpm for 10 min at 4°C to remove precipitates. After 30 min of incubation, the absorbance was measured at 500 nm against the blanks, which comprised L-asparagine and hydroxylamine solutions. One unit of L-asparaginase (U) was defined as the amount of enzyme required to form 1 µmol of L-aspartic acid beta-hydroxamate per minute.

Statistical analysis of L-asparaginase activity

The nonparametric Kruskal–Wallis test was used to determine if there was a statistically significant difference ($p < 0.05$) between the results of L-asparaginase production by endophytic fungi from *M. urundeuva*. This test allows comparison of two or more independent samples of the same or different sizes and does not assume normality for the corresponding residuals. All calculations were performed using R software (R Development Core Team 2015).

Results

In total, 672 leaf fragments from *M. urundeuva* (336 from caatinga and 336 from *brejo de altitude*) were randomly collected from eight different trees in each area. The number of colonized fragments was 35 (10.41 %) from the caatinga and 133 (39.58 %) from the *brejo de altitude* ecosystems. A total of 187 endophytes were isolated (35 from the caatinga and 152 from the *brejo de altitude* ecosystems). Isolates were grouped on the basis of morphological features (macro- and micromorphology) and subjected to DNA extraction and analysis of the ITS rDNA sequences using tool BLASTn in the GenBank database at NCBI, which showed the presence of 12 genera (one exclusively from caatinga, seven exclusively from *brejo de altitude*, and four common to both areas). To confirm the results obtained in the BLASTn searches, phylogenetic analyses based on ML and BI were performed, which showed the presence of endophytes belonging to seven orders in Ascomycota (Botryosphaeriales, Chaetothyriales, Diaporthales, Eurotiales, Glomerellales, Hypocreales, and Pleosporales) and one order in Basidiomycota (Polyporales) (Fig. 1).

The isolates identified as *Diaporthe* were the most frequent in *M. urundeuva* (66 isolates belonging to 15 putative morpho-species), followed by *Phyllosticta* (32 isolates and one putative morpho-species), *Colletotrichum* (17 isolates and one putative morpho-species), *Talaromyces* (10 isolates and one putative morpho-species), *Penicillium* (seven isolates and three putative morpho-species), *Exserohilum* (five isolates and one putative morpho-species), *Sarocladium terricola* (3 isolates), and *Alternaria* (three isolates and two putative morpho-species). In addition, *Earliella scabrosa*, *Neofusicoccum*, *Rhinocladiella*, and a Diaporthales *incertae sedis* (one isolate) were isolated only once (Tab. 1). Endophytes belonging to *Exserohilum*, *Diaporthe*, *Penicillium*, and *Sarocladium* were observed in both areas, whereas *Talaromyces* was obtained only from the caatinga and *Alternaria*, *Colletotrichum*, *Earliella*, *Neofusicoccum*, *Phyllosticta*, *Rhinocladiella*, and a Diaporthales *incertae sedis* were isolated only from the *brejo de altitude* ecosystem. The genus *Talaromyces* (IndVal = 1; $p < 0.001$) was an indicator of the caatinga ecosystem, and the genera *Phyllosticta* (IndVal = 1; $p < 0.001$), *Diaporthe* (IndVal = 90 %; $p < 0.006$), and *Colletotrichum* (IndVal = 79 %; $p < 0.026$) were indicators of the *brejo de altitude* ecosystem.

