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Characterization of White- and Brown-Rot Fungi Applied to the Decay of Caatinga Biome Wood (*Swartzia psilonema* Harms) from Brazil

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Abstract

This study investigated seven fungal species, belonging to the Culture Collection of the Brazilian Forest Service, in the decay of *Swartzia psilonema* wood from *Caatinga* Biome. The fungi were identified by sequencing the ITS regions and partial LSU gene rDNA and evaluated for the potential to produce enzymes of biotechnological interest when grown on *S. psilonema* sawdust as a carbon source. Test results with *S. psilonema* as a fungal substrate (sawdust and wood samples) were compared in an attempt to establish a relation between enzymatic production and wood weight loss. The correct identification of all fungi was confirmed. Therefore, they belong to the species *Coniophora puteana, Gloeophyllum trabeum, Meruliporia incrassata, Phanerochaete chrysosporium, Schizophyllum commune, Trametes versicolor* and *Trametes sanguinea. T. versicolor* showed the highest production of laccases, endo-1,4-β-xylanases and endo-1,4-β-glucanases. *S. psilonema* was classified from moderate to highly resistant, and its highest weight loss was caused by *G. trabeum*. The form, kind and complex composition of the substrate proved to be important factors influencing the extent of fungal decay. These results contribute the identification and characterization of biological resources of the culture collection, as well as offer valuable information related to wood classification as a commercial product and to the crude fungal extracts, since they represent a potential source of enzymes that can be used in cocktails for the woody biomass deconstruction.

Keywords – Basidiomycota – Lignocellulolytic Enzymes – Weight Loss – Wood Characterization – Wood Decay Fungi

Introduction

Fungi are the major decomposers in forest ecosystems (Shirouzu et al. 2016, Pasailiuk et al. 2022). Their community–level interactions make them fundamental to global carbon cycling (Hiscox et al. 2018). These higher fungi have developed complex biochemical systems capable of converting and mineralizing plant cell wall lignocellulose to carbon dioxide and water (Fackler &

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Schwanninger 2012). In addition to their ecological roles, they have important applications in agriculture, forestry and as biotechnological resources in industrial processes (De Mattos-Shipley et al. 2016, Miriyagalla et al. 2022).

A specific group, the wood decay fungi, has its largest number of species among the Basidiomycota, especially in the Agaricomycotina subphylum, distributed in large orders such as Boletales, Agaricales and Polyporales. The latter is an important order in terms of wood-decaying polypores species, with many of these decomposers belonging to genera like *Ganoderma*, *Phanerochaete*, *Pycnoporus* and *Trametes* (Riley et al. 2014, Froese 2018). Due to their ability to break down lignocellulosic biomass, these fungi are used as models in tests to verify the natural durability of wood, allowing indications of various uses for this material. Thanks to this characteristic, they have also been studied as sources of enzymes that can be used in bioprocesses based on the use of lignocellulose to obtain bioproducts such as pulp and paper, bio-fuels, food, animal feed and bio-chemicals (Rytioja et al. 2014).

Given the heterogeneous polymeric constitution of the cell wall, these fungi produce a wide variety of extracellular enzymes with the ability to degrade it, thus converting the polymers to monomers and short oligomers, which constitute their basic sources of carbon (Mäkelä et al. 2014). Cellulose is hydrolyzed by a set of enzymes that act synergistically on it, which are collectively named cellulases. The main ones are endoglucanases, exoglucanases, and beta-glucosidases (Rytioja et al. 2014). In view of the complex constitution of hemicellulose, the arsenal of enzymes necessary for its degradation involves the coordinated action of endo and exoenzymes and auxiliary enzymes. For cleavage of xylan, for example, the action of enzymes such as endo-1,4-β-xylanases and β–xylosidases on the main polysaccharide chain occurs. In addition, other enzymes may be required to cleave the lateral chains of the polymer, which represent barriers to other xylanases reaching the main chain (Rytioja et al. 2014, Carvalho et al. 2009). The degradation of lignin involves enzymes called class II peroxidases with high oxidation potentials, such as lignin peroxidases, manganese peroxidases and versatile peroxidases (Riley et al. 2014, Li et al. 2023) besides the phenol oxidases not dependent of peroxide that directly oxidize phenolic rings, the laccases, which can also act with the aid of low molecular weight mediators on non-phenolic structures (An et al. 2015).

The main decay pathways of this group of fungi are characterized as white and brown rot (Lundell et al. 2014). The fungi that cause white rot have the set of enzymes necessary for the complete degradation of all major constituents of the cell wall of the vegetable fiber, so that the residual wood becomes whitish or yellowish, moist, soft and often fibrous. Most of the wood decay fungi act through this mechanism and are often found on angiosperm wood species (Rytioja et al. 2014). The brown rot fungi make the residual wood dry, brown, powdery and cracking to cubicles. Apparently, they lost their genes that encode ligninases, presenting only a few genes for non-ligninolytic general peroxidases (Lundell et al. 2014). They also use non-enzymatic mechanisms on the cell wall, which has low porosity, through the production of low molecular weight oxidants, leading to diffuse depolymerization with an increase in the alkaline solubility of the wood, a typical feature of brown rot fungi (Presley et al. 2018).

This natural process can be seen in two aspects: negative, related to biodeterioration, when the wood is the product of interest, and positive, when it can be used for industrial purposes, as in the conversion, with high added value, of lignocellulosic waste in biorefineries. In particular, the enzymatic mechanisms characteristic of white rot fungi offers a series of applications in the field of renewable resource biotechnology (Fackler & Schwanninger 2012, Toussaint et al. 2016).

The Brazilian Forest Service (BFS) through its Forest Products Laboratory (FPL) maintains the Xylophagous Fungal Culture Collection as part of its work of technological characterization of wood, thus, it uses fungal species referenced in international standards for assessing the natural durability of this raw material and its derived products. The support given by BFS to sustainable forest management in the Caatinga, an exclusive biome in Brazil, made the FPL able to characterize timber species in that region and thereby make possible their potential uses, according to their physical and biological properties. Among these species, *Swartzia psilonema* provided the

lignocellulosic substrate to the study developed here, which allowed to deepen the knowledge about the wood decay fungi and to assign a new value to the FPL Culture Collection in addition to a service collection, so that it can contribute as a source of biological resources with potential for biotechnological applications.

The species *S. psilonema* belongs to the Fabaceae family, one of the largest among angiosperms, whose genus *Swartzia* has the highest number of species occurring in tropical America, with the Amazon being its dispersion center. They are medium to large sized trees, with some exceptions of shrubs, ranging from around 3 to 30 m in length, in which wood is your most economically valuable product (Angyalossy-Alfonso & Miller 2002, Meirelles & Souza 2015). In some species of *Swartzia* relevant phytochemical characteristics have been described as the presence of isoflavonoids, triterpenic saponins and aromatic diterpenes, chemical components with antimicrobial, antifungal and cytotoxic activity (Marqui et al. 2008).

The objectives of this study were: a) to identify seven isolates of wood decay fungi belonging to the FPL Culture Collection. The cultures have been maintained for about three decades in the FPL Collection and came from the Reference Mycology Culture Collection of the Forest Mycology Research Center (CFMR, Madison, WI) of the United States Department of Agriculture (USDA). The long storage period, accompanied by successive manipulations, motivated the checking of the post-preservation species through molecular techniques of DNA sequencing and phylogeny; b) to characterize these fungal species regarding the production of enzymes of biotechnological interests and their applications in wood degradation; c) to analyze the chemical composition of *S. psilonema* wood from the Caatinga Biome; and d) to classify *S. psilonema* wood for decay resistance according to ASTM Standard D 2017 (2005), thus providing information on the technological potential of this wood.

