



Fungal endophytes from leaves of *Mandevilla catimbauensis* (Apocynaceae): diversity and potential for L-asparaginase production

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Abstract

In this study, we examined endophytic fungi in leaves of *Mandevilla catimbauensis*, an endemic plant species found in the Brazilian dry forest (Caatinga), and endophytic fungi's potential to produce L-asparaginase (L-ASNase). In total, 66 endophytes were isolated, and the leaf-fragment colonisation rate was 11.78%. Based on morphology, internal transcribed spacer (ITS), and partial large subunit (LSU) of ribosomal DNA sequencing, the endophytic fungi isolated belonged to six Ascomycota orders (Botryosphaerales, Capnodiales, Diaporthales, Eurotiales, Marthamycetales, and Pleosporales). *Phyllosticta* species were the most frequent endophytes isolated (23 isolates [45.1%] from two species). The Shannon–Wiener and Fisher alpha index average values were 0.56 and 3.26, respectively. Twenty endophytes were randomly selected for the L-ASNase production test, of which fourteen isolates showed potential to produce the enzyme (0.48–2.22 U g⁻¹), especially *Phyllosticta catimbauensis* URM 7672 (2.22 U g⁻¹) and *Cladosporium* sp. G45 (2.11 U g⁻¹). *Phyllosticta catimbauensis* URM 7672 was selected for the partial optimisation of L-ASNase production because of its ability to generate considerable amounts of enzyme. We obtained the highest L-ASNase activity (3.47 U g⁻¹), representing an increase of 36.02% in enzymatic production, under the following experimental conditions: a pH of 4.2, 1.0% inoculum concentration, and 2.5% L-asparagine concentration. Our study showed that *M. catimbauensis* harbours an important diversity of endophytic fungi with biotechnological potential for L-ASNase production.

Keywords Caatinga forest · Endophytes · Fungal enzyme · *Phyllosticta* · L-asparaginase optimisation

Introduction

Endophytic fungi inhabit intra- and inter-cellular plant tissues without causing harmful effects on plants. These microorganisms may alter gene expression, modulate biosynthetic pathways, mitigate stressful conditions in

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plants, and play an important role in establishing the plant defence system against potential pathogens. Endophytic fungi are a potential resource for biosynthesis, biotransformation, and biodegradation because they produce many secondary metabolites [1, 2]. Therefore, investigating the fungi inhabiting plant species with medicinal properties can lead to the discovery of new metabolites with potential bioactivity [2].

Many studies on endophytic fungi have been conducted in humid and temperate tropical forests [3, 4]. However, arid, semiarid, and desert areas, such as the Brazilian tropical dry forest (Caatinga), are also promising environments in terms of fungal species diversity [5–7]. Studies conducted in Caatinga sites have contributed to the description of new fungal endophytes with biotechnological potential [6–8]. These studies have described new fungal endophytes, suggesting that the real mycobiome richness has been underestimated [2, 5, 9].

The Caatinga is a tropical dry forest covering a large part of northeastern Brazil and harbours multiple numbers of plants, animals, and microorganisms [10–13]. Considering the large-scale destruction because of anthropogenic interference and to guarantee the maintenance of Caatinga diversity, some areas of this ecosystem have been selected to be conservation units. One important conservation unit is the Catimbau National Park (Parque Nacional do Catimbau), the second largest archaeological park in Brazil, which presents a lush landscape and rich diversity [14]. Catimbau National Park harbours several endemic and exclusive plant species, including *Mandevilla catimbauensis* Souza-Silva, Rapini & JF Morales (Apocynaceae), a rare and endemic species that is vulnerable to extinction [15, 16]. *Mandevilla catimbauensis* is an herbaceous and climbing plant mainly recognised by its twining habit and it is only found in the Catimbau National Park [15, 17]. *Mandevilla* is considered the largest neotropical genus of the family Apocynaceae, and it is represented by about 40 species in Brazil [15]. *Mandevilla* species have medicinal importance in secondary chemical compound production with anti-inflammatory, antinociceptive, and antipyretic properties [18].

Several fungi isolated from dry environments have shown a potential for production of biomolecules of industrial interest, such as L-asparaginase (L-ASNase) [6, 7, 9]. L-ASNase has been used as a drug in anti-leukaemia chemotherapy, but there are several adverse effects when this enzyme is produced by bacteria, which has led the scientific community to search for alternative sources [19]. In addition to its clinical use, L-ASNase has been used by the food industry to reduce the formation of acrylamide, a harmful toxin formed during high-temperature food processing [20]. The L-ASNase used by the food industry has a fungal origin [21], and the increased use of the enzyme has motivated the search for

fungal resources capable of meeting the high-L-ASNase demand of pharmaceutical and food industries [19, 22].

