



Fungal endophytic community associated with *Hevea* spp.: diversity, enzymatic activity, and biocontrol potential

Adriene de Oliveira Amaral¹ · Ana Francisca Tibúrcia Amorim Ferreira e Ferreira² · Jânia Lília da Silva Bentes^{1,2}

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Abstract

Plants of the genus *Hevea* present a great diversity of endophytic fungal species, which can provide bioactive compounds and enzymes for biotechnological use, and antagonist agents for plant disease biological control. The diversity of endophytic fungi associated with leaves of *Hevea* spp. clones in western Amazonia was explored using cultivation-based techniques, combined with the sequencing of the ITS rRNA-region. A total of 269 isolates were obtained, and phylogenetic analysis showed that they belong to 47 putative species, of which 24 species were unambiguous. The phylum *Ascomycota* was the most abundant (95.4%), with predominance of the genera *Colletotrichum* and *Diaporthe*, followed by the phylum *Basidiomycota* (4.6%), with abundance of the genera *Trametes* and *Phanerochaete*. Endophytic composition was influenced by the clones, with few species shared among them, and the greatest diversity was found in clone C44 (richness: 26, Shannon: 14,15, Simpson: 9.11). The potential for biocontrol and enzymatic production of endophytes has been investigated. In dual culture tests, 95% of the isolates showed inhibitory activity against *C. gloeosporioides*, and 84% against *C. cassiicola*. Efficient inhibition was obtained with isolates HEV158C and HEV255M (*Cophinforma atrovirens* and Polyporales sp. 2) for *C. gloeosporioides*, and HEV1A and HEV8B (*Phanerochaete* sp. 3 and *Diaporthe* sp. 4) for *C. cassiicola*. The endophytic isolates were positive for lipase (69.6%), amylase (67.6%), cellulase (33.3%), and protease (20.6%). The enzyme index ≥ 2 was found for amylase and lipase. The isolates obtained from rubber trees showed good antimicrobial and enzymatic potential, which can be tested in the future for use in the industry, and in the control of plant pathogens.

Keywords Enzymatic screening · Phylogeny · Microbial antagonisms · Biodiversity

Introduction

The rubber tree (*Hevea brasiliensis*), a species native to the Amazon region, is the main source of natural rubber in the world, used as a raw material for various products, especially for the manufacture of tires in the automobile industry [1, 2]. During the 1840s, Brazil dominated the world market for natural rubber, exploiting native rubber trees distributed along the Amazon River basin [3]. To meet the high demand in the international market, crops began to be exploited in

monoculture, a model successfully implemented in South-east Asian countries; however, this model eventually failed in the Amazon because of the endemic occurrence of fungus *Pseudocercospora ulei* [4], a causal agent of South American leaf blight (SALB), which is the main biological barrier for rubber cultivation in the Amazon biome [5, 6].

The main rubber plantation areas are currently situated in Southeast Asian countries, where *P. ulei* is absent, and they are responsible for supplying the world market of natural rubber [7]. In Brazil, some strategies have been adopted for the production of rubber: planting of rubber trees outside the common geographic range of *P. ulei*, crossbreeding between *Hevea* species, and crown grafting of resistant genotypes in order to obtain clones that are productive and highly resistant to SALB [3–8]. In Brazilian State of Amazonas, ongoing grafting and hybridization experiments with *H. brasiliensis*, *H. guianensis*, *H. pauciflora*, *H. nitida*, and other species of *Hevea* aim to obtain interspecific hybrids with high latex yield and resistance to SALB that can be planted directly

✉ Jânia Lília da Silva Bentes
jlbentes@ufam.edu.br

¹ Universidade Federal do Amazonas, Programa de Pós-Graduação em Ciências Florestais e Ambientais, Manaus, Amazonas, Brazil

² Universidade Federal do Amazonas, Programa de Pós-Graduação em Agronomia Tropical, Manaus, Amazonas, Brazil

or grafted onto highly adapted panels [9, 10]. The selection of superior clones and cultivars with higher levels of resistance to diseases and increased productivity can also impact the recruitment of beneficial microbial species that inhabit the internal tissues of plants, such as endophytic fungi [11].

Endophytic fungi colonize the internal tissues of plants, at some point in their life cycle, without causing apparent damage [12, 13]. Plant hosts and endophytic fungi establish ecological relationships that vary from mild parasitism, to commensalism, and to mutualism, in which the fungi provide protection against pathogens and herbivores and resistance against deleterious abiotic factors, and the plant provides shelter and nutrients [14]. In recent years, several studies conducted in Peru and Brazil have explored the great diversity of endophytic fungi of *Hevea* spp., comparing among different plant organs, collection sites, and native forests and commercial plantations [15–17]. A novel class of ascomycetes, the Xylonomycetes was described with endophytes of *Hevea* spp. in Peru [18], the novel species *Trichoderma amazonicum* was isolated from *H. guianensis* and *H. brasiliensis* in the Amazon [19] and three novel phylogenetic lineages of *Tolypocladium* were isolated from *Hevea* spp. in Brazil, Peru, and Mexico [20]. The endophytes of the rubber tree also represent a rich source of new molecules with biotechnological potential [21, 22].

Endophytic fungi synthesize a large enzymatic arsenal that enable them to colonize and survive in host tissues, including many hydrolytic enzymes such as amylases, cellulases, lipases, and proteases [21]. Extracellular hydrolytic enzymes are used in chemical processes in the textile industry, agriculture, environmental remediation, biomedicine, and energy sectors. These enzymes also play an important role in the mechanisms employed by biocontrol agents against plant pathogens [23]. Selected endophytic fungi used as biocontrol agents are capable of reducing the growth of plant pathogens in vitro and the incidence/severity of the diseases in the field, generally through a combination of mechanisms such as antibiosis — including the production of enzymes, competition for space and nutrients, hyperparasitism, and induction of the plant defense responses [24]. The use of these fungi in biological control can be a viable alternative reducing the dependence of modern agricultural systems on fungicides for disease control, since these can cause environmental pollution, and affect human and animal health [25].

The main objective of this study was to investigate the diversity foliar fungal endophytes of several clones of rubber trees currently tested for resistance against SALB, obtained by interspecific hybridization of *Hevea* spp. followed by grafting on *H. brasiliensis*. The main hypothesis guiding this study is that the plant genotype directly affects the composition of endophytic fungal communities. We also evaluated the production of extracellular hydrolytic enzymes by the

isolated endophytes and their antagonistic effects against two important plant pathogens, *Corynespora cassiicola* and *Colletotrichum gloeosporioides*.

Materials and methods

Sampling, isolation, and preservation of fungi

The collections were carried out in a plantation of rubber tree clones located at Embrapa Amazônia Ocidental (02°53'49.1''S–59°59'07.3''W), Rodovia AM-010, Km 29, Manaus, AM, Brazil. Three adult individuals were randomly selected from each of the following crown clones: C06 (*H. pauciflora* CNS G 124 × *H. rigidifolia* CNS NA 8105), C44 (*H. pauciflora* CNS AM 7745 × *H. rigidifolia* CNS AM 8105), and C45 (*H. guianensis* var. *marginata* Hgm 16 × *H. pauciflora* Baldwin CBA 1); all clones grafted on a CNS AM 7905 panel of *H. brasiliensis* [10]. Twenty healthy leaflets were collected from each plant. The samples were washed in water, and five fragments of 1 cm² were removed from each leaflet. These were subjected to superficial disinfection in 70% ethanol solution for 30 s, 1.5% sodium hypochlorite (2.5% active chlorine) for 2 min, followed by three washes in sterile distilled water. The margins of the disinfected leaf fragments were removed, and five fragments were deposited equidistantly on Petri dishes (90 mm) containing agar-water (17 g of agar; 1 L of distilled water). The disinfestation procedure was checked by plating an aliquot of 100 µL of the water used in the last washing of the disinfestation process on agar-water. The plates were kept in an incubator for 1 week at 27 °C, and hyphae growing from the leaf fragments were transferred to Petri dishes containing PDA (Kasvi, São J. Pinhais, PR, BR) supplemented with the antibiotic chloramphenicol (0.2 g/L⁻¹) and kept at room temperature (± 26 °C) under constant lighting to induce spore production. Monosporic cultures of 269 endophytic isolates were obtained according to the methodology described by Choy et al. [26] and preserved in slant agar tubes containing PDA covered with sterile water (Castellani method [27]) at room temperature (± 26 °C).