The composition of endophytic fungal communities differed significantly between the caatinga and *brejo de altitude* ecosystems based on PERMANOVA analysis ($F = 12.25$, $R2 = 0.47$, $p < 0.0001$; Fig. 2). The species accumulation curve did not reach a *plateau*, but, using the first-order Chao index (Chao 1), the sampling effort was sufficient to recover 96 % and 71 % of estimated richness for the caatinga and *brejo de altitude* ecosystems, respectively (Fig. 3A-B). The richness and diversity of endophytic fungi based on Shannon index were significantly higher for *brejo*

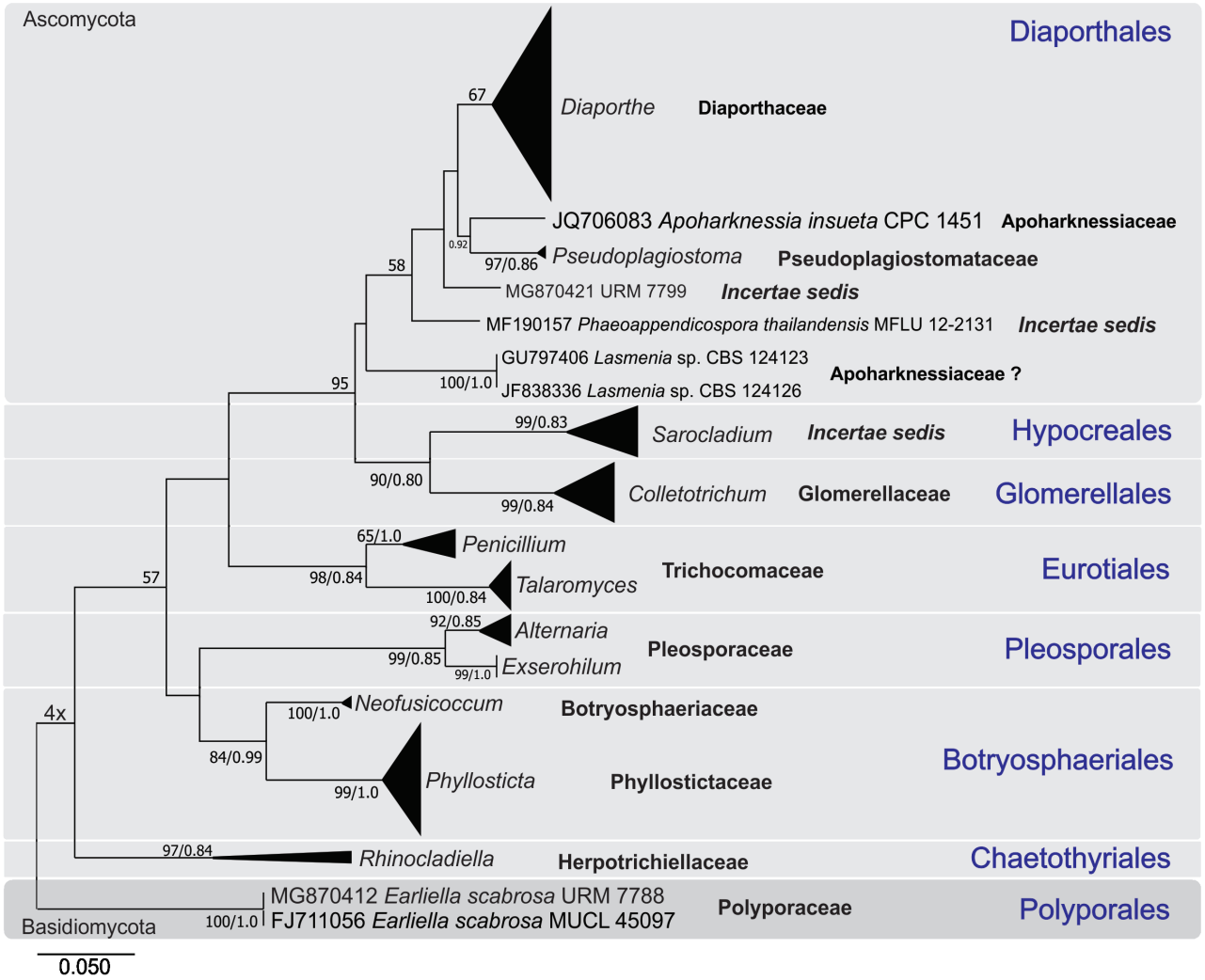

Figure 1. Maximum likelihood (ML) tree obtained from ITS rDNA sequences of 153 endophytic fungi isolated from leaves of the medicinal plant *Myracrodruon urundeuva* growing in a Brazilian tropical dry forest (Caatinga forest). *Earliella scabrosa* (Basidiomycota, URM 7788 and MUCL 45097) was used as outgroup. ML bootstrap support values above 50 % and posterior probabilities from BI above 0.80 are shown at nodes.