Materials & Methods

Fungal Collection and Maintenance

In the current study, seven fungal species from the Xylophagous Fungal Culture Collection of the FPL (Distrito Federal, Brazil) were used as models in the experiments of molecular identification, enzymatic production and accelerated laboratory decay test (Table 1). All species come from pure cultures obtained by the monohyfal cultivation method from the stock cultures stored in a BOD incubator at 5 ºC.

The fungi were grown in Petri dishes containing the solid medium, malt extract agar – MEA (3% malt; 1.8% agar) and incubated at 25 °C for 7 days. Five mycelial disks (Ø 1 cm) were then removed from each fungal culture and inoculated in cryotubes containing 50% (v/v) glycerol solution for maintenance in freezer at -80 °C.

Table 1 List of fungal species from the FPL Culture Collection related to the type of decay in the wood.

Primary Identity	Collection Codes	Decay Type
Coniophora puteana (Schumach.) P. Karst.	FPL–201	Brown rot
Gloeophyllum trabeum (Pers.) Murrill	FPL–203	Brown rot
Meruliporia incrassata (Berk. & M.A. Curtis) Murrill	FPL–205	Brown rot
Phanerochaete chrysosporium Burds.	FPL–105	White rot
Pycnoporus sanguineus (L.) Murrill	FPL–106	White rot
Schizophyllum commune Fr.	FPL–107	White rot
Trametes versicolor (L.) Lloyd	FPL–108	White rot

Molecular Identification

The fungal identification was performed by sequencing nuclear ribosomal DNA markers, covering the entire internal transcribed spacer (ITS) regions and part of the large subunit (LSU)

gene, complemented by phylogenetic data. Initially, the fungi (Table 1) were grown in Petri dishes containing MEA covered with cellophane paper and incubated at room temperature for 10 days in the dark. For genomic DNA extraction, the mycelia of these fungi were collected from the cellophane surface of the cultures in solid medium and transferred to 1.5 ml microtubes containing 30 µl of Tris–EDTA (TE) buffer. Genomic DNA extraction was carried out using the Wizard® Genomic DNA Purification kit (Promega, USA) following manufacturer's instructions. The DNA concentration was determined by spectrophotometry using NanoVue^{TM} Plus (GE Healthcare, UK).

The final volume reaction of 12.5 µL for amplification of ITS and partial LSU sequences was prepared using 6.25 µL of MyTaqTM Mix 2x (Bioline, USA), 0.3 µL (0.24 µM) of each V9G–Foward (5' – 3') primer: TTA CGT CCC TGC CCT TTG TA (Hoog & Ende 1998) and LR5–Reverse (5' – 3') primer: ATC CTG AGG GAA ACT TC (Vilgalys & Hester (1990), 1 µL of genomic DNA (2 ng/µL) and 4.65 µL of ultrapure water.

Polymerase chain reaction (PCR) cycling was conducted in a Thermo Bio-RAD/MyCycler thermocycler under the following conditions: 1 cycle for initial denaturation step at 95 °C for 1 min and 30 s, 35 cycles with one denaturation step at 95 °C for 20 s, one annealing step at 53 °C for 45 s and final extension step at 72 °C for 5 min. The amplified DNA was analyzed by electrophoresis on agarose gel 1% (w/v) in the presence of ethidium bromide (1 µg mL^{-1}), U.V. at 254 nm and standard molecular marker 1 kb DNA Ladder® (Invitrogen) to check its size and purity. The PCR products were purified using ExoSAP-IT® enzyme treatment (USB, Affymetrix, USA) following the manufacturer's recommendation and then sequenced by Sanger procedure (Macrogen Inc., South Korea).

Sequencing data were first analyzed for the quality of the chromatograms obtained for each isolate. The consensus sequence of the sequencing of each isolate was assembled with the aid of Geneious software v.9.1.3. (https://www.geneious.com Biomatters, New Zealand). The consensus sequence was compared against the collection of ITS and partial LSU ribosomal DNA sequences from the Genbank database (Benson et al. 2018) by the BLASTn algorithm (Madden 2013). The sequences with the best e–value parameters and maximum coverage were used to classify the sequence at species level. The multiple alignment of the isolates rDNA sequences was performed by the software MUSCLE v. 3.8.31 (Edgar 2004) using additional ITS and partial LSU rDNA sequences of fungal isolates retrieved from GenBank (Supplementary file). The phylogenetic tree was assembled in Fast Tree v.2.1 (Zhou et al. 2018) by the modified method of maximum likelihood with 1000 bootstraps.

Collect and Sampling of Wood Species

Five native *Swartzia psilonema* trees, popularly known as Blood rosewood, with 23, 23.5, 26, 27.5 and 32 cm in diameter at breast height (DBH), approximately 12 m tree height and 4.5 m commercial tree height, were collected by special authorization given by the State Department of Environment and Water Resources of the State of Piauí, in the municipality of Lagoa do Sítio (Northeast, Brazil), with predominance of typical Caatinga vegetation, at different points in a rural settlement area with a community forest management plan supported by the LPF/SFB. The same number of samples of the reference wood species *Cecropia* sp. and *Pinus* sp. were collected at the Água Limpa Farm from the University of Brasília, Federal District (Midwest, Brazil).

Wood Chemical Characterization

A disc was removed from each tree at the Diameter at Breast Height (DBH). Using a power-driven saw, each wood disc was cut into four equals wedges that included sapwood and heartwood. One part was chipped, milled in a Wiley knife mill and sifting. It was used the material retained between the 40 and 60 mesh screens.

The percentage of extractives was determined in a solution of ethanol/toluene (1:2) according to Tappi standard T 204 om-88 (1996). Ash content determination was done through Tappi standard T 211 om-93 (1993). LAP-003 NREL (1995) was used to quantify insoluble lignin and LAP-004 NREL (1996) to soluble lignin. The values estimate for holocellulose was calculated subtracting the

amounts of extractives, ash, acid-soluble lignin and acid-insoluble lignin from 100%. Each tree was analyzed by triplicate.

Wood Sawdust

The production of lignocellulolytic enzymes in fungi is strongly influenced by the substrate, therefore, we chose as substrate the *S. psilonema* wood sawdust readily available during the process of wood technological characterization by FPL. *S. psilonema* heartwood samples were reduced to small fragments to be crushed in a knife mill and then sieved with the aid of a sieve shaker using two sieves with 4.75 and 0.5 mm openings, as indicated by Simões et al. (2007). The material used in the tests was that collected on a 0.5 mm mesh sieve after five minutes at maximum speed in the shaker.