Extraction of genomic DNA

Total genomic DNA was extracted from monosporic cultures after 10 days of cultivation on PDA following the phenol chloroform protocol [28]. The concentration and quality of the extract DNA were determined using the 260 nm/280 nm absorbance ratio in a Nanodrop® 2000c spectrophotometer (Thermo Fisher Scientific), and the intensity of bands on a 0.8% agarose gel () using the lambda phage weight marker (10 ng) as a reference. The final DNA concentration was adjusted to 100 ng/µl⁻¹, and samples were stored at 4 °C.

PCR and Sanger sequencing

The internal transcribed spacer of the ribosomal RNA gene region (ITS), which is the universal barcode for molecular identification of fungi was selected for amplification [29]. PCR amplifications were performed in final volumes of 12 μL , containing 1 μL of DNA, 2.4 μL of colorless Go Taqbuffer® Flexi(5X), 0.8 μL of MgCl_2 (25 mM), 0.5 μL of dNTPs (10 mM), 0.5 μL of each primer (10 μM), (ITS1F-5'TCCGTAGGTGAACCTGCGG3'/ITS4R-5'TCCGTA GGTGAACCTGCGG3') [30], 0.12 μL of the GoTaq® DNA polymerase (Promega, Madison, WI, USA) and 6.18 μL of ultrapure water. The PCR reactions were performed in a ProFlex™ thermocycler (Applied Biosystems, Foster City, CA, USA), following the amplification protocol used by Gazis and Chaverri (2010) [15]. The generated amplicons were checked on a 1% agarose gel stained with Gel Red™ (Biotium, Fremont, CA, USA), using a 100 base pair molecular weight marker (Synapse Biotechnology, EN, NG).

The PCR products were purified with polyethylene glycol (PEG 20%) following the protocol described by Dunn and Blattner (1987) [31], and the sequencing reaction was performed using the BigDye® Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The reading of the products obtained in the sequencing reactions was performed on the ABI 3500 genetic analyzer automatic sequencer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's guidelines.

Phylogenetic analysis

The electropherograms were analyzed with SeqAssem® [32]. The consensus sequences ranged from 400 to 600 base pairs (bp). The sequences were compared against reference ITS sequences deposited in the Genbank in NCBI (*National Center for Biotechnological Information*) using BLAST (*Basic Local Alignment Search Tool*) and UNITE (Unified system for the DNA based fungal species) [35]. Multiple sequence alignments were composed with ITS sequences of the endophytic fungi of the rubber trees and reference ITS sequences that showed the highest identity scores in the BLAST searches, using the Clustal W algorithm [33] implemented in the MEGA7 program [34].

Phylogenies were constructed for the phyla *Ascomycota* and *Basidiomycota*, and for each genus and/or species individually, using the Maximum likelihood method RAxML-HPC v.8 on XSEDE 8.2.12 [36], on the CIPRES portal [37], and the previously defined parameters underwent rapid bootstrap analysis (1000 replicates) and search for the best-scoring ML tree. The generated trees were rooted with reference sequences and isolates from the present study; for *Ascomycota*, the selected outgroups were *Fomitopsis subtropica* (no.

KR605787.1) and HEV228J, and for *Basidiomycota*, they were *Xylaria cubensis* (no. AB625420.1) and HEV268N. The phylogenetic trees were visualized using the FigTree v1.4.3 software (<http://tree.bio.ed.ac.uk/software/>). The trees were edited using the Inkscape 0.92 software (<https://inkscape.org/>). The sequences obtained from the isolated endophytes of this study have been deposited in GenBank under accession numbers MT470446 – MT470693 for ITS (Table S1).

Diversity and taxonomic composition

The analysis of the taxonomic composition of endophytic fungi using clones was performed using the ggplot2 package [38] in Rstudio version 1.2.5042 [39]. Estimates of richness and diversity were measured using alpha diversity, which is the average diversity of species in locations or habitats on a local scale using the estimation of prediction (extrapolation) and rarefaction (interpolation) curves based on Hill's numbers, which are estimates of empirical data that tend to be an increasing function of the sampling effort and are used to characterize phylogenetic or functional taxonomic diversity [40]. Hill (1973) [41] integrated species richness and abundance into a class of measures that is currently called Hill numbers, where $q=0$ corresponds to species richness, $q=1$ Shannon–Wiener entropy, and $q=2$ Simpson. The analyses were performed using the Vegan [42] and iNEXT [43] packages in software R.

Dual culture tests

A total of 102 representative isolates were used in antagonism tests against the plant pathogens *Corynespora cassiicola* (from *Solanum lycopersicum*) and *Colletotrichum gloeosporioides* (from *Capsicum chinense*), using the in vitro dual culture plate assay [44]. Antagonists and pathogens were cultured for 7 days in Petri dishes (90 mm) containing PDA medium, maintained at a temperature of ± 26 °C. A 5-mm disc of each antagonist (endophytes) and the pathogens were deposited in the opposite directions, 2 cm from the margin of the plate with PDA medium, and they were kept at a temperature of ± 26 °C. Plates containing only pathogens represented the negative controls. The evaluations were carried out every 48 h until the control colonies reached 50% of their growth, and the experiment was carried out in triplicate. The diameter of the colonies was obtained and growth inhibition (%) of the pathogen was determined by the formula: inhibition (%) = $R - r/R * 100$, where R is the radial growth of the pathogen in the control plate and r is its growth in dual culture tests [45]. The antagonistic activity of endophytic fungi was initially classified according to inhibition percentage, and the isolates present in the largest classes were analyzed using analysis of variance (ANOVA), followed by Tukey's test (5%) using the Agricolae package [46] in R.

Determination of enzymatic activity

The endophytic isolates used in the antagonism test were evaluated for their ability to synthesize extracellular hydrolytic enzymes: amylases, cellulase, lipase, and protease.

Amylase production was detected Petri dishes (90 mm) with Glucose Yeast Peptone (GYP) medium supplemented with soluble starch (dextrose 10.0 g, yeast extract 0.1 g, peptone 0.5 g, agar 15 g, soluble starch 2 g, distilled water 1 L, pH 6). After 7 days of incubation at 25 °C, plates were flooded with iodine solution (1% iodine; 2% potassium iodide; distilled water 100 ml) for 10 min. The presence of a bluish-black color and the formation of a halo indicated the presence of amylase [47].

For cellulase detection, the GYP medium was supplemented with 0.5% carboxy-methyl-cellulose (CMC). After 7 days of incubation at 25 °C, plates were flooded with 20 ml of the 0.2% Congo red solution (0.2 g of Congo red, 100 mL of distilled water) during 20 min and subsequently washed with NaCl (1 M) for 15 min. In areas where cellulose was hydrolyzed, discoloration occurred, marked by the presence of a halo around the colony, thus indicating the activity of this enzyme [48].