Table 1. Absolute and relative (%) frequency of endophytic fungi isolated from leaves of the medicinal plant *Myracrodruon urundeuva* in the Caatinga and *brejo de altitude* ecosystems in Brazil.

Endophytic fungi	Caatinga		brejo de altitude	
	f	fr	f	fr
Alternaria spp.			5	2.67
Colletotrichum sp.			17	9.09
Diaporthe spp.	11	5.88	55	29.41
Earliella scabrosa			1	0.53
Exserohilum sp.	5	2.67	3	1.60
Neofusicoccum sp.			1	0.53
Penicillium spp.	1	0.53	5	2.67
Phyllosticta sp.			34	18.18
Rhinocladiella sp.			1	0.53
Sarocladium terricola	2	1.06	1	0.53
Talaromyces sp.	10	5.34		
Diaporthales *incertae sedis*			1	0.53

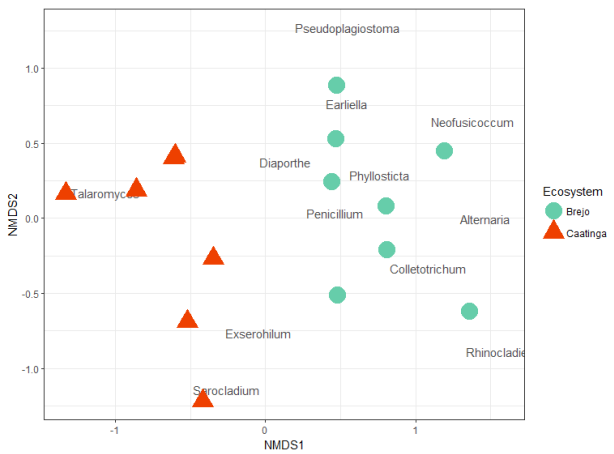

Figure 2. Nonmetric multidimensional scaling of endophytic fungi assemblages from leaves of *Myracrodruon urundeuva* in the caatinga and *brejo de altitude* ecosystems, Brazil.

de altitude than for caatinga (Fig. 4A-B), but there was no difference in diversity based on Fisher's index between the two areas (Fig. 4C).

Twenty endophytes were randomly selected for verification of their capacity to produce L-asparaginase in a liquid medium, of which four did not grow in the pre-fermentation liquid medium (*Exserohilum* sp. URM 7780, *Colletotrichum* sp. URM 7796, *Earliella scabrosa* URM 7788, and *Phylosticta* sp. URM 7797), three others did not show L-asparaginase activity and 13 demonstrated the ability