Production of Lignocellulolytic Enzymes

Initially, a screening was performed to detect enzymatic activity in each fungal strain by a solid medium plate assay. MYG solid media (malt extract 5 g L^{-1}, yeast extract 2.5 g L^{-1}, glucose 10 g L^{-1} and agar 20 g L^{-1}) was used for the tests. For detection of laccase activity, the MYG agar plate was added with Remazol Brilliant Blue indicator (0.3 M). For detection of xylanase and endoglucanase activities, the MYG agar plate was supplemented with 0.5% (w/v) oat-spelt xylan (Sigma- Aldrich, USA) or 0.5% (w/v) carboxymethylcellulose (CMC) (Dinâmica, Brazil), respectively, as carbon sources (Garcia 2006, Gomes 2017). All isolates were incubated for 4 days in BOD at 25 ± 2 ºC and 73 ± 2% RH, in the dark. After growth, the plates supplemented with carbohydrates were stained with 0.1% (w/v) congo red indicator and then bleached with NaCl solution (0.5 M) for detection of enzymatic activity, visualized by the lighter colored halo, as previously described by Carder (Carder 1986). Laccase activity was detected by transparent halo formation, according to Garcia (2006). The degradation halo was determined according to Hankin and Anagnostakis (Hankin & Anagnostakis 1975). The fungi that showed the highest degradation halos were selected for the enzymatic production experiments.

Enzymatic production was carried out by inoculating the fungi in conical flasks containing 50 mL of minimum mineral media (0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.16% $(NH_4)_2SO_4$ at pH 7.0) supplemented with 0.5% (w/v) of sawdust from *S. psilonema* wood as carbon source. The cultures were incubated at 28 °C in a rotary shaker under constant agitation of 120 rpm for 10 days. Two flasks were removed every 24 hours, the cultures filtered under vacuum, and the filtrates centrifuged at 10,000 × g for 10 min at 4 °C. The supernatants from the cultures of each fungal strain were used as sources of holocellulases and laccases for determination of enzymatic activity.

Enzymatic Assay and Protein Quantification

The enzymatic activities were evaluated by quantification of reducing sugar released from the filter paper (Whatman Nº 1), carboxymethylcellulose (CMC) 2% (w/v) and oat spelt xylan 1% (w/v) as substrates according to the method described by Miller (1959). Total cellulase, endo-1,4–β–glucanase and endo–1,4–β–xylanase activities were performed as described in Hamann et al. (2015). The absorbance in the colorimetric reaction was read in a spectrophotometer at 540 nm. One enzymatic unit (IU) was defined as the amount of enzyme required to produce 1 μmol of reducing sugar (glucose or xylose) per minute of reaction. Laccase activity was determined by oxidation of the ABTS substrate (2,2'–azino–di–3–ethylbenzthiazoline sulfonic acid) according to the method described in Garcia (2006). The absorbance reading was performed at 420 nm. One unit of laccase activity (IU) was defined as the amount of enzyme which leads to the oxidation of 1 μmol of substrate per minute.

The Bradford method was used to determine the protein concentration of the enzyme samples from the supernatants using the BioRad Quick Start™ kit (Bio–Rad Laboratories Inc., USA) following the manufacturer's instructions. The bovine serum albumin protein was used as standard. The absorbance reading was performed at 595 nm. All assays were performed in triplicate and the

data were presented as mean values ± standard deviation (SD). Statistical analyzes were performed using SigmaPlot v.12.0 (Systat Software Inc, USA) and the statistical significance was defined as $p \leq 0.05$.

Accelerated Laboratory Decay Test

The *S. psilonema* wood species was tested at the accelerated laboratory test with some adaptions according to ASTM Standard (2005) at the FPL. The species of *Cecropia* sp. and *Pinus* sp. were used as reference and control of the degradation to white and brown rot fungi, respectively.

The trees were cut into boards, air-dried and sawed into blocks with dimensions of 25 × 25 × 9 mm^3 in the radial, tangential and longitudinal sections (R × T × L). The blocks were randomly selected from each wood species, being 30 samples per fungus. The specimens were conditioned at 63 ± 2% relative humidity (RH) and temperature of 22 ± 2 °C at the climatization room, to a constant weight at 12% moisture content, before and after fungi attack.

Glass bottles with 250 mL capacity, were filled with 130 g of sieved B horizon soil, collected in Água Limpa farm from UnB, previously sieved and with pH adjusted to 6.0 by limestone addition. The soil was moistened to reach about 130% of its moisture holding capacity by the addition of 65 mL of distilled water. In each glass bottle was placed a feeder strip of *Cecropia* sp. or *Pinus* sp. (3 x 29 x 35 mm^3) for exposure to white rot and brown rot fungi, respectively, as described by Da Silveira (Da Silveira et al. 2019). These glass bottles were steam sterilized at 121 ºC for 30 minutes, cooled and incubated for 7 days until the initiation of the test. The fungi listed in Table 1 were cultivated in a malt extract solution, under rotation of 100 rpm at room temperature for 24h, and, in the sequence, were incubated at 25 ± 2 ºC and 75 ± 2% RH for three weeks until the inoculation stage of the wood samples.

The obtained fungal cultures were blended in a mixer and the glass bottles inoculated with 3 mL of liquid culture medium. In the sequence, the glass bottles were incubated at 25 ± 1 ºC and 73 ± 2% RH for four weeks until the complete colonization of the feeder strips. The wood samples were steam sterilized at 121 ºC for 30 min, and aseptically inserted in the bottles already inoculated and incubated at 25 ± 2 ºC and 75 ± 2% RH for up to 16 weeks. From the 8th week of the test, the control samples were weighed weekly. When this mass loss reaches a value equal to or greater than 50% of the initial mass, the experiment for each fungal species was ended. The fungal mycelium was removed from the samples and conditioned at 22 ± 2 ºC and 63 ± 2% RH until they reach the stability of mass variation. The classification of natural durability of the wood was made based on the difference between the initial and final mass, after the fungal attack.

Statistical analysis

In the analysis of the mass loss averages, the presence of outliers and their subsequent removal was done according to Hoaglin et al. (1986). Considering that the data did not present normality, the non-parametric test of Kruskal-Wallis was used at a 5% significance level through the software SPSS v. 21, to assess the statistical differences among them.

Results

Fungal Identification

Seven ITS and partial LSU rDNA fungal sequences were generated for *Trametes sanguinea*, *T. versicolor*, *Phanerochaete chrysosporium*, *Schizophyllum commune*, *Coniophora puteana*, *Gloeophyllum trabeum* and *Meruliporia incrassata* that were deposited in the GenBank database (Table 2). The ITS and partial LSU rDNA sequences of the previously identified fungi as *S. commune*, *P. chrysosporium*, *T. versicolor*, *C. puteana*, *P. sanguineus* and *G. trabeum* presented percentages of similarity between 95% and 99% with the ITS and partial LSU rDNA sequences of isolates of the same species, listed in the GenBank.

Five distinct main clades were formed in the phylogenetic tree, represented by the orders such as Boletales, Agaricales, Gloeophyllales, Polyporales and Auriculariales, all of the Agaricomycetes

class, subphylum Agaricomycotina of the phylum Basidiomycota (Fig. 1). The fungal species previously identified as *M. incrassata*, *C. puteana*, *S. Commune*, *G. trabeum*, *P. sanguineus*, *T. versicolor* and *P. chrysosporium* formed arrangements with isolates of the same species, in distinct groups, distributed among the main clades. The species of the genera *Meruliporia*, *Serpula* and *Coniophora* formed distinct groups within the Boletales clade. The ITS and partial LSU rDNA sequence of the fungus previously identified as *M. incrassata* showed identity with ITS and partial LSU rDNA sequences of the species *Meruliporia incrassata* (AJ419912) and *Serpula incrassata* (GU187541), both corresponded to the homotypic synonyms in the taxonomic database of the GenBank. All the isolates of the *S. commune* species were collected within the Agaricales clade. *G. trabeum* MN783219 was positioned with another *G. trabeum* isolates within the Gloeophyllales clade. The largest clade formed was the Polyporales, which brought together representatives of the genera *Pycnoporus*, *Trametes*, *Ganoderma*, *Ceriporia*, *Hydnopolyporu*s and *Phanerochaete* (Fig. 1). The clade formed by species of the genus *Pycnoporus* originated two subgroups composed of *Pycnoporus sanguineus* isolates, with *Trametes sanguinea* being a homotypic synonym of the species in the taxonomic database of the GenBank. Phylogenetic tree analysis arranged *T. versicolor* MN783213 together with *T. versicolor* isolates in a distinct group from other *Trametes* species, as well as, positioned *P. chrysosporium* MN783215 in a clade composed only by *P. chrysosporium* isolates (Fig. 1).