The lipase production was evaluated in culture medium using Tween 20 (1%) as substrate (peptone 10 g, NaCl 5.0 g, CaCl₂·2H₂O 0.1 g, agar 15 g, distilled water 1 L, pH = 6.0). After the incubation period (7 days at 25 °C), the lipase activity was observed by the presence of a white flocculent precipitate around the mycelial layer, where calcium crystals were formed [49].

The protease activity was evaluated using a culture medium containing skimmed milk (0.25 g KH₂PO₄, 0.125 g KCl, 0.05 g MgSO₄·7H₂O, 0.025 g CaCl₂, 6.25 mL 22.5% skimmed milk, 2.5 g glucose, 250 mL distilled water, 3 g agar, pH 5.4). The presence of lighter concentric halos around the colonies indicated proteolytic activity [50].

The enzymatic index (EI) was calculated according to the formula: $EI = R/r$, where R is the diameter of the halo around the colony and r is the diameter of the colony [51]. Fungi were classified as good producers ($EI \geq 2$), moderate producers ($EI < 2$ and > 1) weak producers ($EI \leq 1$) [52]. The isolates with the highest EI underwent analysis of variance (ANOVA), followed by Tukey's test (5%) using the Agricolae package [46] in RStudio.

Results

Isolation and molecular identification

A total of 269 endophytic isolates were obtained from 300 leaflet fragments from CPAA clones C06 (86 isolates), C44 (84 isolates), and C45 (99 isolates) from *Hevea* spp.

According to the analysis of Maximum parsimony using sequences from the ITS rRNA-region, the endophytic isolates were grouped into 47 putative species, distributed in two phyla, three classes, nine orders, and 20 genera (Table S2, Figs. 1 and 2). Thirty putative species corresponded to rare species, with a single representative present in the samples. Most of the endophytic isolates belong to the phylum *Ascomycota* (95.4%), and within this phylum, the most abundant orders were Glomerellales (63.60%), Diaporthales (18.77%), Botryosphaeriales (5.74%), and Pleosporales (3.83%). The most abundant genera were *Colletotrichum* (62.45%), *Diaporthe* (19.70%), and *Phyllosticta* (4.83%), and other genera known as plant pathogens to *Hevea*, such as *Curvularia* and *Corynespora*, were isolated in this study. Within the phylum *Basidiomycota*, the isolates belonged to a single order, Polyporales (4.6%).

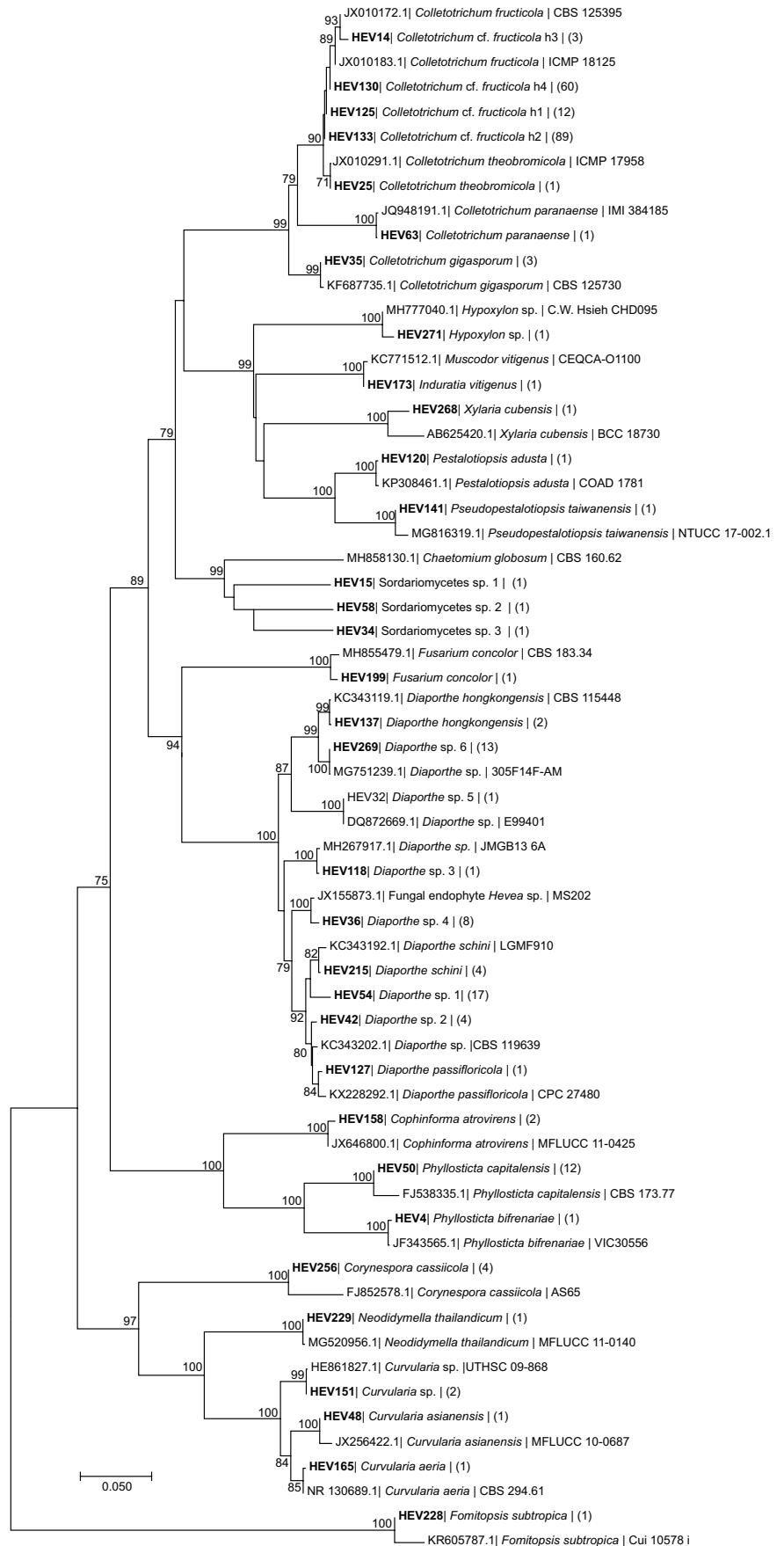
Nineteen species belonging to the phylum *Ascomycota* were named unambiguously with high statistical bootstrap support and correspond to the genera *Colletotrichum*, *Cophinforma*, *Corynespora*, *Curvularia*, *Diaporthe*, *Fusarium*, *Induratia*, *Multiguttulispora*, *Neodidymella*, *Phyllosticta*, *Pestalotiopsis*, *Pseudopestalotiopsis*, and *Xylaria* (Fig. 1, Fig. S1). Some isolates had low statistical support (bootstrap < 70), and they cannot be nominated at the order level (*Sordariomycetes*, sp. 1, 2, and 3), of species (*Hypoxylon* sp., *Diaporthe* sp. 1, 2, 3, 4, 5, and 6, and *Curvularia* sp. 1), and class (*Ascomycota* sp. 1 and sp. 2). Within the species *Colletotrichum fructicola*, the isolates were divided into 4 haplotypes according to point mutations observed at positions 22, 474, and 475 in the sequence alignment (haplotype 1: 22 / C, 474 / G, 475 / -; haplotype 2: 22 / C, 474 / A, 475 / -; haplotype: 22 / C, 474 / A, 475 / C; haplotype 4: 22 / T, 474 / A, 475 / C) (Fig. S5).