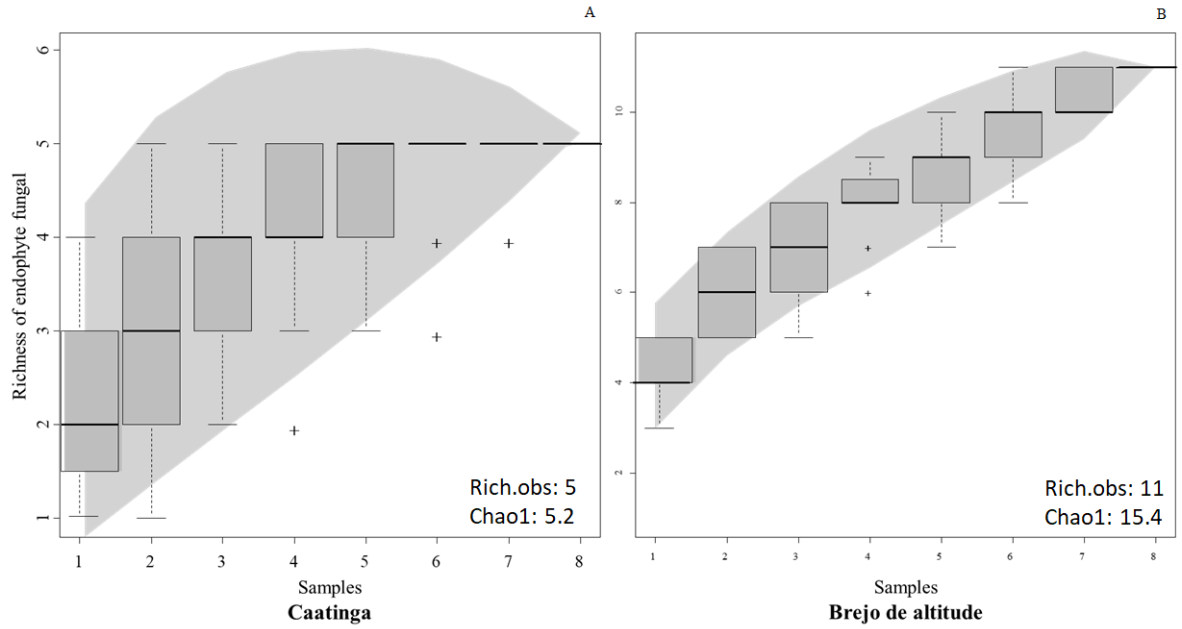

Figure 3. Species accumulation curves for endophytic fungi recovered from leaves of *Myracrodruon urundeuva*, showing the richness registered (Rich.obs) and estimated (Chao1) in the caatinga (**A**) and *brejo de altitude* (**B**) ecosystems, Brazil.