Table 2 Taxa and GenBank accession numbers of the nucleotide sequences generated through this investigation.

Taxon	ITS and partial LSU GenBank Accession no.
Trametes sanguinea	MN604696
Trametes versicolor	MN783213
Phanerochaete chrysosporium	MN783215
Schizophyllum commune	MN783217
Coniophora puteana	MN783218
Gloeophyllum trabeum	MN783219
Meruliporia incrassata	MN783220

Chemical Characterization of Brazilian Caatinga Wood Species

The results of chemical analysis of *Swartzia psilonema* wood showed 4.01% of extractives, 0.39% of ashes, 32.43% of total lignin with 31.27% insoluble lignin and 1.16% soluble lignin, and 63.17% of holocellulose (cellulose and hemicellulose).

Lignocellulolytic activity

Trametes sanguinea MN604696, *Trametes versicolor* MN783213, *Phanerochaete chrysosporium* MN783215, *Schizophyllum commune* MN783217, *Coniophora puteana* MN783218, *Gloeophyllum trabeum* MN783219 and *Meruliporia incrassata* MN783220 grew on culture media containing carboxymethylcellulose, oat spelt xylan and glucose plus the Remazol Brilliant Blue indicator, as carbon sources and degraded these substrates, with exception of *M. incrassata* MN783220 which did not degrade the substrate with Remazol Brilliant Blue indicator (Fig. 2g). The fungi that showed the highest degradation halos were *T. versicolor* MN783213, *G. trabeum* MN783219 and *M. incrassata* MN783220 (Fig. 2a–f, h, i).

T. versicolor MN783213, *G. trabeum* MN783219 and *M. incrassata* MN783220 grew in a liquid medium containing *S. psilonema* sawdust as carbon source and produced total cellulases, endo-1,4–β–glucanases and endo–1,4–β-xylanases (Fig. 3). Only *T. versicolor* MN783213 produced laccases (Fig. 4).

Maximal production of laccases (44.22 U/mg) (Fig. 4), endo–1,4–β–glucanases (4.15 U/mg) (Fig. 3b), total cellulases (0.64 U/mg) (Fig. 3a) and endo–1,4–β–xylanases (8.02 U/mg) (Fig. 3c)

were detected in the white rot fungus *T. versicolor* MN783213 after two, six and eight days of growth.

In brown rot fungi, the maximal production of endo–1,4–β–xylanases (1.42 U/mg) (Fig. 3c), total cellulases (0.37 U/mg) (Fig. 3a) and endo–1,4–β–glucanases (2.12 U/mg) (Fig. 3b) by *G. trabeum* MN783219 were obtained after two, eight and nine days, already in the *M. incrassata* MN783220 the highest activity values of endo–1,4–β–glucanases (0.37 U/mg) (Fig. 3b), total cellulases (0.80 U/mg) (Fig. 3a) and endo–1,4–β–xylanases (1.36 U/mg) (Fig. 3c) were detected on the 5th, 7th and 9th days of growth.

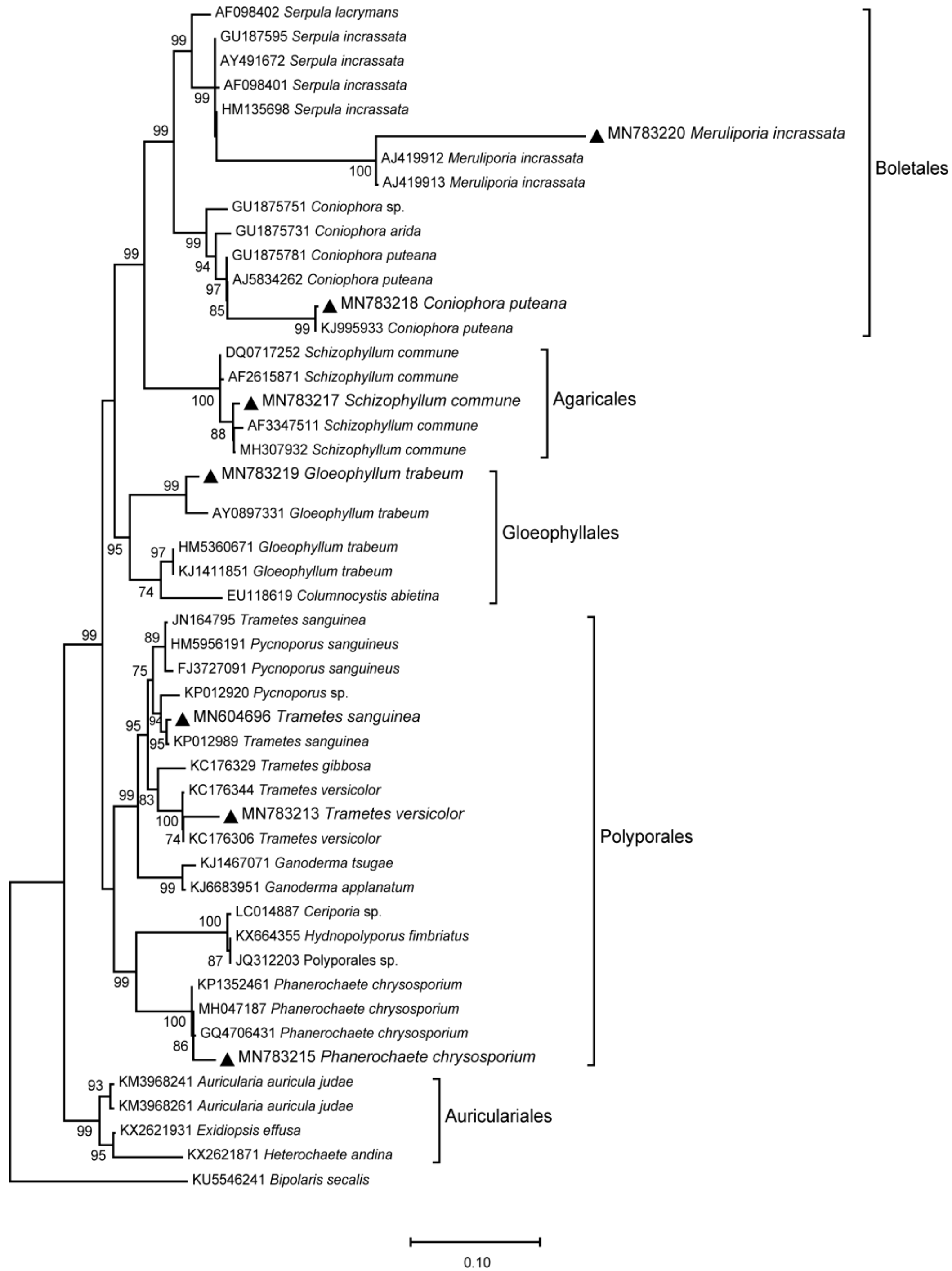

Fig. 1 – Phylogenetic tree of ITS regions and partial LSU rDNA gene from fungal isolates causing wood decay. Fungal sequences from the FPL Culture Collection were highlighted with the triangle symbol. The tree was constructed using Fast Tree v.2.1 with 1000 bootstraps. Values below than 70% were not shown. The *Bipolaris secalis* KU5546241 sequence was used as outgroup.