The phylogenetic tree with isolates of the phylum *Basidiomycota* (Fig. 2) showed 7 distinct clades distributed into a single class, Agaricomycetes, and 5 species belonging to the genera *Fomitopsis*, *Rigidoporus*, *Tinctoporellus*, and *Trametes* were named with high statistical support (100% bootstrap support). Within the genus *Phanerochaete*, isolates HEV60M, HEV196G, and HEV1A could not be identified at the species level, and isolates HEV259M (*Russulales*, sp.1), HEV40H (*Polyporales*, sp. 1), and HEV255M (*Polyporales*, sp.2) were identified only at class level (Fig. 2).

Taxonomic composition and alpha diversity

The composition of the endophytic communities was influenced by the different clones evaluated (Fig. 3A). Clones C06 and C45 showed a more similar composition, with greater relative abundance of the genera *Colletotrichum* and *Diaporthe* (class *Sordariomycetes*), while in clone C44, in addition to these two genera, the genus *Phanerochaete* (*Agaricomycetes*) was predominant (Fig. 3A). The total number

Fig. 1 Maximum likelihood phylogenetic tree based on the sequencing of the ITS rDNA-region, showing the phylogenetic relationship of the endophytic fungi belonging to the phylum *Ascomycota*, isolated from different clones of *Hevea* spp. Isolates from this study are highlighted in bold. The scale bar shows the number of replacements per site, and bootstrap support values ≥ 70 are displayed on the nodes. The species *Fomitopsis subtropica* was used as an outgroup



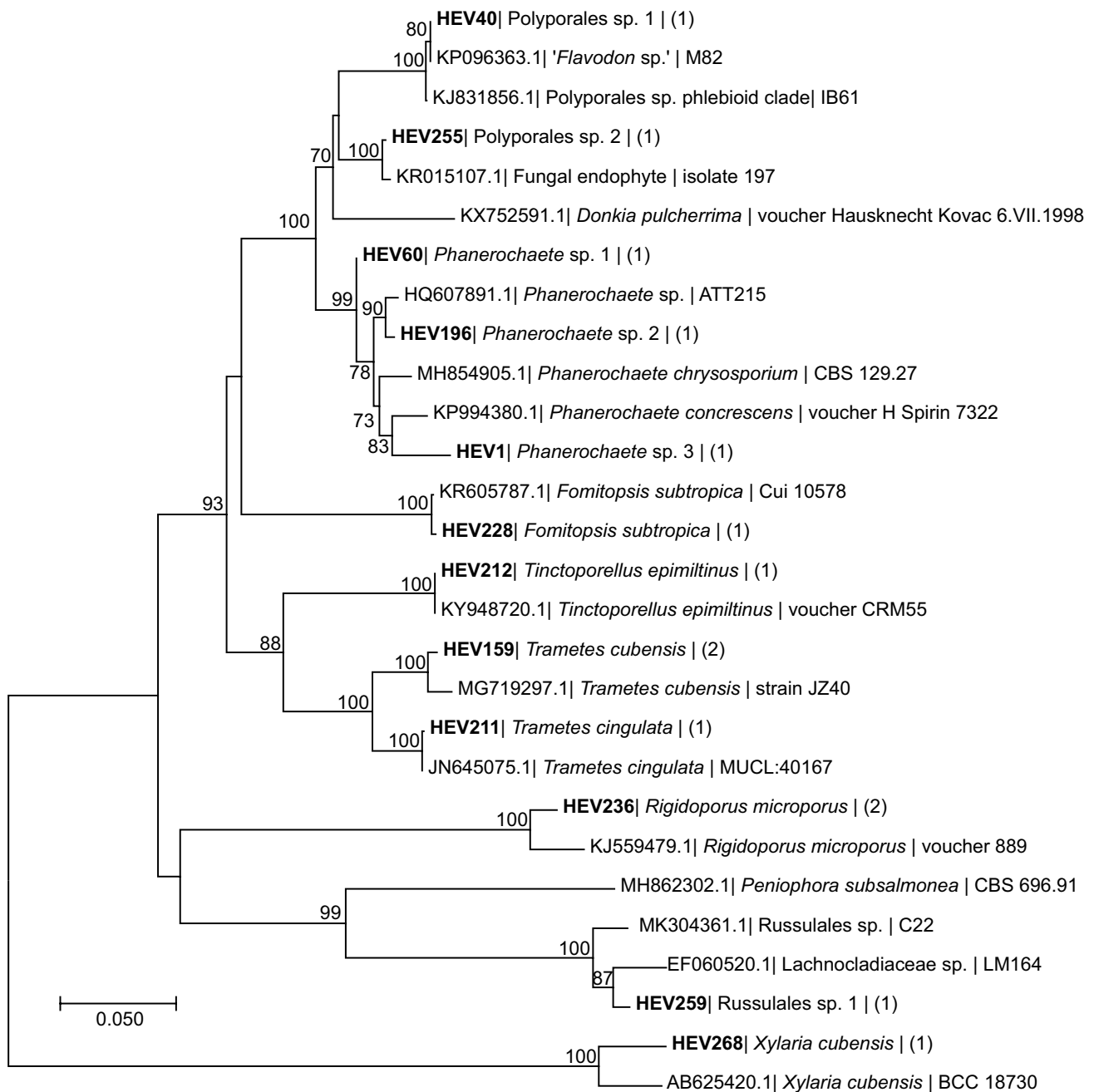


Fig. 2 Phylogenetic tree of maximum likelihood based on the sequencing of the ITS rDNA-region, showing the phylogenetic relationship of the endophytic fungi belonging to the phylum *Basidiomycota*, isolated from different clones of *Hevea* spp. Isolates from

this study are highlighted in bold. The scale bar shows the number of replacements per site, and bootstrap support values ≥ 70 are displayed on the nodes. The species *Xylaria cubensis* was used as an outgroup

of species found/unique in each clone was 26/15 (C44), 21/11 (C06), and 18/9 (C45).

A small number of species was shared among the clones, which reflects a specificity in the recruitment of endophytic fungi by *Hevea* clones. Clones C06 and C44 shared three species (*Diaporthe* sp. 3, *Rigidoporus microporus*, and *Colletotrichum gigasporum*); C44 and C45 shared two

species (*Corynespora cassiicola* and *Curvularia* sp. 1); and between clones C06 and C45, only one species was shared (*C. fructicola* h3). The species *C. fructicola* h1, *C. fructicola* h2, *C. fructicola* h4, *Diaporthe* sp. 1, *Diaporthe* sp. 2, and *Diaporthe* sp. 6, were detected in all sampled clones.

The analysis of alpha diversity in the three clones showed that clone C44 has greater richness and diversity in all the

