Research, Society and Development, v. 11, n. 2, e13211225325, 2022
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v11i2.25325

Evaluation of enzymatic production of hydrolases and oxyredutases by *Fusarium pseudocircinatum* and *Corynespora torulosa* isolated from caesarweed (*Urena lobata* L., 1753)

Avaliação da produção enzimática de hidrolases e oxirredutases por *Fusarium pseudocircinatum* e *Corynespora torulosa* isolados de malva (*Urena lobata* L., 1753)

Evaluación de la producción enzimática de hidrolasas y oxiredutasas por *Fusarium pseudocircinatum* y *Corynespora torulosa* aisladas de malva (*Urena lobata* L., 1753)

Received: 01/03/2022 | Reviewed: 01/08/2022 | Accept: 01/18/2022 | Published: 01/21/2022

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Abstract

This work evaluates the enzymatic production of hydrolases and oxireductases by endophytic fungi isolated from leaves of the caesarweed (*Urena lobata* L.), which is a perennial plant that is well adapted to the Amazon floodplains region, being and is widely used in the production of hessian sacks and other products of natural fiber. Fungi strains *Fusarium* sp. (1290) and *Corynespora* sp. (1291) were reactivated, and had their DNA extracted and sequenced to obtain molecular identification. For enzymatic production, dried and ground caesarweed was used as a substrate in the mineral salt solutions Manachini and GLBN 40 over during 10 days of submerged cultivation (CS) under agitation. The CA was vacuum filtered daily with a 0.22 µm Millipore membrane to obtain enzymatic extracts, from which the activities of FPase, xylanase, CMCase, β-glycosidase, pectinase, laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) were evaluated. Lines strains 1290 and 1291 were identified as *F. pseudocircinatum* and *C. torulosa*, respectively, the latter being the best producer of laccase (8,691 U/L), MnP (5,353 U/L), β-glycosidase (0.328 U/mL) and CMCase

(0.351 U/mL) and the fungus *F. pseudocircinatum* was the best producer of FPase (1.294 U/mL), xylanase (12.052 U/mL) and pectinase (0.183 U/mL). No LiP activity was detected for either of the strains. The results showed that the strains used are promising for the production of seven of the eight quantified enzymes, and these enzymes are of interest to several industrial sectors.
Keywords: *Urena lobata;* Endophytic fungi; Hydrolases; Oxidoreductases.

Resumo
Este trabalho avaliou a produção enzimática de hidrolases e oxirredutases por fungos endofíticos isolados da folha da malva (*Urena lobata* L.), a qual é uma planta perene bem adaptada à região da várzea na Amazônia e é muito utilizada na produção de sacarias e outros produtos de fibra natural. Os fungos *Fusarium* sp. **(**1290) e *Corynespora* sp. (1291) foram reativados, e tiveram seus DNAs extraídos e sequenciados para obter a identificação molecular. Para a produção enzimática utilizou-se a malva seca e triturada como substrato nas soluções de sais minerais Manachini e GLBN 40 ao longo de 10 dias de cultivo submerso (CS) sob agitação. O CS foi filtrado à vácuo diariamente com uma membrana Millipore 0,22 µm para a obtenção dos extratos enzimáticos, dos quais foram avaliadas as atividades de FPase, xilanase, CMCase, β-glicosidase, pectinase, lacase, manganês peroxidase (MnP) e lignina peroxidase (LiP). As linhagens 1290 e 1291 foram identificadas como *F. pseudocircinatum* e *C. torulosa*, respectivamente, sendo este último o melhor produtor de lacase (8.691 U/L), MnP (5.353 U/L), β-glicosidase (0,328 U/mL) e CMCase (0,351 U/mL) e o *F. pseudocircinatum* foi o melhor produtor de FPase (1,294 U/mL), xilanase (12,052 U/mL) e pectinase (0,183 U/mL). Nenhuma das linhagens apresentou atividade de LiP.Os resultados demonstraram que as linhagens utilizadas são promissoras para a produção de sete das oito enzimas quantificadas, sendo estas enzimas de interesse para diversos setores industriais.
Palavras-chave: *Urena lobata;* Fungos endofíticos; Hidrolases; Oxirredutases.