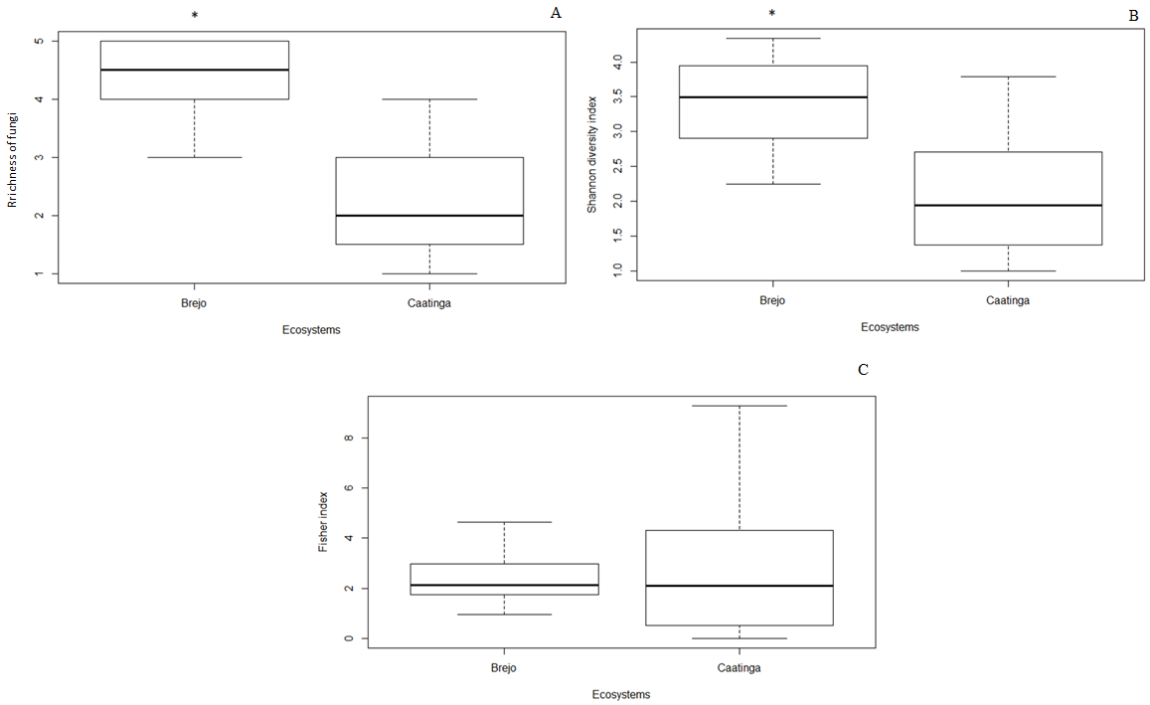

Figure 4. Boxplot indicating the richness (**A**) and diversity based on Shannon–Wiener (**B**) and Fisher indices (**C**) of endophytic fungi from leaves of *Myracrodruon urundeuva* in the caatinga and *brejo de altitude* ecosystems, Brazil. Asterisks (*) indicate significantly higher values of the evaluated attribute based one-way ANOVA. Median (central dot), quartile (box), maximum and minimum (whiskers) are shown.

to produce the enzyme (Tab. 2). The best L-asparaginase producers were *Diaporthe* sp. URM 7793, which showed an average production of 2.41 U/g, followed by *Diaporthe* sp. URM 7779 (2.00 U/g), *Talaromyces* sp. URM 7785 (1.91 U/g), and *Diaporthe* sp. URM 7792 (1.47 U/g), whereas other *Diaporthe* strains URM 7784, URM 7794, and URM 7795 did not produce the enzyme. Other isolates, such as *Sarocladium terricola* URM 7778 and *Diaporthe* sp. URM 7791, produced an average of ≤1.37 U/g of the enzyme. The Kruskal–Wallis test showed a statistically significant difference between L-asparaginase production by endophytes from *M. urundeuva* (p = 0.0144; Tab. 2). When L-asparaginase production by different isolates was compared, no significant difference was observed among the averages obtained for *Diaporthe* sp. URM 7793, *Diaporthe* sp. URM 7779, *Talaromyces* sp. URM 7785, and *Diaporthe* sp. URM 7792. However, there was a significant difference between the enzyme production obtained from the isolate *Diaporthe* sp. URM 7793 and other isolates with a production of less than or equal to 1.37 U/g.

Table 2. L-asparaginase activity obtained by endophytic fungi isolated from leaves of the medicinal plant *Myracrodruon urundeuva* in the Caatinga and *brejo de altitude* ecosystems in Brazil.

Endophytic fungi	URM	Ecosystem*	Activity 1 (U/g)	Activity 2 (U/g)	Average§ (U/g)
Diaporthe sp.	7793	C	2.25	2.57	2.41^{a}
Diaporthe sp.	7779	C	2.63	1.38	2.00^{ab}
Diaporthe sp.	7792	B	1.36	1.59	1.47^{abc}
Sarocladium terricola	7778	C	1.24	1.51	1.37^{bc}
Diaporthe sp.	7791	C	1.30	1.19	1.25^{cd}
Phylosticta sp.	7787	B	0.58	0.55	0.57^{fg}
Talaromyces sp.	7785	C	1.78	2.05	1.91^{ab}
Colletotrichum sp.	7782	B	0.69	0.72	0,71^{ef}
Penicillium sp.	7790	B	0.70	0.70	0.70^{ef}
Altenaria sp.	7783	B	0.70	0.76	0.73^{e}
Exserohilum sp.	7789	B	0.73	0.77	0.75^{de}
Penicillium sp.	7786	C	0.93	0.57	0.75^{ef}
Colletotrichum sp.	7781	B	0.80	0.75	0.77^{de}
Diaporthe sp.	7784	B	0.00	0.00	0.00^{g}
Diaporthe sp.	7794	B	0.00	0.00	0.00^{g}
Diaporthe sp.	7795	B	0.00	0.00	0.00^{g}

* Ecosystems: C = Caatinga and B = brejo de altitude.

§ Average followed by the same letter do not differ significantly ($p > 0.05$).