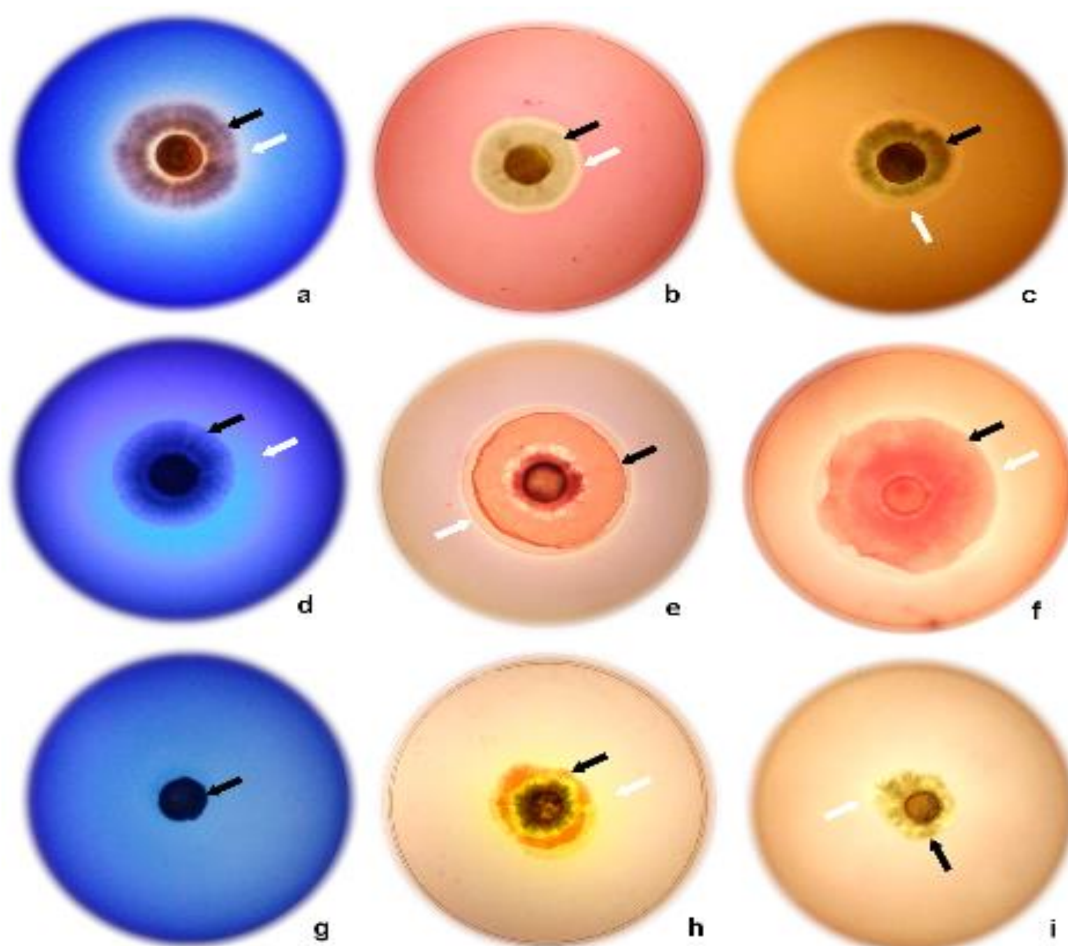

Fig. 2 – Wood decay fungi in agar-plate modified assay. (a–c) *Trametes versicolor* MN783213: a Culture in MYG + Remazol Brilliant Blue. b Culture in MYG + Carboxymethylcellulose. c Culture in MYG + Xylan Oat Spelt. (d–f) *Gloeophyllum trabeum* MN783219: d Culture in MYG + Remazol Brilliant Blue. e Culture in MYG + Carboxymethylcellulose. f Culture in MYG + Xylan Oat Spelt. (g–i) *Meruliporia incrassata* MN783220: g Culture in MYG + Remazol Brilliant Blue. h Culture in MYG + Carboxymethylcellulose. i Culture in MYG + Xylan Oat Spelt. Black arrows = edge of fungal colonies. White arrows = Enzymatic activity areas

Accelerated Laboratory Decay Test

The weight loss in the *Swartzia psilonema* wood and in the *Cecropia* sp. and *Pinus* sp. reference woods by accelerated decay with *T. versicolor* MN783213, *T. sanguinea* MN604696, *G. trabeum* MN783219, *P. chrysosporium* MN783215 and *M. incrassata* MN783220 are shown in Table 3. *S. commune* MN783217 and *C. puteana* MN783218 did not show satisfactory development on the feeder strips wood under the conditions determined by the standard test method.

All the fungi in Table 3 degraded the *Cecropia* sp. and *Pinus* sp. woods causing them significant weight loss, as expected for each decay type. In the investigation of *S. psilonema* degradation, all the fungi in Table 3 caused it weight loss. However, its highest decomposition rate was observed in *G. trabeum* MN783219 (29.72%) which allowed it to be classified as moderately resistant to this brown rot fungus. Statistically similar weight loss (18.55%) made the *S. psilonema* wood classified as resistant to both white rot fungi, such as *T. versicolor* MN783213 and *T. sanguinea* MN604696. The weight loss was less than 10% for *P. chrysosporium* MN783215 and *M. incrassata* MN783220 and the *S. psilonema* wood was classified as highly resistant to both white and brown rot fungi, respectively.