Resumen
Este trabajo tiene como objetivo evalúar la producción enzimática de hidrolasas y oxireductasas por hongos endofíticos aislados de la hoja de malva (*Urena lobata* L.), que es una planta perenne bien adaptada a la región de la llanura aluvial amazónica, siendo ampliamente utilizada en la producción de sacos y otros productos de fibra natural. Se reactivaron los hongos *Fusarium* sp. (1290) y *Corynespora* sp. (1291), se extrajo su ADN y se secuenciaron para obtener la identificación molecular. Para la producción enzimática, se utilizó malva seca y molida como sustrato en las soluciones de sales minerales Manachini y GLBN 40 durante 10 días de cultivo sumergido (CS) bajo agitación. Se realizaron filtraciones a vacuo diariamente con una membrana Millipore 0.22 µm para obtener extractos enzimáticos que fueron evaluados de acuerdo a la actividad FPasa, xilanasa, CMCasa, β-glicosidasa, pectinasa, lacase, peroxidasa de manganeso (MnP) y peroxidasa de lignina (LiP). Los linajes 1290 y 1291 fueron identificadas como *F. pseudocircinatum* y *C. torulosa*, respectivamente, siendo esta última la mejor productora de lacasa (8.691 U/L), MnP (5.353 U/L), β-glicosidasa (0,3285 U/mL) y CMCase (0,351 U/mL) y el hongo *F. pseudocircinatum* fue el mejor productor de FPasa (1,294 U/mL), xilanasa (12,052 U/mL) y pectinasa (0,183 U/mL). Ambos linajes no tenían actividad de LiP detectada. Los resultados mostraron que los linajes utilizados son prometedores para la producción de siete de las ocho enzimas cuantificadas, y estas enzimas son de interés para varios sectores industriales.
Palabras clave: *Urena lobata*; Hongos endofíticos; Hidrolasas; Oxidorreductasas.

1. Introduction

Urena lobata L., 1753 (Malvaceae), which is popularly known as caesarweed, is an agricultural crop that is the raw material used in the production of screens, ropes, book covers, and sacks used in the exports of agricultural products, such as coffee (Cao et al., 2020; Cunha et al., 2021). In the Amazonas state, Brazil, caesarweed has adapted to the floodplain areas of whitewater rivers, mainly in the channels of the Solimões and Amazon Rivers, and is concentrated in the municipalities of Anamã, Anori, Beruri, Caapiranga, Coari, Codajás, Iranduba, Itacoatiara, Manacapuru and Parintins. The total cultivated area is 7,684 hectares, and production in the Amazonas state reached 6,770 tons in 2018, with Manacapuru being the largest producer in the state, with 4,042 tons (Cunha et al., 2021; Maciel et al., 2019).

Since it is an important crop in the Amazon, there is interest in the study of its microbial diversity in order to find promising microorganisms for bioprocesses, in this case, producers of enzymes and secondary metabolites. The microorganisms of greatest interest are the endophytes, which live in symbiotic association and confer mutual benefits in this interaction, i.e., the plant offers protection and nutrients and the endophytes produce metabolites that help in the plant's growth as well as providing protection against pests and diseases (Deng & Cao, 2017).

Research involving enzymes has intensified and this is due to their recognized catalytic capacity, which is applied in the optimization of industrial processes. Enzymes are active and versatile, and perform reactions quickly, especially under mild reaction conditions (Sharma et al., 2020). Hydrolases and oxyredutases are the classes of enzymes that have the greatest potential in various industries such as those of food, textile, cellulose, in addition to use in effluent treatment (Naik et al., 2019).

Endophytic species of *Trichoderma reesei*, *Penicillium oxalic* and *Aspergillus niger* are among the main producers of cellulases used in the hydrolysis of cellulose and sugar production (Li et al., 2021). Divided into endoglucanases (which act on the inner region of the cellulose fiber that produce oligosaccharides formed by glucose units), exoglucanases (which act on the ends of the fiber that release cellobiose, and formed by two glucose units) and β-glycosidases (which act on the cellobioses that release glucose molecules) (Marques et al., 2018; Gaete et al., 2020).