Endophytic fungi are a limitless source of novel metabolites, and endophytes from plants growing in particular ecological niches may have the ability to produce a variety of secondary metabolites [1]. In this study, we investigated, for the first time, the diversity and biotechnological potential of endophytic fungi from the plant *M. catimbauensis*, which grows in the Caatinga dry forest. We investigated the hypothesis that leaves of *M. catimbauensis* harbour a rich and diverse endophytic fungal community with the potential to produce L-ASNase. In order to test this hypothesis, our study aimed to analyse the diversity of endophytic fungi living in healthy *M. catimbauensis* leaves and verify the capacity of the endophytes to produce L-ASNase.

Materials and methods

Study area

This study was carried out at the Parque Nacional do Catimbau (8° 24' 00" and 8° 36' 35" S; 37° 09' 30" and 37° 14' 40" W), a federal conservation unit in the Caatinga dry forest, Pernambuco State, Brazil (Fig. 1a). The climate is semiarid and is classified as BSh according to Köppen's scale [23]. It has an average annual temperature and rainfall of 23 °C and 650–1100 mm, respectively, with March and October being the wettest and driest months, respectively. The park contains heterogeneous vegetation, with four distinct vegetation types: rupestrian grasslands, semi-deciduous and evergreen vegetation, and small deciduous trees and shrubs [12].

Plant collection and isolation of endophytic fungi

Mandevilla catimbauensis leaves (Fig. 1b) were sampled in May 2015 during one of the longest drought periods in a sedimentary area of the Caatinga dry forest from Serra de Jerusalém (at an altitude of 910 m), Catimbau National Park, Buíque municipality (Fig. 1a). Leaves from 20 individuals were randomly collected, packed in paper bags, and processed in the laboratory within 24 h. The endophytic fungi were isolated by processing the leaves according to the method proposed by Bezerra et al. [24]. After disinfection, the leaf fragments were placed in Petri dishes containing potato dextrose agar (PDA) medium supplemented with chloramphenicol (100 mg L⁻¹) and tetracycline (50 mg L⁻¹) to restrict bacterial growth. The Petri dishes were incubated at 28 ± 2 °C for up to 30 days under a natural light cycle. Fungal endophyte growth was observed every day, and any colony found was isolated, purified, and maintained on PDA for later identification. The efficacy of the surface sterilisation process was verified by inoculating 1 mL of water used