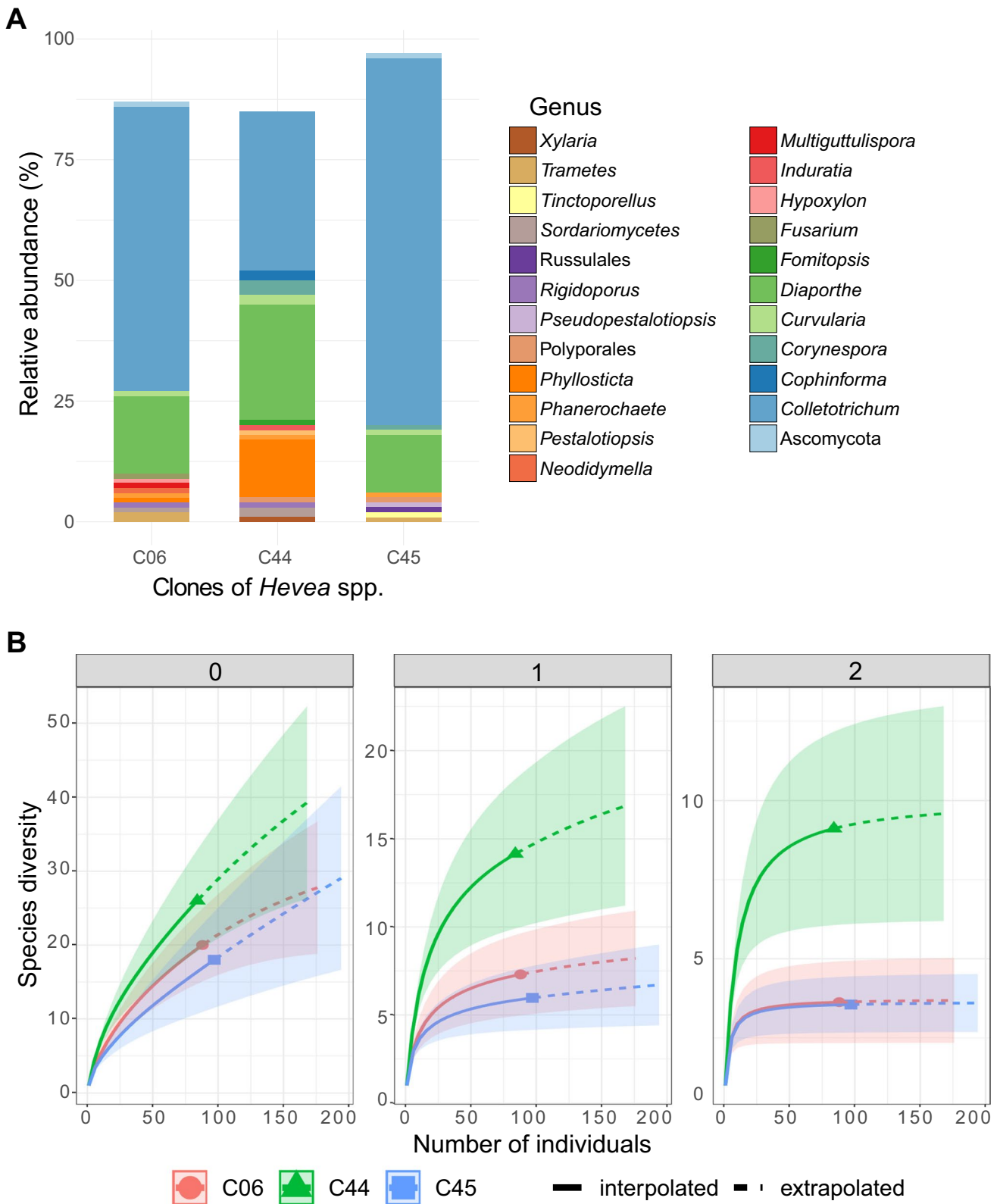


Fig. 3 Taxonomic composition and analysis of alpha diversity within clones C06, C44, and C45 of *Hevea* spp. **A** Relative abundance of the identified genera of endophytic fungi found in the different clones. **B** Rarefaction curve (solid line) and extrapolation (dashed line) with 95% confidence intervals (shaded areas) for data on endophytic fungi

of *Hevea* spp. The curves compare the value of species richness (0), Shannon diversity (1) and Simpson dominance (2) of endophytic fungi. Relative percentages do not sum up to 100 because a proportion of strains could not be identified

ecological indexes tested (Table 1, Fig. 3A and B). High values of richness and Shannon index reveal that clone C44 harbors a greater number of species when compared to C06 and C45 (Table 1). However, the high value of Simpson's dominance index demonstrates that this clone is dominated by few species of endophytic fungi. The rarefaction curves demonstrate a continuous increase, without reaching the plateau ($q=0$), indicating that not all diversity present in the clones was captured (Fig. 3B). The same pattern was found for clone C44 in the Shannon ($q=1$) and Simpson ($q=2$) indices, while the curves for clones C06 and C45 in these indices reached the plateau.

In vitro antagonism tests

The antagonistic activity of 102 fungal endophytes of *Hevea* spp. against the plant pathogens *C. cassiicola* and *C. gloeosporioides* was evaluated in paired culture tests (Table S3), and there were inhibitory activities by competition at 4 days of testing. The inhibitory activity against *C. gloeosporioides* was found in 95% of isolates (1.05–66.40% inhibition), and 84% against *C. cassiicola* (1.29–44.32% inhibition). The grouping of endophytic isolates by classes according to the percentage of inhibition of plant pathogens showed that 42% of the isolates were able to inhibit 10–20% of the mycelial growth of *C. cassiicola* (Fig. 4A), while 32.3% inhibited 20–30% mycelial growth of *C. gloeosporioides* (Fig. 4B).

The Tukey's test carried out with the endophytic isolates grouped in the highest classes of percentage of inhibition, showed that the species *Cophinforma atrovirens* (HEV158C), Polyporales sp. 2 (HEV255M), and *Phanerochaete* sp. 3 (HEV1A) showed significant antagonistic activity against *C. gloeosporioides* ($p < 0.05$), with the highest values of the percentage of inhibition of this pathogen. The species *Phanerochaete* sp. 3 (HEV1A) and *Diaporthe* sp. 4 (HEV8B) were the ones that best inhibited the mycelial growth of *C. cassiicola* (Table 2).

Enzymatic activity

The endophytic isolates of *Hevea* spp. were investigated for the synthesis capacity of the enzymes amylase, cellulase, lipase, and protease (Fig. 5, Table S3, Fig. S2). Lipolytic activity was found in 69.61% (71 isolates), amylolytic

activity in 67.65% (69 isolates), cellulolytic activity in 33.33% (34 isolates), and proteolytic activity, in 20.59% (21 isolates) of the 102 isolates tested. The isolates HEV129A (*Colletotrichum* cf. *fruticola*), HEV239K (*Diaporthe* sp. 6), HEV253K (*Diaporthe* sp. 1), and HEV51K (*Hypoxylon* sp. 1) were the only ones that secreted all the enzymes evaluated in this study.

The classification of fungi according to the enzyme index (EI) showed that no isolate had a good producer profile for protease ($EI \geq 2$). For cellulase, only HEV228J isolate obtained $IE > 2$. The opposite trend occurred in the amylase and lipase tests, in which most of the tested isolates proved to be moderate producers of these enzymes ($EI < 2 > 1$), and 7% and 2% of the isolates were considered to be good producers ($EI \geq 2$) (Fig. S2).

The statistical analysis with the 10 largest producers of each enzyme is shown in Fig. 5. The highest enzyme indices (4.29, 4.17, and 3.64) were found in the lipase tests, with the isolates HEV259M (*Russulales* sp. 1), HEV15C (*Sordariomycetes* sp. 1), and HEV34G (*Sordariomycetes* sp. 3). In addition, the isolates HEV50K, HEV242L, and HEV174E, which belong to the species *Phyllosticta capitalensis*, were considered good producers of this enzyme ($EI \geq 2$). For amylase, higher enzyme levels were obtained by isolates of the species *C. cf. fruticola* (haplotype 1 and 2), and among these, the HEV170D isolate ($EI: 3.35$) showed significant differences ($p < 0.05$) in the production of this enzyme (Fig. 5) compared to the other isolates of the species. The species *Microsporus rigidoporus* also showed amylolytic activity (1.79), considered to be a moderate producer ($EI: 1 < 2$).

There was greater diversification of species in terms of cellulolytic activity. The isolates that had the best production performance of this enzyme belong to 5 genera, *Fomitopsis*, *Corynespora*, *Diaporthe*, *Sordariomycetes*, and *Hypoxylon* (Fig. 5). Among the 10 isolates, 5 belong to the genus *Diaporthe*, with an enzyme index ranging from 1.41 to 1.99 (moderate producer). However, the HEV228J isolate (*Fomitopsis subtropica*) presented the highest enzyme index (2.31) for this enzyme ($p < 0.05$). The endophytic isolates showed moderate proteolytic activity ($EI: 1.16$ to 1.48), and the isolates HEV268N (*Xylaria cubensis*), HEV137B (*Diaporthe hongkongensis*), and HEV59M (*Trametes cubensis*) had the highest enzyme indexes ($p < 0.05$).