Xylanase enzymes are important bioconverters of lignocellulosic compounds into sugars, and hydrolyze β-(1,4) bonds of the xylan molecule. These enzymes are widely used in paper bleaching, in the production of bread, juices, as well as being used as an additive in animal feed, and in the production of xylitol and ethanol (Amobonye et al., 2021).

Pectinases form a group of enzymes that promote the decomposition of polysaccharides that form pectic substances. They are also used to reduce excessive bitterness in citrus peels, restore aroma lost during drying and improve the firmness of peaches and processed pickles. The fungus *A. niger* is the most widely used for the industrial production of pectinases (Patidar et al., 2018; Noguchi et al., 2020).

Ligninases are enzymes of the oxyreductase group that can alter the properties of lignin and partially remove it from biomass by facilitating the hydrolysis process. They play a fundamental role in the degradation and detoxification of industrial waste and in the treatment of xenobiotics and colorants, as well as being also used in bioremediation of soil and water (Garcia, 2018; Kumar & Chandra, 2020).

In this regard, the application of enzymes of microbial origin in various sectors of modern society has stimulated the market for enzymes, and has made the continuous and extensive bio-prospection of new enzymes and/or selection of more efficient processes, thus contributing to the discovery of efficient biocatalysts. In this context, the present study proposes to evaluate the enzymatic production of hydrolases and oxyreductases by endophytic fungi isolated from caesarweed.

2. Methodology

2.1 Microorganisms

In this study, we used the strains *Fusarium* sp. (1290) and *Corynespora* sp. (1291), which were both isolated from the leaves of *U. lobata* and are deposited in the fungi collection at the Laboratory of Microorganisms and Bioassays of the Amazon (LABMICRA), at the Federal University of Amazonas (UFAM) registered in SisGen A91107E. The fungi were reactivated in PDA for 8 days at 28 °C, then a conidial suspension was prepared for the 1290 strain (anamorphic form), scraping the conidia were scraped and covered inserted in sterile cryogenic tubes with 20% glycerol, at the concentration No. 6 of the McFarland turbidity scale. The suspensions were then frozen at -20° C for later use. For the 1291 strain, the inoculum is described in item 2.3.

2.2 Morphological and molecular identification

The strains were identified from macro and micromorphological observations of characteristics such as the color of the surface and the back of the colonies, as well their texture and pigment. To discriminate the vegetative and reproductive structures of the strains, microcultures were incubated for 48 hours at 28 °C and then stained with lactophenol blue and the results obtained were compared with the specific literature (Barnett & Hunter, 1972; Kiffer & Morelet, 1999; Watanabe, 2002).

2.2.1 DNA extraction

Fungi were grown in 125 mL Erlenmeyer flasks, containing 50 mL of BDY medium according to Souza et al. (2004). The cultures were subjected to incubation at 28 °C for 24-48 hours, under an agitation of 120 rpm. Mycelial masses were obtained through vacuum filtration and the fungal DNA extraction procedure followed the protocol of the Zymo Research Fungal/Bacterial DNA MicroPrep™ kit, with adjustments (Silva-Filho et al. 2021). At the end, the extracted product was quantified in a spectrophotometer (NanoDrop, Thermo Scientific), and the integrity of the extracted product was evaluated by electrophoresis using 0.8% agarose gel. All the extracted material was then stored at -20 °C.

From the total DNA, an internal fragment of approximately 700 bp was amplified from the *Its*-1 and 2 regions of the *rDNA* using the primers Its1 (5'-TCCGTAGGTGAACCTGCGG-3') and Its4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990) and the PCR Kit (Illustra™ PuReTaq Ready-to-Go™ PCR beads, GE Healthcare) with a final volume of 25 µL [19 µL of H_2O milli-Q, 2 µL of DNA and 2 µL of each primer (10 pmol/µL) in a thermocycler (SuperCycler®, Kyratec)]. An initial cycle of 94 °C for 4 minutes and 35 cycles of 94 °C for 2 minutes (denaturation); 55 °C for 2 minutes (annealing) and 72 °C for 2 minutes (amplification), which was maintained at 4 °C for an undetermined period at the end of amplification (Souza, 2006). The PCR product was verified using electrophoresis in 1% agarose gel stained with GelRed™ (Invitrogen® plus) using the Plus DNA Ladder marker (Invitrogen®) of 1 Kb.