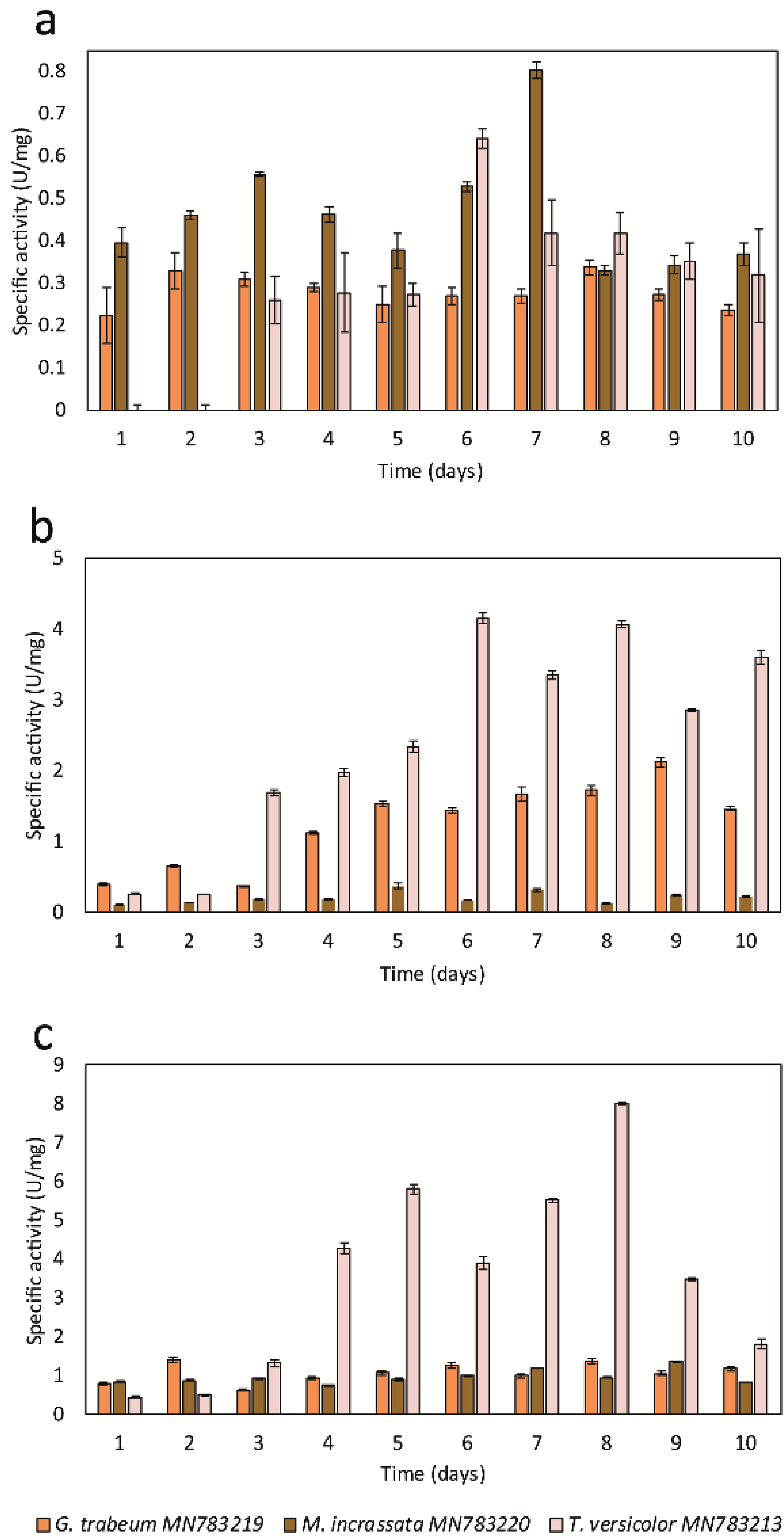

Fig. 3 – Production of total cellulases. a endo–1,4–β–glucanases. b and endo–1,4–β-xylanases. c by wood decay fungi when grown on 0.5% (w/v) as a carbon source *Swartzia psilonema* wood sawdust. Statistical significance was defined as $p \leq 0.05$.

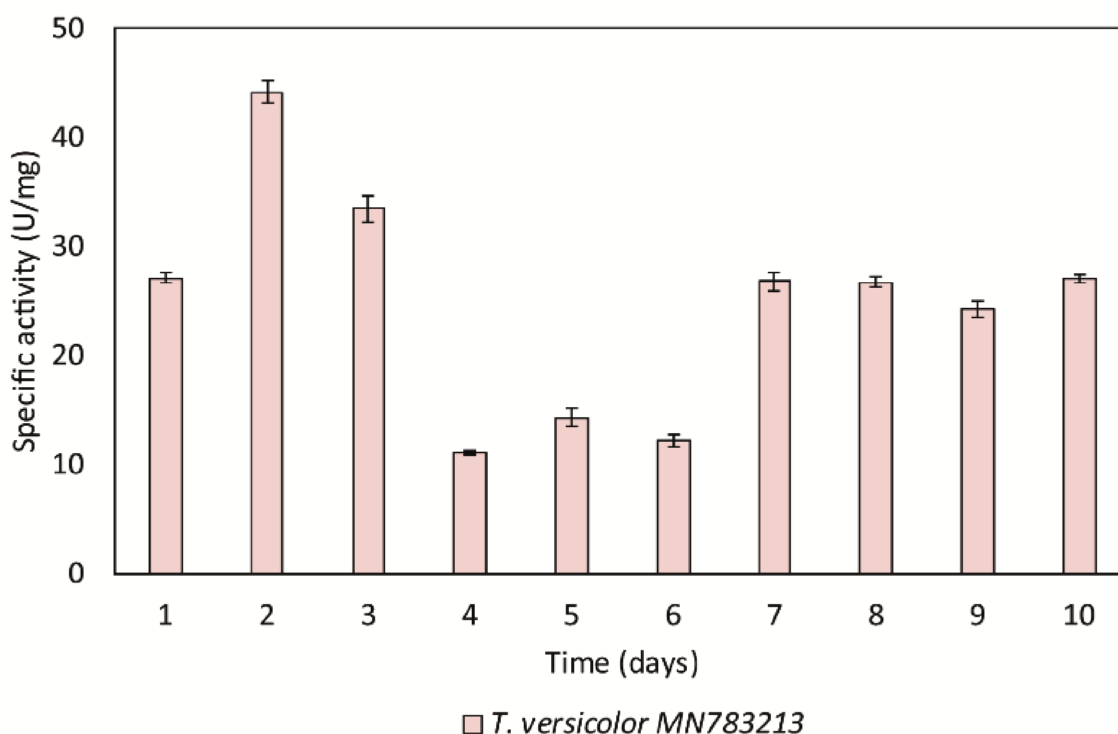

Fig. 4 – Production of laccases by *T. versicolor* MN783213 when grown on 0.5% (w/v) as a carbon source *Swartzia psilonema* wood sawdust. Statistical significance was defined as $p \leq 0.05$.

Discussion

The fungal identification carried out by this investigation is essential data given to the FPL Culture Collection, considering that the correct identification of the species used in standardized accelerated decay tests is fundamental to the reproducibility and reliability of its results to access the natural durability of woods (Plaschkies et al. 2014, Raja et al. 2017, Miriyagalla et al. 2022). The objective of expanding the functionality of the FPL Culture Collection beyond a service collection, acting as a source of biological resources with potential for biotechnological applications, also makes this data crucial, in addition to contributing to composing national lists of fungi with the aim of cataloging and protecting fungal biodiversity (Pasailiuk et al. 2022). The results of this study promoted the insertion of the Xylophagous Fungal Culture Collection of the FPL in the Information System on Brazilian Biodiversity (SiBBr) a node of the Global Biodiversity Information Facility (GBIF) in Brazil. The sequencing of the ITS region and the LSU gene of the ribosomal DNA has been reported positively in the identification of wood decay fungi (Schmidt et al. 2012), however, in our study we observed that there is not a large number of sequences covering the ITS region and the partial LSU gene at the same time for the species investigated by this research in the database employed. This fact has also been previously reported in studies involving the complete sequencing of the ITS region, which state that there is a significant proportion of fungal species that do not have complete ITS sequence data in public databases such as GenBank, one of the main repositories of fungal sequence data (Yahr et al. 2016). One of the contributions of our study consists of providing data on the complete sequencing of the ITS region and partial LSU gene ribosomal DNA of the studied fungi, linked to the reference cultures, to the GenBank public database. In addition to resolving doubts regarding the correct identity of these reference cultures, which have been manipulated for more than three decades during the application in laboratory decay tests aiming to classify wood.