Table 1 Alpha diversity metrics (Richness, Shannon, and Simpson indices) of endophytic isolates present in different clones of *Hevea* spp. in the western Amazon

Clones of <i>Hevea</i> spp.	No. of isolates	Richness (q0)		Shannon (q1)		Simpson (q2)	
		Observed	SD	Observed	SD	Observed	SD
C06	86	20	±12.33	7.30	±1.57	3.63	±0.77
C44	84	26	±32.84	14.15	±4.13	9.11	±1.81
C45	99	18	±96.08	5.97	±2.03	3.55	±0.51

SD standard deviation for clones of *Hevea* spp

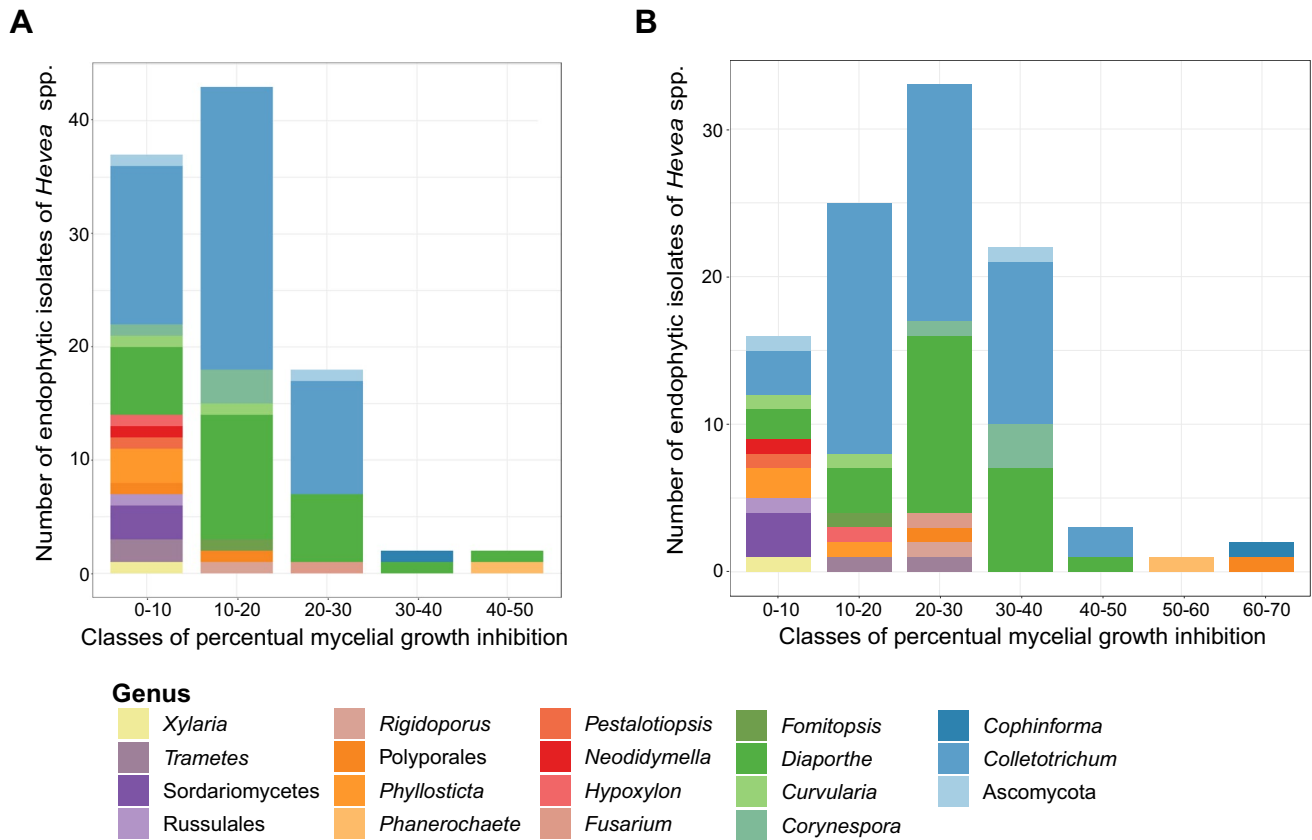


Fig. 4 Classes of mycelial growth inhibition of (A) *Corynespora cassiicola* and (B) *Colletotrichum gloeosporioides* by endophytic isolates of *Hevea* spp. in dual culture tests

Table 2 Mean percentage of mycelial growth inhibition of *Colletotrichum gloeosporioides* and *Corynespora cassiicola* by selected endophytic isolates in dual culture tests

Isolate	Species	Mean percentage of mycelial growth inhibition (%)	
		<i>C. cassiicola</i>	<i>C. gloeosporioides</i>
HEV1A	<i>Phanerochaete</i> sp. 3	44.32 (±0.87) ^a	53.51 (±1.21) ^b
HEV8B	<i>Diaporthe</i> sp. 4	43.71 (±0.16) ^a	27.48 (±3.14) ^d
HEV158C	<i>Cophinforma atrovirens</i>	32.19 (±2.39) ^b	66.39 (±0.70) ^a
HEV208H	<i>Diaporthe schini</i>	31.84 (±1.68) ^b	38.97 (±2.26) ^c
HEV170D	<i>Colletotrichum</i> cf. <i>fructicola</i> h1	21.31 (±0.74) ^c	42.85 (±1.44) ^c
HEV240H	<i>Diaporthe</i> sp. 6	19.17 (±1.79) ^c	42.12 (±0.92) ^c
HEV255M	Polyporales, sp. 2	18.25 (±1.89) ^c	61.29 (±2.24) ^{ab}
HEV100T	<i>Colletotrichum</i> cf. <i>fructicola</i> h4	18.06 (±1.49) ^c	40.93 (±1.55) ^c

Means values with the same letter in the column did not differ significantly according to Tukey’s test ($p < 0.05$). Values in parentheses correspond to the standard deviation

Discussion

Species of the genus *Hevea*, in particular *H. brasiliensis*, are known worldwide for being the main source of natural rubber. However, in recent years, it has been playing another role: that of harboring a vast and diverse community of

endophytic fungi that can not only perform different biotechnological functions in plants but also be used as biocontrol agents against phytopathogens [11–16, 53]. The present study addressed the diversity, and the enzymatic and biocontrol-related potential of foliar endophytic fungi present in three different clones of *Hevea* spp. (CPAA C06, C44, and C45), located in the Western Amazon.