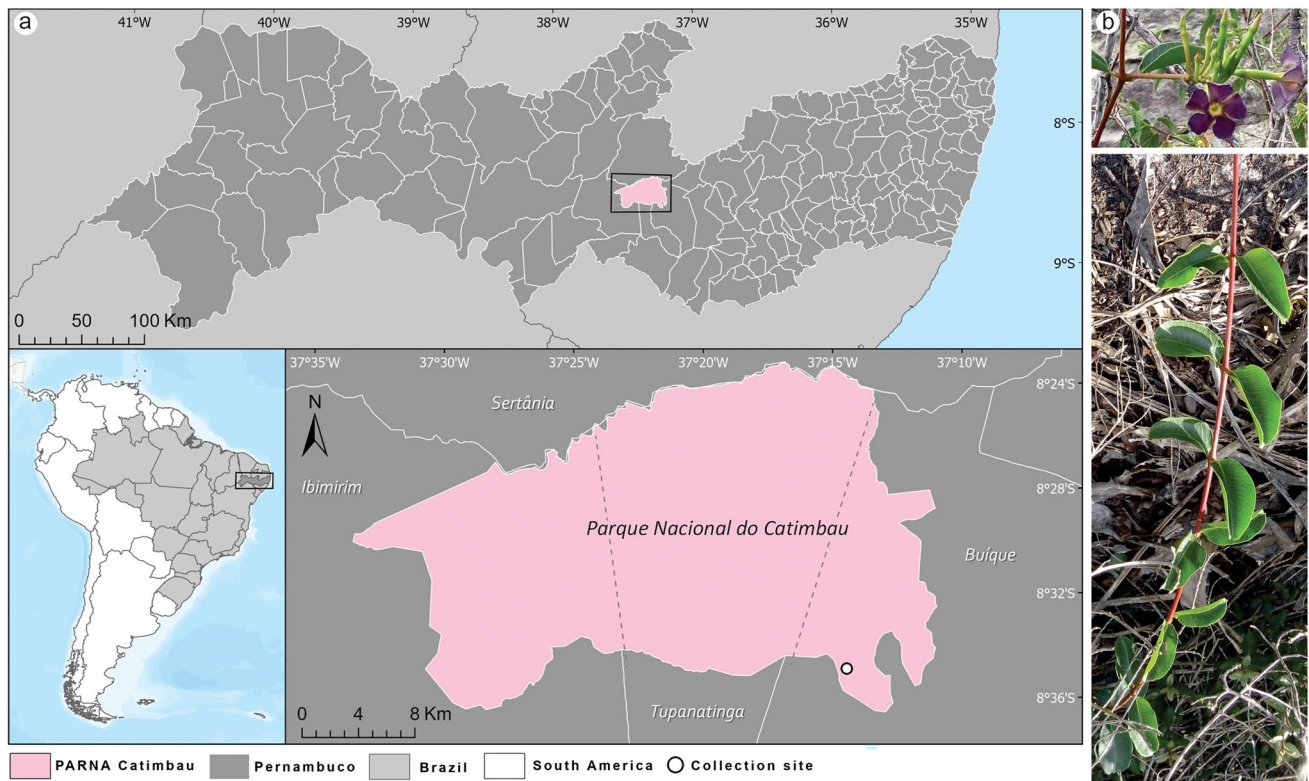


Fig. 1 a The geographical location of the Parque Nacional do Catimbau (Catimbau National Park), Brazilian tropical dry forest (Caatinga). b *Mandevilla catimbauensis* in the Catimbau National Park

during the last rinses onto Petri dishes containing the same medium, followed by incubation under the same conditions.

A specimen of *M. catimbauensis* has been deposited in the Herbario UFP – Geraldo Mariz under number 81.210, and representative cultures of the endophytes are deposited in the URM culture collection (Micoteca URM Profa. Maria Auxiliadora Cavalcanti). Both collections are located at the Universidade Federal de Pernambuco (Recife, Brazil). The plant collections were authorised by MMA/ICMBio (SIS-BIO number 48492–1).

DNA extraction, PCR, sequencing, and phylogenetic analyses

All the isolated endophytic fungi were cultured on PDA, and the DNA was extracted using the Wizard® Genomic DNA Purification kit (Promega, USA) according to the manufacturer's instructions. The internal transcribed spacer (ITS) and part of the nuclear ribosomal small subunit (LSU) regions of the rDNA were used to identify endophytic fungi. PCR, sequencing, and sequence analyses were then performed as described by Bezerra et al. [25].

Searches using the BLASTn tool in the GenBank database at NCBI were used to verify the phylogenetic relationships

among all the ITS and LSU rDNA sequences. Subsequently, selected sequences deposited in GenBank were aligned with the ITS sequences obtained in this study to verify their relationships based on maximum likelihood (ML) analysis. Sequence editing, alignments, and phylogenetic analysis were conducted according to the procedures described earlier [25], and the ML analysis used 1000 bootstrap replicates and GTR + I + G as the best nucleotide model.

The sequences obtained in this study were deposited in the GenBank database (ITS = MT569893–MT569930 and LSU = MT569931–MT569974) (Online Resource Table 1). The sequence alignment used to perform the phylogenetic analysis was deposited in TreeBASE (study S26389).

Colonisation rate and absolute and relative frequencies

The endophytic colonisation rate (CR) was calculated by considering the ratio between the number of colonised fragments (Nf) and the total number of fragments (Nt) taken from the plant tissue ($CR = Nf / Nt \times 100$). The absolute frequency represents the total number of endophytes isolated, and the relative frequency is the number of isolates of each genus divided by the total number of isolates and multiplied by 100.

Ecological analyses

We calculated the richness (S), Shannon–Wiener diversity index (H'), and Fisher's alpha value for all samples. Richness was defined as the number of species in each sample, and the Shannon–Wiener diversity index was calculated using the following equation: $H' = -\sum(P_i \ln(P_i))$, where $P_i = n_i / N$ and n_i = number of endophytic isolates of taxon i and N = total number of endophytic isolates of all taxa [26]. For statistical purposes, the H' values were converted to $\text{Exp}(H')$. Fisher's alpha value was calculated using the equation: $S = \alpha \times \ln(1 + n / \alpha)$, where S is the number of taxa, n is the number of isolates, and α is Fisher's alpha value [27]. Species accumulation curves were determined, and observed richness was compared to estimated richness using the first-order Jackknife (Jackknife 1). All analyses were performed using R software v.3.5.0 (R Development Core Team 2018) and the “agricolae” [28], “vegan” [29], and “iNEXT” packages [30].

L-ASNase production in liquid medium

The L-ASNase production process was based on the studies by Loureiro et al. [31] and Silva et al. [6]. During the pre-fermentative stage, biomass production was induced using Czapek Dox's Medium (CDM) [32], as modified by Pádua et al. [7] [glucose (14.0 g L^{-1}), L-asparagine (10.0 g L^{-1}), KH_2PO_4 (1.52 g L^{-1}), KCl (0.52 g L^{-1}), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.52 g L^{-1}), $\text{CuNO}_3 \cdot 3\text{H}_2\text{O}$ (0.01 g L^{-1}), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g L^{-1}), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g L^{-1}), and $(\text{NH}_4)_2\text{SO}_4$ (2.0 g L^{-1}) at pH 6.2]. Erlenmeyer flasks (250 mL) containing 100 mL of CDM were inoculated with five discs (5 mm) of fungal mycelium that had been allowed to grow for 7 days on malt extract agar. These flasks were incubated at $30 \text{ }^\circ\text{C}$ for 96 h at 120 rpm. The cultures were filtered using Whatman no. 1 filter paper, and the biomass obtained was used to determine enzyme production. During the fermentative stage, the biomass obtained in the previous stage was inoculated into the modified CDM medium (as described above), and the modifications were that the glucose concentration was adjusted to 2.0 g L^{-1} and no $(\text{NH}_4)_2\text{SO}_4$ was added. The inoculated media were incubated at $30 \text{ }^\circ\text{C}$ for 96 h at 120 rpm. Finally, the cultures were filtered using Whatman no. 1 filter paper. The enzymatic activity of the obtained biomass was quantified as described below.