Discussion

Several surveys have demonstrated the high diversity of endophytic fungi associated with medicinal plant species worldwide (Venieraki *et al.* 2017). In Brazil, some researchers have also demonstrated the diversity and the biotechnological potential of these fungi to produce biomolecules with economic importance in the agricultural, industrial, and medical sectors (Siqueira *et al.* 2011; Bezerra *et al.* 2015; Santos *et al.* 2015a; Azevedo & Quecine 2017). However, studies examining the diversity and biotechnological potential of endophytes from semiarid regions of Brazil, such as the Caatinga forest and the *brejo de altitude* ecosystem, are either incipient or have not been conducted (Bezerra *et al.* 2012; 2013; 2017a; b; c; Freire *et al.* 2015; Pires *et al.* 2015; Santos *et al.* 2015a).

The medicinal plant *M. urundeuva* has an important ecological role in the caatinga and *brejo de altitude* ecosystems, where this species contribute with native pollinators (Gaino *et al.* 2011); however, the diversity of endophytic fungi from this species has not been studied in these two ecosystems, to the best of our knowledge, this study is the first report of endophytes and their biotechnological potential to produce L-asparaginase. Although some surveys (*e.g.* Freire & Gonçalves 2012; Cavalcanti *et al.* 2017) have reported the isolation of endophytes from the tissues of *M. urundeuva*, the main goal of these studies was to evaluate the biotechnological potential of these endophytic fungi. In Brazil, Bezerra *et al.* (2015) studied the diversity of endophytic fungi from different tissues of the medicinal plant *Bauhinia forficata* and obtained 95 endophytes, of which 18 were isolated from leaves. Huang *et al.* (2008) reported an isolation rate of approximately 74 % in a study of endophytic fungi from leaves, stems, flowers, fruits, and roots of traditional Chinese medicinal plants.

Other studies of endophytic fungi isolated from different medicinal plants, such as the works of Kharwar *et al.* (2008) in India (*Catharanthus roseus*; colonization rate, 34 %), Banhos *et al.* (2014) in Brazil (*Myrcia guianenses*, 53.3 %), and Siqueira *et al.* (2011) in Brazil (*Lippia sidoides*, 50.41 %), obtained a colonization rate similar to that observed in this study. Recently, Toghueo *et al.* (2017) studied the composition of endophytic fungal community of three medicinal plants (*Cananga odorata*, *Terminalia catappa*, and *Terminalia mantaly*) in Cameroon and obtained a leaf colonization rate of 20.0 %–100.0 %, demonstrating a variation in the colonization of the same tissue in different plants. Another study by Mussi-Dias *et al.* (2012) reported the isolation of only 20 endophytes from 11 medicinal plants in Brazil, contradicting the results obtained from leaves of *M. urundeuva* and other medicinal plants, from which several endophytic fungi were isolated. In Brazil, Bezerra *et al.* (2015) obtained 95 endophytes from 180 fragments of different tissues of the medicinal plant *B. forficata*, and Nascimento *et al.* (2015) found a colonization rate of 32.1 % in leaves of *Calotropis procera*, from which 156 endophytes were isolated, mainly from the oldest leaves (74 fungal isolates and 11 species). These results are similar to those obtained in the present study: the colonization rate was 10.41 % in fragments from the caatinga and 39.58% in those from the *brejo de altitude* ecosystem.

The composition of endophytic fungi assemblages is strongly influenced by the type of ecosystem in which they are found. The number of endophytic fungi isolated from the *brejo de altitude* ecosystem was higher than that