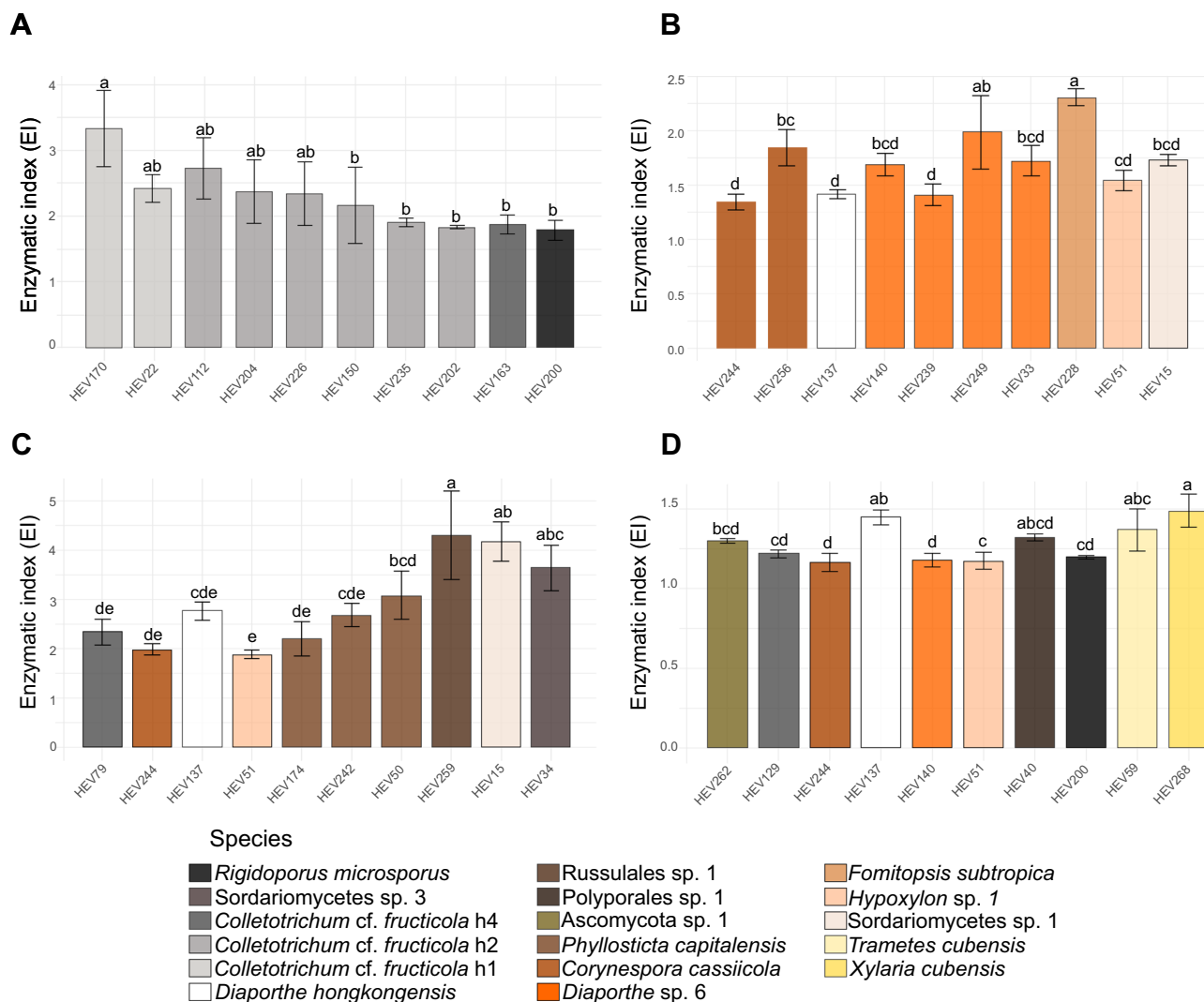


Fig. 5 The ten endophytic fungi of *Hevea* spp. that most produced the enzymes (A) amylase, (B) cellulase, (C) lipase, and (D) protease. The letters in the top bar represent the differences between mean values in the Tukey test

A total of 269 fungal isolates were recovered, and although this number is high, compared to other studies on *Hevea* [17, 54], the rarefaction curves showed that the sampling was insufficient to capture the richness present in the different clones. This finding is similar to the result found for other tropical species, such as *Paullinia cupana* (Sapindaceae), *Theobroma cacao* (Malvaceae), and *H. guianensis* and *H. brasiliensis* (Euphorbiaceae) [16, 17, 55, 56]. Tropical trees harbor a great diversity of fungal species; however, such diversity is commonly underestimated owing to the limitations of cropping-dependent techniques (e.g., isolation method, growth medium, and incubation conditions) [17, 57]. On the other hand, these techniques offer further insights into morphological taxonomy, phenotypic assessments, and time-efficient molecular identification of fungal isolates [58].

Forty-seven putative species were identified by sequencing the ITS rDNA-region, 30 of which represent singletons. The prevalence of rare species (singletons) in different genotypes contributes to the increase in richness and diversity of endophytic fungi [64]. In this case, the highest diversity values were found in the clone with the smallest number of recovered isolates (clone C44), probably influenced by rare species, while clones C45 and C46 presented lower richness, with greater dominance of the species *Colletotrichum* cf. *fructicola*.

The diverse composition of fungi among hosts of the same species may be associated with factors such as climate, mode of fungal dispersion, and the composition of communities surrounding the host plants [59]. In this study, the sampled plants were grown in the same environment and under the same climatic conditions, thus

suggesting that the variation in diversity is host-derived. Different cultivars can actively recruit specific endophytic fungal communities, which act in physiological functions and in response to abiotic and biotic stress [60]. The sampled clones have a distinct constitution; they are derived from interspecific crosses between the species. *H. pauciflora*, *H. rigidifolia*, and *H. guianensis*, which not only produce great amounts of latex but are also resistant to fungus *P. ulei* [10]. Thus, it is likely that the host is influencing the foliar endophytic fungi communities observed in this study.

Phylogenetic analyses using the ITS region showed that some fungal isolates had low statistical support (bootstrap < 70) and were not grouped with reference isolates. These results may correspond to new endophytic species in *Hevea*, considering that this genus harbors a great diversity of endophytic fungi, as reported in the studies of Gazis et al. (2012) [18] and Chaverri and Gazis (2011) [19], which described new species and classes. Another factor is related to the ITS region. Although it is the universal barcode for fungi [29], it has low resolution to discriminate species in certain taxonomic groups, such as species belonging to *Phanerochaete* and Polyporales (*Basidiomycota*), and Sordariomycetes and *Diaporthe* (*Ascomycota*), as noted in the present results.

The phylum *Ascomycota* was predominant in this study (95%). The classes Sordariomycetes and Dothideomycetes were the most representative in the clones, corroborating the studies of Arnold, 2007, in which 75% of the isolated endophytes were found to belong to these classes. The genera *Colletotrichum* and *Diaporthe* were dominant among *Hevea* clones. In recent studies with endophytes of *H. brasiliensis* and *H. guianensis*, this genus was constantly isolated, showing a dominance rate of 47.62 and 38%, respectively [17, 54]. In addition, native and planted trees of *Hevea* exhibit distinct patterns of diversity, and the presence of pathogenic species, such as *Colletotrichum*, *Diaporthe*, *Pestalotiopsis*, and *Corynespora*, found in the present study, are typical of monoculture plantations of *Hevea* [16].

Colletotrichum and *Diaporthe* cause diseases in different crops, with wide geographic distribution and low host specificity, and may be present in several species as a latent pathogen [17, 61]. The abundance of these genera in the samples may be due to the environment and the management of these clones in the collection area, which is surrounded by other crops such as guarana (*Paullinia cupana* var. *sorbilis*), host of *Colletotrichum* and *Diaporthe*.

One hundred and sixty-four isolates were grouped within the species *Colletotrichum* cf. *fruticola*. In the world, *C. fruticola* is widely distributed, causing anthracnose in several plants, such as members of the family Euphorbiaceae as *H. brasiliensis* [62], and as endophytic species, it has

been reported in Brazil, China, Korea, Panama, and Thailand [63]. The high presence of the species may indicate a truly endophytic habit in *Hevea*, since the leaves were healthy and showed no symptoms, or it could be part of the species life cycle, as a strategy of infection in the host, since many species of the genus *Colletotrichum* use hemibiotrophic parasitism to colonize the host, with an initial biotrophic phase and, later, a necrotrophic phase [64].