Table 3 Weight loss (%) and natural decay resistance classification of three forest wood species according to ASTM Standard D 2017–05.

Fungal strains	Weight loss (%)		
	***Cecropia* sp.**	***Pinus* sp.**	***Swartzia psilonema***
T. versicolor MN783213	47.82b	25.34bc	18.55b
	NR	MR	R
T. sanguinea MN604696	47.42b	23.73b	18.55b
	NR	R	R
G. trabeum MN783219	50.31b	53.22d	29.72c
	NR	NR	MR
T. versicolor MN783213	47.82b	25.34bc	18.55b
	NR	MR	R
P. chrysosporium MN783215	44.64b	20.26b	6.47a
	MR	R	HR
M. incrassata MN783220	42.60a	57.06d	8.26a
	MR	NR	HR

The same letter in weight loss columns indicates that they do not differ statistically from each other by the peer comparison test at the 5% probability level. HR, Highly Resistant; R, Resistant; MR, Moderate Resistance; NR, Not Resistant

In the evaluation of enzymatic production, the absence of plate activity described in *M. incrassata* MN783220 in the culture medium with Remazol Brilliant Blue is in accordance with that described brown rot fungi as to the mechanisms employed by them in the selective removal of carbohydrates in wood. This is an evolutionary process that was accompanied by losses of genes related to lignin degradation (Floudas et al. 2012, Presley et al. 2018). However, in *G. trabeum* MN783219 plate activity was detected. This characteristic has been previously described in the literature for this and other brown rot fungi such as *Gloeophyllum sepiarium*, *Coniophora puteana* and *Antrodia vaillantii* that reacted positively in these tests by discoloring the polymeric dye Poly R-478, suggesting a different mechanism of depolymerization for this group of fungi (Freitag & Morrell 1992). In subsequent publications, it was demonstrated that these fungi can modify lignin by a different mechanism from those employed by white rot fungi, by promoting diffuse depolymerization of cell wall constituents through the action of low molecular weight oxidants (Presley et al. 2018).

T. versicolor, *G. trabeum* and *M. incrassata* are species reported as strong producers of holocellulases during growth on lignocellulosic biomass (Rytioja et al. 2014, Baldrian & Valášková 2008). However, our study reports for the first time the production of these enzymes and laccases by these fungi during growth on *S. psilonema* wood sawdust as a carbon source. The enzymatic production data obtained here contribute to elucidating the possible steps involved in the wood degradation by decay fungi.

T. versicolor MN783213 was the best producer of laccases, endo–1,4–β–glucanases and endo–1,4–β–xylanases. After the second day of growth, maximum laccase activity was observed accompanied by low activities of endo–1,4–β-glucanases and endo–1,4–β–xylanases. Laccases are among the main catalytic oxidoreductases enzymes on lignin degradation in Basidiomycota (Hassett et al. 2009, Schmidt-Dannert 2016). The biocatalytic activities detected at the beginning of growth suggest a higher enzymatic activity on lignin. This proposes an efficient action mode of this fungus: to

overcome the recalcitrance of lignin to gain access to the carbohydrates of the cell wall, a critical step in view of the high content of total lignin in *S. psilonema*. These data provide relevant information about the fungus activity on lignocellulose, since there is an intense field of research focusing on the use of wood decay fungi in the pretreatment step of lignocellulose in biorefineries, concentrated mainly in the search for genera and species with the potential to delignify biomass without degrading cellulose at this step of the bioconversion process (Capolupo & Faraco 2016). In agreement with our results, other *T. versicolor* species showed high laccases production during the initial stage of wood colonization (Machuca & Ferraz 2001, Lekounougou et al. 2008). On the other hand, previous studies have related the high laccases production in the initial stage of wood colonization by *T. versicolor* to the presence of aromatic compounds in the wood extractives, suggesting a detoxification function for laccases (Lekounougou et al. 2008). It is possible, therefore, that among 4.01% of *S. psilonema* extractives, there were aromatic compounds that functioned as substrates for laccases. This is important data to be considered in the wood degradation process by fungus, since the quantity and type of extractive are the main factors that confer resistance to wood against fungal degradation (Da Silveira et al. 2019).

In the current study, it was observed that the lignocellulolytic capacity of decay fungi could be influenced by factors associated to the substrate, related to its basic composition and kind (wood samples or wood sawdust). The relation with the substrate is crucial as to the type and amount of enzymes secreted (Doria et al. 2014, Ullah et al. 2019). The type of carbon source in the medium may be the most relevant factor affecting the target enzyme synthesis. Previous studies reported activity peaks of laccases (0.93 U/ml), endoglucanases (0.7 U/ml) and xylanases (5.2 U/ml) in *T. versicolor* using wheat bran as a carbon source. This substrate is considered excellent in promoting the growth and lignocellulolytic enzyme production in a large number of Basidiomycota fungi (Irbe et al. 2014). With this in mind, the study with *S. psilonema* wood sawdust as a carbon source proved to be relevant due to the production peaks described here for these same enzymes.

The chemical composition of the *S. psilonema* wood is consistent with the description in the literature about hardwoods, which have a slightly higher amounts of cellulose and hemicellulose than those of softwoods (Santana & Okino 2007, Rytioja et al. 2014). The hardwoods are also known to have a higher proportion of xylan in hemicellulose (Rytioja et al. 2014). This data can be considered here within the high percentage of holocellulose found in *S. psilonema* wood and would partly explain the higher production of endo–1,4–β–xylanases by *T. versicolor* MN783213 compared to the other fungal strains, favored by the relation of the fungus to its preferred substrate, given those white rot fungi are commonly isolated from hardwoods (Wymelenberg et al. 2011, Rytioja et al. 2014). However, the relatively lower production of endo-1,4-β-xylanases by the remaining fungal strains may have been compensated by the production of other enzymes acting on holocellulose. Such behavior was similarly observed in the brown rot fungus *Daedalea quercina* in the study of *Populus* sp. wood degradation (Doria et al. 2014).

G. trabeum MN783219 was the second in endo–1,4–β–glucanase activity. In agreement with our results, the previous paper demonstrated that *G. trabeum* produced hydrolytic activities during the entire wood degradation and showed a prompt capacity to convert carboxymethylcellulose to reducing sugars, suggesting the predominance of endoglucanase activity (Aguiar et al. 2013, Li et al. 2023). There is an increase in the production of endo–1,4–β–glucanases by *G. trabeum* MN783219 from the 4th day of growth, reaching its maximum value on the 9th day. Endo-1,4-β-xylanases were earlier produced in comparison to cellulases, reaching its maximum value on the 2nd day. This activity pattern is in accordance with previous researches that suggested the preferential degradation of hemicelluloses by brown rot fungi as an efficient way to access cellulose microfibrils (Goodell 2003, Aguiar et al. 2013). However, the endo–1,4–β–xylanases production by *G. trabeum* MN783219 was relatively lower than that by *T. versicolor* MN783213. The substrate preference of brown rot fungi for softwoods can partly explain this production pattern. Softwoods have a higher mannan content compared to hardwoods. It was observed that during the evolution of the brown rot fungal lifestyle, the number of genes encoding xylanases was reduced, so that they have slightly lower numbers of xylanolytic enzymes than the white rot fungi, at the same time that several copies of genes encoding mannanases

were identified in the genomes of brown rot fungi probably helping them to colonize softwoods (Rytioja et al. 2014, Song et al. 2015).