L-ASNase activity

The L-ASNase activity was determined according to Drainas et al. [33], as modified by Silva et al. [6] and Pádua et al. [7]. A total of 1.5 mL Tris–HCl buffer (20 mM, pH 8.6) and 0.1 g of

fungal biomass from each culture obtained after the fermentation step were vortexed. Then, 0.2 mL of L-asparagine solution (100 mM) and 0.2 mL of stock hydroxylamine solution (1 M, pH 7.0) were added, and the samples were incubated for 30 min at $37 \text{ }^\circ\text{C}$ and 150 rpm. The reaction was stopped by adding 0.5 mL of FeCl_3 reagent [10% (w/v) FeCl_3 plus 5% (w/v) trichloroacetic acid in 0.66 mol L^{-1} HCl] to all the samples, including the blank samples (Tris–HCl and mycelium). The reaction mixtures were then centrifuged for 15 min at 6000 rpm and $4 \text{ }^\circ\text{C}$ to remove the precipitates. Absorbance was measured at 500 nm against the blank samples that received L-asparagine and hydroxylamine solutions after 30 min of incubation. One unit of L-ASNase was defined as the amount of enzyme that releases $1 \text{ } \mu\text{mol}$ of β -hydroxamic aspartic acid per minute.

Partial optimisation of L-ASNase production

A statistical experimental design (2^3 factorial design with four central points) [34, 35] was used to partially optimise L-ASNase production. We used a factorial design comprising 12 trials, three variables (concentration of L-asparagine, pH, and inoculum concentration), and four replications at the central point. The pre-fermentation conditions were the same as described above (see the “L-ASNase production in liquid medium” section). The modified CDM was also used for fermentation, where the variables used were adjusted according to the results of the experimental design, and the flasks were incubated at 120 rpm for 120 h at $30 \text{ }^\circ\text{C}$. After the incubation period, the fungal biomass was filtered and used to determine enzyme activity [33 modified by 6 and 7]. Based on the factorial design results, additional experiments were carried out to increase the enzymatic production.

Statistical analysis of L-ASNase activity

The non-parametric Kruskal–Wallis test was used to determine if there was a statistically significant difference ($p < 0.05$) or a marginally significant difference ($p < 0.1$) between the results for L-ASNase production by endophytic fungi from *M. catimbauensis*. The results obtained from a 2^3 factorial design and the non-parametric Kruskal–Wallis test were processed using R software. This test was carried out to verify and evaluate the relationship between the selected independent variables and enzyme production. All calculations were randomly performed.

Results

A total of 560 leaf fragments from *M. catimbauensis* were randomly collected from 20 individuals. The colonisation rate for the leaf fragments was 11.78%, and a total

of 66 endophytic fungi were isolated; however, 15 isolates did not grow after isolation. We identified 13 genera based on their morphological characteristics (macro and micromorphology) and analysis of the ITS or LSU rDNA sequences. The isolated endophytes belonged to six orders in Ascomycota (Botryosphaerales, Capnodiales, Diaporthales, Eurotiales, Marthamycetales, and Pleosporales) (Online Resource Fig. 1). *Phyllosticta* isolates were the most frequent (45.10%) in *M. catimbauensis*, followed by *Diaporthe* (11.76%). *Cercospora*, *Cladosporium*, *Marthamyces*, *Paracercosporidium*, *Paraconiothyrium*, *Parapyrenochaeta*, *Phaeophleospora*, and *Preussia*, which were only present at low frequencies (one or two isolates) and were considered to be rare endophytes (Table 1).

The total richness of the endophytic fungi was 18 taxa, and the estimated richness per individual *M. catimbauensis* varied from one to seven species, with an average of 1.9. The total diversity of the endophytic fungi, according to the Shannon index, ranged from 0.69 to 1.90, with an average of 0.56. The Fisher index values varied from 0.79 to 26.78, and the average was 3.26. The species accumulation curve did not reach a plateau, and the sampling effort was not sufficient to recover all the estimated richness using the first-order Chao Jackknife index (Chao Jackknife 1) (Fig. 2).