2.2.2 Bioinformatics sequencing and analysis

The PCR products were purified with the enzyme ExoSAP-IT (GE Healthcare) and adjusted to the concentration of 10 ng/µL. The BigDye Terminator kit (Applied Biosystems) was used for sequencing. Taxonomic identification was performed using the online tool BLASTn, considering the result of the highest score, against the rRNA/ITS (ITS from fungi type and reference material) database, which is available at the National Center for Biotechnology Information (NCBI). For the phylogenetic analysis, the MEGA-X v10.2.5 program was used, and the sequences were aligned using ClustalW, while the phylogenetic tree was constructed using the maximum parsimony (MP) method, with a heuristic search using the TBR ("tree-bisection-reconnection") algorithm, which considered the most efficient MP method and with a bootstrap of 1000 repetitions.

2.3 Submerged cultivation and obtaining of enzyme extracts

An enzyme production curve was performed in triplicate using 5% caesarweed (previously dried at 40 °C and ground) in 250 mL Erlenmeyer flasks containing 50 mL of Manachini solution (Manachini et al., 1987), pH 4.5 and solution GLBN 40, pH 5 autoclaved for 20 minutes at 121 °C.

Five fragments of approximately 1 cm^2 of strain 1291 (grown for 8 days) and 25 µl of the conidia suspension of strain 1290 were used as the inoculum. The assay was subjected to shaking in a shaker incubator at a temperature of 28 °C for 10 days. Filtration was performed daily in a vacuum filtration system with a 0.22 µ membrane (Millipore) and the enzymatic extracts were stored in 50 mL Falcon® tubes at a temperature of -20 °C.

2.4 Determination of enzymatic activities

2.4.1 Determination of total cellulase activity (FPase)

The activity of total cellulases (FPase) covers exo- and endoglucanases, and is determined from the degradation of the paper filter. This dosage was based on the methodology of Ghose (1987) and Miller (1959), with modifications. An aliquot of 20 µL of enzyme extract, a 0.6 cm diameter circle of Whatman No. 1 filter paper and 40 µL of sodium citrate buffer (50 mM, pH 4.8) was added to a PCR plate, and left for 60 minutes at 50 ºC. This was performed in triplicate.

To control the enzyme, the paper circle was removed, while the other reagents remained. To control the substrate, 60 µL of sodium citrate buffer and a circle of filter paper were added, and for the blank, only 60 µL of sodium citrate buffer were added. After the reaction time, 120 µL of 3,5-dinitrosalicylic acid (DNS) were added to the samples that were subjected to boiling bath for 15 minutes. Then, the samples were cooled and 20 µL of this reaction mixture was transferred to a 96-well plate (ELISA) containing 180 µL of ultrapure water. The samples were read using a UV spectrophotometer at 540 nm. A standard curve was constructed with a 1 mg/ml glucose solution. One U equals the quantity necessary to release 1 µmol of glucose/min.

2.4.2 Determination of β-glycosidase (BGL) activity

The quantification of β-glycosidase activity was carried out using the methodology of Silva (2013) with some modifications. In a PCR plate, 25 µL of 1% cellobiose substrate was homogenized in sodium citrate buffer (50 mM, pH 5) and 25 µL of enzyme extract heated for 30 minutes at 50 ºC. An aliquot of 10 µL was transferred to microtubes containing 1,000 µL of the enzyme reagent (RGT) of the Glucose Liquicolor kit (In Vitro Diagnóstica) and left to reacting for 5 minutes at 37 ºC.

For the control of the substrate, the enzyme extract was replaced, and for the control of the enzyme, the substrate was replaced, both with ultrapure water. For the blank, 10 µL of ultrapure water was added to 1,000 µL of the RGT and, for the standard, 10 µL of the glucose standard (STD) contained in the kit was added to 1,000 µL of the RGT. The samples were read using a UV spectrophotometer at 500 nm. One U equals the quantity necessary to release 1 µmol of glucose/min.