M. incrassata MN783220 secreted more endo–1,4–β–xylanases compared to the other enzymes evaluated during the entire cultivation period, with production values close to each other and whose maximum value secretion was detected on the 9th day. It presented the highest conversion rate of filter paper to reducing sugars, reaching the peak of total cellulase activity on the 7th day. However, endo-1,4-β-glucanase activity was discreet regarding to the other strains evaluated. Particularly on the 7th day of growth, a simultaneously higher activity of the three evaluated enzymes was detected. Since brown rot fungi are known to have caused the most destructive forms of wood decay by rapidly removing carbohydrates from the cell wall, only modifying the lignin (Kleman-leyer & Kirk 1994, Schmidt-Dannert 2016) this result can be related to the fact that brown rot fungi initiate cellulose degradation with highly reactive oxidants such as low molecular weight free radicals, including hydroxyl radicals formed through the Fenton reaction (Rytioja et al. 2014). However, the detection of low endo-1,4-β-glucanase activity during all wood decay may correlate with the activity of this enzyme on CMC, since in a previous study it was demonstrated that among the endoglucanases produced by *M. incrassata* in the cotton cellulose decay, there was one isolated whose activity on cellulose was detected but which did not release reducing sugars from CMC nor did it significantly reduce the viscosity of CMC solutions (Kleman-leyer & Kirk 1994). On the other hand, the lower activity of endo-1,4-β-glucanases may have been compensated by the activity of other cellulases, considering that *M. incrassata* MN783220 presented the best result in the evaluation of total cellulase activity. Nevertheless, it is worth mentioning that there are few reports in the literature regarding the enzymatic production of *M. incrassata*. Research in this regard has received considerable attention (Schilling et al. 2012, Hegnar et al. 2019). The data found here contribute in this sense, since more emphasis has been placed on *Serpula lacrymans*, its European pendant, an important species of wood decay fungi very common in the central continent (Schmidt 2007, Nurika et al. 2020).

There was variability in *S. psilonema* wood weight loss with values ranging from 6.47% to 29.72% under conditions of laboratory decay tests. Our result is in line with those of other studies which state that this constitutes a direct reflection of the morphophysiological differences among the fungal species tested, differences closely linked to the decay type, as observed in the *Pinus* sp. wood decay by *M. incrassata* MN783220, which promoted the highest weight loss (57.06%) in the test (Zabel & Morrel 1992, Da Silveira et al. 2019). However, the natural durability, mainly attributed to the chemical property of the wood, such as the extractive content, is the fundamental basis of this variation as an endogenous factor of the plant, giving it protection against biological decay (Carneiro et al. 2009, Sundararaj et al. 2015, Da Silveira et al. 2019). This occurs in a peculiar way for each fungal agent, since for *P. chrysosporium* MN783215, *S. psilonema* wood was highly resistant, with the test's lowest weight loss (6.47%), however, it was shown to be susceptible to *G. trabeum* MN783219, whose decay activity caused it the highest weight loss (29.72%). *S. commune* MN783217 and *C. puteana* MN783218 have already been used in accelerated decay tests in previous studies carried out by the FPL, so a hypothesis that may be related to the fact that they did not participate in the accelerated decay test in the present study is that the genetic characteristics related to their ability to cause wood mass loss have not been properly conserved in them during the Collection maintenance process.

The high levels of enzymatic activities detected in *T. versicolor* MN783213 in the *S. psilonema* wood sawdust assay did not correlate directly with the extent of weight loss in the *S. psilonema* wood samples. This is not uncommon. Machuca and Ferraz (Machuca & Ferraz 2001) found in their studies with *Eucalyptus grandis* that the high enzymatic activities produced by wood decay fungi were not equivalent to a higher wood weight loss and they attributed this to the fact that the enzymes could not penetrate the cell wall easily to this form of substrate. Giorgio et al. (2012), in a similar study, evaluated that *Pinus taeda* in the form of wood sawdust was the substrate that allowed the best growth and production of hydrolytic and oxidative enzymes by white rot fungi. With this in mind, Doria et al. (2014) stated that the form and kind of the substrate (sawdust

or chips) may influence the fungal growth and extent of wood degradation, since the compacting of the chip makes the initial fungal attack difficult in a short time, while sawdust has finer and easily colonized particles, stimulating a higher enzymatic production. However, the correlation found here between enzymatic activity and weight loss may have been conditioned by other factors, such as the non-enzymatic mechanisms used by brown rot fungi, which would partly explain the higher *S. psilonema* wood decay by *G. trabeum* MN783219 in accelerated decay test, since previous studies claim that brown rot fungi combine Fenton-based oxidation and enzymatic hydrolysis to degrade wood (An et al. 2015, Presley et al. 2018), and the influence of *S. psilonema* extractives on the fungi, so that it appears to be less efficient against *G. trabeum* MN783219 in regard to *T. versicolor* MN783213 and *M. incrassata* MN783220.

The complex composition and natural durability characteristics of *S. psilonema* wood makes the use of its sawdust as a potential source for the enzymes production interesting in the investigation of alternative lignocellulosic biomass, as previously discussed. Therefore, few studies take into account the durability in choosing this material, however, it is an impact factor on the fungal lignocellulolytic capacity, as seen here in the use of wood in compacted form. Some studies used enzyme extraction methods during the accelerated decay test itself (Hwang et al. 2008, Aguiar et al. 2013, Irbe et al. 2014). This is an important field of investigation that can be deepened in the sense of a more critical analysis of how natural durability may or may not be affecting the production and recovery of enzymes through this testing type.

Further studies on lignocellulases production by the evaluated fungi are needed, since there is a synergism in their action in the cell wall deconstruction. Cultivation parameters such as pH, temperature and incubation time also need to be investigated, as they significantly influence the production of these enzymes by microorganisms (Leitão et al. 2017, Ticona et al. 2020).

It is worth mentioning that the data produced by this study also integrate the FPL pioneer studies in the chemical and technological characterization of the *S. psilonema* species, contributing to a better knowledge and use of its wood.

Conclusion

The fungi *Trametes versicolor*, *Schizophyllum commune*, *Meruliporia incrassata*, *Phanerochaete chrysosporium*, *Gloeophyllum trabeum*, *Coniophora puteana* and *Pycnoporus sanguineus* were identified at the species level and corresponded to primary identifications contained in the culture deposit carried out more than three decades in the FPL. *Trametes versicolor* MN783213, *Gloeophyllum trabeum* MN783219 and *Meruliporia incrassata* MN783220 were able to grow in the presence of *Swartzia psilonema* wood sawdust and produced lignocellulolytic enzymes. The present study is the first report of lignocellulases production by these decay fungi during growth on *S. psilonema* wood sawdust as a carbon source. *T. versicolor* MN783213 proved to be the best producer of laccases, endo–1,4–β–glucanases and endo–1,4–β–xylanases. By means of the accelerated decay test, *S. psilonema* wood was classified as resistant to *T. versicolor* MN783213 and its highest weight loss reported to *G. trabeum* MN783219. The form, kind and composition, including the extractives, of the lignocellulosic substrate were presented as important factors that influenced the extent of fungal degradation. These results contribute to the preparation of reports on the natural durability of commercial woods and to the biological resources characterization of the FPL Culture Collection, providing valuable information on crude fungal extracts as sources of enzymes with potential for application in bioprocesses using woody biomass.

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