Of the 20 endophytic fungi tested in the liquid medium, 14 isolates showed a capacity to produce the enzyme L-ASNase, with the enzymatic activity varying between 0.48 and 2.22 U g⁻¹ (Table 2). *Phyllosticta catimbauensis* URM 7672 and *Cladosporium* sp. G45 had the best results for the intracellular enzyme production (2.22 and 2.11 U g⁻¹, respectively) (Table 2). Based on these results, the endophyte *P. catimbauensis* URM 7672 was selected for partial optimisation of L-ASNase production.

L-ASNase production using the experimental 2³ factorial design varied between 0.61 and 2.25 U g⁻¹ (Table 3). The highest L-ASNase production (2.25 U g⁻¹) was obtained at pH 5.0, 1.5% L-asparagine, and 1.5% inoculum (Assay 6, Table 3). The analysis of variance results showed that the increase in L-asparagine production was statistically significant, whereas the pH and the interaction among the three explanatory variables were marginally significant, and the inoculum concentration was not significant (Online Resource Table 2). The lack of adjustment was not statistically significant, and the determination coefficients (R^2) and adjusted R^2 were 0.8418 and 0.7514, respectively. Based on the independent variables' effects (Online Resource Fig. 2), an experimental sequence was performed with the adjusted variables to obtain a greater enzymatic production (Table 4). In this experimental design, 3.5 U g⁻¹ of L-ASNase was obtained using 3.5% L-asparagine, pH 4.2, and 1.0% inoculum (Assay 5, Table 4). Table 4 shows the Kruskal–Wallis analysis,

which resulted in a p -value of 0.0155. The best values for enzymatic production were produced by assays 4 (3.47 U g⁻¹) and 5 (3.50 U g⁻¹). According to the test results, there were no significant differences between these values. Assays 4 and 5 had the same pH (4.2) and inoculum concentration (1.0 g), but had different L-asparagine concentrations (assay 4 = 2.5 g and assay 5 = 3.5 g). The increase in L-ASNase enzymatic activity was 36.02% and 36.57% in assays 4 and 5, respectively.

Discussion

In this study, we isolated 66 endophytic fungi from the leaves of *M. catimbauensis*, a plant species belonging to the Apocynaceae family. Studies on the endophytic fungi associated with this plant family have recorded between 11 and 22 endophytes [36]. The low recorded fungal colonisation rate (11.78%) in the leaves of *M. catimbauensis* may be because of the presence of latex in the plant composition, as latex has antibacterial and antifungal properties [37]. Another study on endophytic fungi isolated from the stems and roots of *Nerium indicum* (Apocynaceae) reported a total of 11 endophytic fungi isolated from healthy tissues of the plant (five from stems and six from roots) [38].

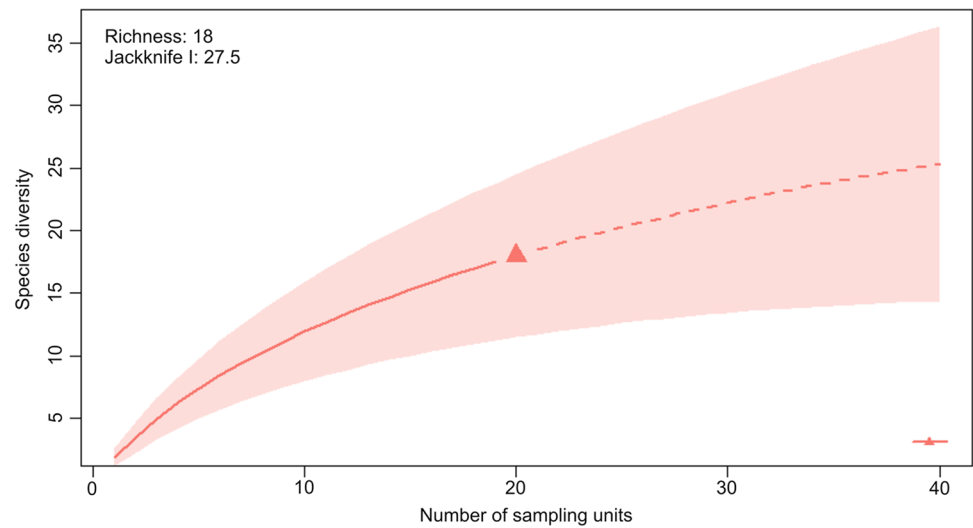
Different factors may have influenced the low diversity of fungal endophytes recovered from *M. catimbauensis* leaves ($H' = 0.69$ to 1.90 and $F-\alpha = 0.79$ to 26.78), for example, plant tissue type and age, micro-habitats, the relationship between fungal hosts, climate, vegetation changes, and human impact [39–41]. Variations in solar intensity and exposure are relevant factors associated with the composition of the endophytic fungal community [42]. Furthermore, ultraviolet radiation in semiarid regions, such as the Caatinga dry forest in Brazil, can negatively influence the diversity of these fungi [7].