isolated from the caatinga ecosystem. Some authors have demonstrated that fungal endophyte diversity may be influenced by diverse biotic and abiotic factors, such as the type and age of plant tissues; heterogeneous profile of micro-habitats; relationship between fungi, hosts, and substrates and climate and vegetation changes (Nascimento *et al.* 2015; Koide *et al.* 2017). According to Rodriguez *et al*. (2009), variations in solar intensity and exposure are a determining factor in the composition of the endophytic fungal community. Moreover, ultraviolet radiation in semiarid regions, such as the Caatinga forest in Brazil, can have a negative influence on the richness and diversity of these fungi. The importance of pigmented endophytes in these areas was pointed out by Bezerra *et al*. (2013; 2017a) when they highlighted that these fungi from caatinga can confer host plant tolerance to extreme environmental conditions, including ultraviolet radiation. In this scenario, *brejos of altitude*, a humid forest in the middle of the dry forest (Caatinga) is an ecosystem more favourable to the maintenance of a diverse community of endophytic fungi.

Endophytes belonging to *Diaporthe*, *Phyllosticta*, and *Colletotrichum* are frequently isolated (Murali *et al.* 2007; Singh *et al.* 2017; Bezerra *et al.* 2018). Species of these genera are commonly reported as endophytic fungi from medicinal crops; endemic, natural, and invasive plants; and economically important plants. Members of these genera are also reported to be the causes of several serious plant diseases on fruits (e.g. *Citrus* and banana), grains, cereals, ornamentals, and forest plants (Bezerra *et al.* 2017a; Dissanayake *et al.* 2017; Guarnaccia *et al.* 2017).

Interestingly, endophytes identified as *Colletotrichum* and *Phyllosticta* were isolated only from the *brejo de altitude* ecosystem. Bezerra *et al*. (2012) and Freire *et al*. (2015), studying a Cactaceae species from the caatinga ecosystem, did not isolate species belonging to these two genera. In a temperate forest in Japan, Hata *et al*. (2002) reported species of *Colletotrichum* and *Phyllosticta* as the most common endophytes. Another group of isolates, *Diaporthe* species, were isolated in both ecosystems but were more frequently recovered in *brejo de altitude*. Species of *Diaporthe* are widely reported as endophytes of several plants living in dry environments (Murali *et al.* 2007; Gomes *et al.* 2013; Bezerra *et al.* 2017a), and recently, Crous *et al*. (2016) proposed a *Diaporthe* species, named *D. caatingaensis*, to accommodate some strains isolated from the cactus *Tacinga inamoena* in Brazil. The dominance of the same endophytic fungal genus may influence mycodiversity, as was pointed out by Murali *et al*. (2007), who found that species of *Colletotrichum*, *Phomopsis* [*Diaporthe*], and *Phyllosticta* were dominant in dry thorn and dry deciduous forests of India. *Diaporthe* species were also dominant in the fungal endophyte community of the tree *Tectona grandis* in a tropical forest in India (Singh *et al.* 2017). Some endophytes, such as *Earliella scabrosa*, *Rhinocladiella* sp., and a Diaporthales *incertae sedis*, were isolated only once and were also reported as rare isolates in other surveys (e.g. Silva-Hughes *et al.* 2015; Ferreira *et al.* 2017).

The diversity of endophytic fungi observed in this study was similar to that in other surveys conducted in dry environments (Fisher *et al.* 1994; Khidir *et al.* 2010; Bezerra *et al.* 2012; 2013; Loro *et al.* 2012; Silva-Hughes *et al.* 2015). The exception was the *brejo de altitude* ecosystem, which shared a higher degree of diversity similarity with humid forests than with the caatinga ecosystem.

The community of endophytic fungi from *M. urundeuva* is very diverse, as indicated by the mean values of diversity indices (caatinga: H′ = 2.10 and Fisher-Alpha value = 2.30; *brejo de altitude*: H′ = 3.41, Fisher-Alpha value = 2.40). Some surveys of endophytes conducted in the caatinga ecosystem with Cactaceae species demonstrated diversity index values similar to those obtained in our study (H′ = 2.991 and Fisher-Alpha value = 9.167–12.52, Bezerra *et al.* 2013). Verifying the diversity and enzymatic potential of endophytes from the medicinal plant *Bauhinia forficata* in Brazil, Bezerra *et al*. (2015) showed a Shannon-Wiener index value of 1.842 in leaves and 1.677–2.206 in other plant organs (sepals, seeds and stems). Verifying the diversity and anticancer and antimicrobial potential of fungal endophytes associated with the medicinal plant *Monarda citriodora* in India, Katoch *et al*. (2017) observed an index value of H′ = 2.890 in leaves, while the Fisher-Alpha index did not indicate the presence of diversity. These results indicate that the diversity of endophytic fungi associated with the leaves of *M. urundeuva* is high and that it is influenced by the ecosystem in which the plant is collected.