Inhibition of mycelial growth of *C. cassiicola* and *C. gloeosporioides*

The major mechanism used by endophytes of *Hevea* spp. against the phytopathogens *C. cassiicola* and *C. gloeosporioides* was inhibition by competition (space and nutrients), but there was no inhibition by antibiosis. The choice of the dual culture method may have favored the competition inhibition mechanism, since this test can easily detect direct antagonism, but not indirect antagonism [65]. Another factor is the growth rate among the different endophytic isolates, which may have influenced the antagonism of phytopathogens by competitive exclusion, since fungi that have accelerated growth quickly colonize the substrate, depleting nutrients, and occupying a niche that could be used by the phytopathogen [53].

The percentage of inhibition of phytopathogens by endophytic fungi of rubber trees did not exceed the rate of 66.4% (*C. gloeosporioides*) and 44.32% (*C. cassiicola*) in vitro. In a controlled and favorable environment, both the pathogen and the antagonist tend to grow at similar rates; however, the behavior observed in vitro is susceptible to change in the presence of the host plant, and may have an accentuated or reduced inhibitory effect, as demonstrated in the study by Chow et al. (2018) [66], who found a negative effect of co-inoculation of endophytes *Diaporthe phaseolorum*, *Trichoderma asperellum*, and *Penicillium citrinum*, in the infection of *Ganoderma boninense* in palm oil (*Elaeis guineenses* Jacq.), despite the success of these isolates in paired culture tests.

The species *Cophiniforma atrovirens* showed better performance in inhibiting *Colletotrichum gloeosporioides*, in vitro, and it has been linked to diebacks and stem rot in a restricted plant base, including cashew nuts in Brazil [67]. Low-frequency isolation of endophytic *Cophiniforma* sp.1 was performed in the studies of Vaz et al., (2020) [54], using leaves from rubber trees native to the Eastern Amazon; however, its action as a biological control agent is still poorly understood. The species Polyporales sp. 2 also showed good inhibition of *Colletotrichum*, and although the genus could not be determined, the species forms a sister clade with species of the genus *Phanerochaete*, which also effectively inhibited the two tested phytopathogens.

Endophytic species of the genus *Phanerochaete* are known to produce bioactive compounds with the power to inhibit or control several phytopathogens in vivo and in vitro. Khruengsaï et al. (2019) [68] made an in vitro evaluation of the crude extracts of *Phanerochaete* sp. (strain MFLUCC16-0609) isolated from *Ocimum basilicum* var. *thyrsoiflora*, against phytopathogens *Trichoderma reesei* and *Lasiodiplodia theobromae*, and detected two effective antifungal compounds in the control, sclareolide (42.03%) and muzigadial (19.44%). On the other hand, in vivo trials in the field with *Phanerochaete chrysosporium* (strain B-22) in chrysanthemum, showed that *P. chrysosporium* markedly inhibited the causal agent of chrysanthemum wilt (*Fusarium oxysporum*), in the soil and was able to reduce molecules that stimulate the propagation of the fungus, such as p-hydroxybenzoic acid (HA) [69].

Diaporthe sp. 4 effectively inhibited *C. cassicola* in the antagonism experiment. The species of this genus are used as a source for production of natural products, e.g., tyrosol, with antifungal and antibacterial effects [70, 71]. However, even with the success of these endophytes in the initial screening, additional studies are needed to determine the mode of inhibition and the efficacy of endophytes in controlling the pathogens tested.

Production of extracellular enzymes

The ability to synthesize extracellular enzymes was detected in 91 endophytic isolates of *Hevea*. Of these, a greater number of isolates were able to synthesize amylase and lipase in vitro, grouped as good producers (EI > 2), while there was an opposite trend for cellulase and protease. Endophytic fungi produce an arsenal of extracellular enzymes, which are involved in the process of hydrolysis and biodegradation during colonization of host tissues [72], in the suppression of plant defense mechanisms, as well as for nutrient uptake [73].

In this study, the differentiated synthesis of enzymes by endophytes may be related to the isolation source material, as reported in the studies by Shubba and Srinivas (2017) [74], in which a greater number of endophytes of *Cymbidium aloifolium* obtained from the flowers synthesized lactase, amylase, lipase, and protease, while the endophytes obtained from the root synthesized cellulase and protease. Thus, as the endophytes of the present work were isolated from the leaves, a greater synthesis of amylase and lipase is expected.

Isolates from the species *Colletotrichum* cf. *fructicola* synthesized high levels of amylase (EI > 2). Species of *C. fructicola* are recognized for being good amylase producers, as an adaptive strategy to colonize and interact with

their hosts [75]. In the absence of the living host, *C. fructicola* survives saprophytically in senescent plants, and uses amylase synthesis to degrade available starch into simpler carbohydrates, which will be assimilated by fungi [76]. High amylolytic production can also be a characteristic of the genus *Colletotrichum*, since Shubba and Srinivas (2017) [74] found similar results to those of this study with the species *C. gloeosporioides*.

Most isolates with EI > 2 for lipase belong to the species *Phyllosticta capitalensis*, while the highest enzyme index in this study was found for Russulales, sp.1 and Sordariomycetes, sp. 1 (EI: 4.14). Microorganisms under adverse conditions efficiently synthesize lipases; they are effective in solid and aqueous culture media, and known to withstand extreme temperature and pH values [77], which can be used in various industrial bioprocesses [78].

To date, the role of lipase synthesis in *P. capitalensis* is unknown, but the synthesis for species of the genus has already been reported [79]. In members of the order Russulales, such as ectomycorrhizas, high lipase synthesis is involved with adaptive evolutionary mechanisms, e.g., the expansion of protease and lipase genes throughout the genome, which facilitated the transition of this order from a saprophyte habit to a symbiont one [80].

The enzyme cellulase was detected in only 33.33% of the fungi, and only the species subtropical *Fomitopsis* achieved the best IE (> 2). This enzyme is produced by these fungi to degrade plant materials, as they are responsible for wood decomposition and contribute to biomass recycling [79–81].

The genus *Fomitopsis* is one of the genera that has potential for large-scale production of cellulose, and several studies with species of this genus are seeking to optimize the production of this enzyme [81]. A study with filamentous fungi of cocoa (*Theobroma cacao*) seeds evaluated the synthesis of cellulose by the fungus *F. subtropical*, and this was considered a good producer of this enzyme (IE > 2), a similar result to that of the present study [17].

Production of protease by the isolates was classified as moderate according to their EI, with emphasis on *Xylaria cubensis*. Species of the genus *Xylaria* have been reported as good protease producers in enzyme screening studies [73, 82]. Protease and chitinase are synthesized by endophytic fungi in nature when they compete with other microorganisms for an ecological niche, as these enzymes degrade the hyphae cell walls of pathogenic microorganisms [83].

Cellulase and protease enzymes were detected in less than 40% of endophytic fungi. This may be related to the fact that the plant-endophyte interaction was highly dependent on host species, tissue, plant age, and climatic conditions, which can influence the biology of the fungi and, consequently, the production of these enzymes [82]. The synthesis of these enzymes by endophytic isolates of *Viola odorata* [84] and medicinal

plants [82] demonstrated that the production of cellulase and protease by fungi did not exceed 33 and 37%, respectively, in both studies.

Conclusions

The diversity of endophytic fungi from different rubber tree clones is dependent on the genotype, and they are promising antagonists by competition with plant pathogens, and resources for industrial applications owing to their ability to hydrolyze several enzymes. These microorganisms can be a source for new metabolites used in biocontrol, and enzymes of industrial interest [85]. Studies aimed at increasing the production of enzymes by these fungi can provide highly efficient strains that will be used in industrial processes in the future.

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