Most of the endophytic fungi isolated in this study were pigmented, and *Phyllosticta* was the most frequently found genus. Pigmented fungi have been isolated in studies on various plant species from arid environments, and they may confer host plant tolerance to extreme environmental conditions, including ultraviolet radiation [43, 44]. Suryanarayanan et al. [43] studied the occurrence and distribution of endophytic fungi in tropical forests and observed that the most frequently isolated genera were *Colletotrichum*, *Phyllosticta*, and *Diaporthe* [Phomopsis]. *Phyllosticta* has been frequently isolated as endophytes from the leaves of various tropical plants [44], and endophytes belonging to *Diaporthe* are also frequently isolated [5, 7]. For example, Pádua et al. [7] reported that *Diaporthe* and *Phyllosticta* were the most common endophytes isolated from *M. urundeuva* occurring in the Caatinga forest of Brazil.

Table 1 Absolute (F) and relative (fr) (%) frequency, number of isolates, richness (S), and Shannon–Wiener (H') and Fisher's alpha (F- α) indexes diversity of endophytic fungi isolated from leaves of the medicinal plant *Mandevilla catimbauensis*, an endemic plant of the Caatinga dry forest, Brazil

Endophytic fungi	<i>Mandevilla catimbauensis</i> individuals																				F	fr
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
<i>Alternaria</i> sp. (<i>A. tomato</i> species complex)	1									1		1									3	5.88
<i>Cercospora</i> sp.																	1				1	1.96
<i>Cladosporium</i> sp. (<i>C. cladosporioides</i> species complex)									1	1											2	3.92
<i>Diaporthe caatingaensis</i>													1								1	1.96
<i>Diaporthe cf. acaciarium</i>			1									2									3	5.88
<i>Diaporthe heveae</i>									1												1	1.96
<i>Diaporthe</i> sp. 1													1								1	1.96
<i>Marthamyces renga</i>																	1				1	1.96
<i>Phaeophleospora eucalypticola</i>																					1	1.96
<i>P. hymenocallidicola</i>																					1	1.96
<i>Paracercosporidium</i> sp.																	1				2	5.88
<i>Paraconitomyrium</i> sp.																			1		1	1.96
<i>Parapyrenochaeta</i> sp.																					1	1.96
<i>Phyllosticta capitataensis</i>																					1	1.96
<i>P. catimbauensis</i>																					1	1.96
<i>Preussia</i> sp.																					1	1.96
<i>Pseudofusicoccum stromaticum</i>																					1	1.96
<i>Talaromyces</i> sp. (section <i>Helici</i>)																					1	1.96
Isolates	3	2	2	2	2	2	1	1	3	3	3	2	9	4	2	8	6	2	2	51		
Richness (S)	3	2	2	2	2	2	1	1	3	3	3	2	7	3	1	3	3	3	1			
Shannon–Wiener (H')	1.09	0.69	0.69	0.69	0.69	0.69	1.09	1.09	1.09	1.09	1.09	1.09	1.90	1.04	0.90	0.86	0.86	0.86	0.86			
Fisher's alpha (F- α)																						

Fig. 2 Species accumulation curve for endophytic fungi recovered from leaves of *Mandevilla catimbauensis*, an endemic plant of the Caatinga dry forest, Brazil



Similar to our study, most investigations of microbial diversity have not sufficiently recovered all estimated species richness [7, 45–49]. For example, investigations of endophytic fungi associated with different plant hosts in dry tropical forests (Caatinga) in Brazil were not able to represent the estimated species richness [7, 47, 49]. Similarly, Oliveira

et al. [48] did not isolate all expected endophytic fungi from the leaves of *Cocos nucifera* occurring in coconut crops in a region of the Atlantic Forest in Brazil. In a large study from the Canadian Arctic to the lowland tropical forest of central Panama, Arnold and Lutzoni [46] also demonstrated an inability to isolate all estimated endophytic species from the leaves of 28 hosts, showing that the expected species remarkably exceeded the numbers observed. Based on 1403 endophytes isolated from representative arctic, boreal, temperate, and tropical plants, the authors observed approximately 250 species, whereas 350 species were expected.