The accumulation curves obtained in this study showed that the endophytic fungal community was not recovered in its entirety. Surveys on endophyte diversity conducted in dry environments, including the Caatinga forest, also demonstrated that the endophytic fungi diversity could not be entirely recuperated (Bezerra *et al.* 2013; Silva-Hughes *et al.* 2015; Dastogeer *et al.* 2018). Arnold & Lutzoni (2007), studying tropical humid forest, also demonstrated the incapacity of isolation of all the estimated endophytic species living in a host. Although the fungal endophytic community was closely recovered from leaves of *M. urundeuva*, future studies accessing this diversity may isolate some new endophytes not reported in this survey.

Endophytic fungi from medicinal plants have the capacity to produce similar biological compounds that have been reported from plant tissues (Venieraki *et al.* 2017). In this study, endophytes from caatinga were the most important producers of L-asparaginase. Santos *et al*. (2015a), verifying the capacity of endophytes isolated from the cactus *Cereus jamacaru* in caatinga to produce L-asparaginase, pointed out that species of *Aspergillus*, *Fusarium*, and *Penicillium* were the most important endophytes indicated to produce L-asparaginase, highlighting *A. sydowii* (29.02 U/ml) as the best producer. *Diaporthe* isolates were the highest L-asparaginase producers in this study. Some surveys have

highlighted that species of *Diaporthe* have a great capacity to produce several metabolites, including anticancer drugs (Udayanga *et al.* 2011; Abreu *et al.* 2012).

Other strains, such as *Talaromyces* sp. URM 7785 and *Sarocladium terricola* URM 7778, also demonstrated a high capacity to produce L-asparaginase. Species of *Talaromyces* are widely reported to have the biotechnological capacity to produce different metabolites. For example, Krishnapura & Belur (2016) reported that *T. pinophilus* isolated as an endophyte from the rhizomes of *Curcuma amada* was the best producer of L-asparaginase, exhibiting up to 98% of total activity. Similar results were obtained by Silva *et al.* (2018), who related the L-asparaginase activity from endophytes isolated of the bromeliad *Tillandsia catimbauensis*. The authors verified *Talaromyces* cf. *cecidicola* as the best L-asparaginase producer (2.30 U/g). *Sarocladium* species are often reported in association with grasses, as parasites and as mutual endophytes (Summerbell *et al.* 2011), and have also been reported in association with the Chinese medicinal plant *Salvia miltiorrhiza* (Lou *et al.* 2013). In the literature, we did not find evidence of *S. terricola* as a producer of L-asparaginase, making this the first study to report this. A few other studies were also conducted with endophytes isolated from Thai medicinal plants (Theantana *et al.* 2007; 2009) and from seaweeds in India (Thirunavukkarasu *et al.* 2011) to verify the capacity of endophytic fungi to produce L-asparaginase.

Our results of studying the fungal endophyte community from leaves of *M. urundeuva* in the caatinga and *brejo de altitude* ecosystems in Brazil suggest that this native medicinal plant growing in its natural environment harbours a remarkable diversity of endophytic fungi. The *ex situ* preservation of these endophytes in the URM Culture Collection in Brazil can contribute to other studies for the optimization of L-asparaginase production. These endophytes may perform important mechanisms to guarantee the plant's ability to live in different areas, and they are also sources of bioactive molecules, such as the enzyme L-asparaginase, which could contribute to several biotechnological applications in the food and drug industries.

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