Table 2 L-asparaginase activity produced by endophytic fungi isolated from leaves of the medicinal plant *Mandevilla catimbauensis*, an endemic plant of the Caatinga dry forest, Brazil

Endophytic fungi	URM or isolate numbers	L-asparaginase activity (U g^{-1})
<i>Alternaria</i> sp.	G22	0.00 ^h
<i>Alternaria</i> sp.	G7	0.00 ^h
<i>Cladosporium</i> sp.	G45	2.11 ^a
<i>Diaporthe</i> cf. <i>acaciarum</i>	G25	0.56 ^{fg}
<i>D. heveae</i>	URM 8162	0.80 ^b
<i>Marthamyces renga</i>	G48	0.00 ^h
<i>Phyllosticta capitalensis</i>	URM 8159	0.00 ^h
<i>P. capitalensis</i>	G21	0.76 ^{cd}
<i>P. catimbauensis</i>	URM 7672	2.22 ^a
<i>P. catimbauensis</i>	G12	0.48 ^g
<i>P. catimbauensis</i>	URM 7673	0.99 ^{bc}
<i>P. catimbauensis</i>	G20	0.75 ^{de}
<i>P. catimbauensis</i>	URM 7674	0.57 ^{ef}
<i>Pseudofusicoccum stromaticum</i>	G6	0.61 ^{ef}
<i>P. stromaticum</i>	G41	0.00 ^h
<i>Paracercosporidium</i> sp.	G46	0.48 ^g
<i>Parapyrenochaeta</i> sp.	URM 8160	0.79 ^{bc}
<i>Preussia</i> sp.	URM 8161	0.95 ^b
<i>Talaromyces</i> sp.	URM 8163	1.11 ^b
<i>Talaromyces</i> sp.	URM 8164	0.00 ^h

Values followed by the same letter do not differ significantly ($p > 0.05$) using the non-parametric test of Kruskal–Wallis

Most (70%) of the tested endophytic fungal isolates were able to produce L-ASNase. *Phyllosticta catimbauensis*, a species described in 2017 [50], had the highest ability to produce L-ASNase (URM 7672, 2.22 U g^{-1}). Some studies have demonstrated that endophytic fungi have a high

Table 3 Production of L-asparaginase by *Phyllosticta catimbauensis* URM 7672 in the 2^3 factorial experimental design

Assay	L-asparaginase (%)	pH	Inoculum concentration (biomass %)	L-Asparaginase activity (U g^{-1})
1	0.5	5.0	0.5	0.61
2	1.5	5.0	0.5	2.03
3	0.5	7.0	0.5	1.07
4	1.5	7.0	0.5	1.38
5	0.5	5.0	1.5	1.46
6	1.5	5.0	1.5	2.25
7	0.5	7.0	1.5	0.67
8	1.5	7.0	1.5	1.81
9	1.0	6.0	1.0	1.70
10	1.0	6.0	1.0	1.85
11	1.0	6.0	1.0	1.46
12	1.0	6.0	1.0	1.54

Table 4 Production of L-asparaginase by *Phyllosticta catimbauensis* URM 7672 obtained through an experimental sequence. Inoculum concentration was fixed in 1.0% fungal biomass

Assay	L-asparagine (%)	pH	L-asparaginase activity
1	1.5	5.0	2.33 ^{bc}
2	1.5	4.6	2.49 ^b
3	2.5	4.6	1.13 ^c
4	2.5	4.2	3.47 ^a
5	3.5	4.2	3.50 ^a
6	4.5	4.2	2.91 ^{ab}

Non-parametric test of Kruskal–Wallis ($p=0.0155$). Values followed by the same letter do not differ statistically from each other

production capacity for L-ASNase [6, 7]. Furthermore, previous research has shown that some *Phyllosticta* species can produce novel bioactive metabolites [51], including anticancer drugs [7]. Other endophytes, such as *Cladosporium* sp. G45 and *Talaromyces* sp. URM 8163, have also demonstrated the capacity for L-ASNase production (2.11 U g⁻¹ and 1.11 U g⁻¹, respectively). Kumar et al. [52] reported *Cladosporium* sp. isolated from soil samples as the best producer of L-ASNase (3.74 U g⁻¹). Species of *Talaromyces* have a huge biotechnological capacity to produce several metabolites. Krishnapura and Belur [53] partially purified and characterised the L-ASNase of *T. pinophilus*, an endophyte isolated from the rhizomes of *Curcuma amada* (Zingiberaceae), and they considered it a potential candidate for industrial and clinical trials because of its biochemical properties and high efficiency (174 U mL⁻¹).

Similar to the present study, Pádua et al. [7] used biomass to quantify enzymatic activity based on the formation of β -hydroxamic aspartic acid. The authors reported that *Diaporthe* sp. URM 7793 was one of the best producers (2.41 U g⁻¹), followed by *Diaporthe* sp. URM 7779 (2.00 U g⁻¹) and *Talaromyces* sp. URM 7785 (1.91 U g⁻¹). In addition, Pádua et al. [7] demonstrated the potential of *Phyllosticta* sp. URM 7787 to produce L-ASNase with an average of 0.57 U g⁻¹. Silva et al. [6] evaluated the L-ASNase activities of endophytes isolated from the bromeliad *Tillandsia catimbauensis* and identified *Talaromyces* cf. *cecidicola* URM 7826 as the best producer (2.30 U g⁻¹). In another study, Da Rocha et al. [54] evaluated 32 filamentous fungi isolated from the Caatinga and obtained an activity of 1.58 U g⁻¹ using *Aspergillus terreus* URM 7732. However, Costa-Silva et al. [55] evaluated the conditions of biomass production by *A. terreus* CCT 7693 and obtained a production of 13.50 U g⁻¹ after optimisation (the best condition was CDM containing glucose 2 g L⁻¹, L-proline 10 g L⁻¹, and an inoculum concentration of 4.8×10^8 spores mL⁻¹ adjusted to pH 9.49 at 34.6 °C) of the enzyme production process. The superior

production found by Costa-Silva et al. [55] is a basis for further studies on the conditions of L-ASNase production by *P. catimbauensis* URM 7672.

In this study, 2.5% L-asparagine and 1.0% fungal inoculum at pH 4.2 were the optimal parameters for enzyme production. Furthermore, the experimental design increased enzyme activity by 36.02%. According to Thakur et al. [56], the initial pH of the culture medium can influence enzyme production because it can affect nutrient availability. Kumar et al. [52] discovered that pH 5.8 was optimal for L-ASNase production by *Cladosporium* sp. isolated from soil. In another study, Farag et al. [57] optimised the fermentation parameters and obtained optimum conditions for L-ASNase production by *Aspergillus terreus* at pH 6.0 using dextrose and L-asparagine as sources of carbon and nitrogen, respectively. Similarly, Vieira et al. [58] optimised L-ASNase production by *Penicillium* sp., and the optimum conditions were pH 5.1, 1.2 g L⁻¹ L-asparagine, and 3.0 g L⁻¹ glucose. However, Dias and Sato [59] reported that 2% proline, 0.5% glucose, 0.2% L-asparagine, and pH 8.0 were ideal conditions for enzymatic production by *Aspergillus oryzae*.

Here, we present the first study on endophytic fungi associated with *M. catimbauensis*, an endemic species in the Brazilian tropical dry forest. Our findings showed that *M. catimbauensis* leaves are host to endophytic species with a capacity to produce L-ASNase. Among the 14 endophytic isolates that exhibited enzymatic activity (0.48–2.22 U g⁻¹), *P. catimbauensis* URM 7672 (2.22 U g⁻¹) and *Cladosporium* sp. G45 (2.11 U g⁻¹) were the most promising enzyme producers. During the partial optimisation stage for L-ASNase production by *P. catimbauensis* URM 7672, L-asparagine concentration was found to be significant, and pH was found to be marginally significant, whereas the inoculum concentration was not significant. Furthermore, L-ASNase production by *P. catimbauensis* URM 7672 varied between 1.13 and 3.50 U g⁻¹ when using the optimal experimental design conditions. The best conditions for L-ASNase production by *P. catimbauensis* URM 7672 (3.47 U g⁻¹) were 2.5% L-asparagine and 1.0% inoculum at pH 4.2, considering that lower concentrations of L-asparagine did not produce statistically significant differences for the highest enzyme activity (3.50 U g⁻¹). The production of secondary metabolites by endophytic fungi greatly impacts modern pharmaceutical products, which led us to explore this valuable biological resource to make the process more advantageous for human use [60]. The industrial importance of L-ASNase means that a search for new enzyme production sources is needed. Any new sources can contribute to biotechnological applications in the food and drug industries. These results suggest that the endophyte *P. catimbauensis* URM 7672 should be subjected to further optimisation

studies that will investigate ways of optimising the production of L-ASNase. In addition, genetic manipulation, mutations, and other recombinant DNA techniques can contribute to obtaining higher L-ASNase production, as has been done for other substances [60]. Our results highlight the importance of protecting plant species in their natural environments to guarantee diversity and to allow their potential contribution to future biotechnological studies for promotion of a future eco-friendly and sustainable bio-economy.

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Author contribution Gianne R. Araújo-Magalhães, Cristina M. Souza-Motta, and Keila A. Moreira implemented the project; Gianne R. Araújo-Magalhães collected the plants; Gianne R. Araújo-Magalhães and Leticia F. da Silva performed the fungal endophytes isolation and L-asparaginase production parts of the study; Gualberto S. Agamez-Montalvo designed the experiment and statistically analysed the L-Asparaginase production results; Iolanda R. da Silva performed the ecological analysis; Jadson D.P. Bezerra performed the phylogenetic analysis; Gianne R. Araújo-Magalhães, Marília H.C. Maciel, and Jadson D.P. Bezerra wrote the paper. All the authors reviewed and approved the final version of the paper.

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Data availability Plant specimen is deposited in the Herbário UFP – Geraldo Mariz and endophytic fungi cultures in the URM culture collection, both collections at the Universidade Federal de Pernambuco (Recife, Brazil). DNA sequences are deposited in the GenBank database and sequence alignment in TreeBASE.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

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Conflict of interest The authors declare no competing interests.

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