2.4.3 Determination of the enzymatic activity of xylanase and CMCase

The quantification of xylanase/CMCase was carried out using the methodology of Ghose (1987) and Miller (1959), with modifications. A total of 180 µL of the substrate (birchwood xylan/carboxymethylcellulose) was added to 1% in sodium acetate buffer (50 mM, pH 5.0) and 20 µL of enzyme extract in a PCR plate, which was heated for 10 minutes at 50 °C. An aliquot of 200 µL of DNS was added and the samples were subjected to a boiling bath for 5 minutes and then cooled in an ice bath. Then, 40 µL of the reaction mixture was transferred to an ELISA plate and 160 µL of ultrapure water was added.

For the control, first, the DNS was added and, last, the enzymatic extract and, for the blank, 200 µL of distilled water and 200 µL of DNS were added. The samples were read using a UV spectrophotometer (540 nm). A standard curve was constructed to correlate the absorbance values with the amount of product formed, using a solution of D-xylose 1 mg/ml. One U equals the quantity necessary to release 1 µmol of D-xylose/min.

2.4.4 Determination of pectinase activity

The quantification of pectinase was carried out using the methodology of Stock (2013) with some modifications. In a PCR plate, 25 µL of the substrate citrus pectin (D-galacturonic acid) to 1% sodium acetate buffer (50 mM, pH 5) and 25 µl of the enzyme extract was added and then left for 10 minutes, at 40 °C. An aliquot of 50 µL of DNS was added to the samples, which were then subjected to a boiling bath with boiling water for 5 min. The plate was cooled in an ice bath and 40 µL of the solution was transferred to an ELISA plate containing 160 µL of distilled water.

For the control, the enzyme extract was added after the DNS. Only 50 µL of distilled water and 50 µL of DNS were added to the blank. The samples were read using a UV spectrophotometer (540 nm), and a standard curve was constructed correlating the absorbance values with the amount of product formed, using a solution of D-galacturonic acid 1 mg/ml. One U equals the quantity necessary to release 1 µmol/mL of D-galacturonic acid/min.

The calculation of the enzymatic activity of the hydrolases was carried out using the equation of Inforsato and Porto (2016) shown below:

$$U = \frac{DxC\ (\mu mol.\ mL^{-1}) x\ Vt\ (mL)}{T\ (min) x\ Ve\ (mL)}$$

Where:

D - Dilution (performed when it was necessary to dilute the enzyme solution)

C - Concentration determined from DNS method

Vt - Total reaction Volume

T - Reaction time

Ve - Volume of enzyme solution

U = Enzyme Unit (µmol/min)

2.4.5 Determination of enzymatic activity of laccase

The quantification of the laccase activity was carried out using the methodology of Bourbonnais et al. (1998) with some modifications. In a PCR plate, 60 µl of citrate-phosphate buffer (50 mM, pH 5), 20 µL of ultrapure water, 20 µL of ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] (1 mM) and 100 µL of the enzyme extract were added and the plate was left for 10 minutes, at 30 °C.

For the control (Abs_i), the extract, which had been boiled for 10 minutes in order to degrade the enzymes, was used and, for the blank, the enzyme extract was replaced with ultrapure water. The sample readings were performed in UV spectrophotometer at 420 nm after 10 minutes of reaction (Abs_f). One U equals the quantity necessary to oxidize 1 µmol ABTS/min.

2.4.6 Determination of manganese peroxidase (MnP) enzymatic activity

The quantification of MnP was carried out based on the methodology of Khindaria, Grover and Aust (1994) with some modifications. To a PCR plate, we added 60 µL of phosphate-citrate buffer (50 mM, pH 4.5), 60 µL of sodium lactate (50 mM), 20 µL of $MnSO_4$ (1.0 mM), 20 µL of phenol red (0.1%), 10 µL of bovine albumin (1.8%), 10 µL of H_2O_2 (2.0 mM) and 100 µL of enzyme extract, which was left for 10 minutes at 35 °C. After the reaction time, 13 µl of NaOH (6.5 m) were added.

For the control (Abs_i), the degraded enzyme extract, which had been boiled for 10 minutes, was used. For the blank, the sample was replaced with ultrapure water. The readings were performed in a UV spectrophotometer at 610 nm after the interruption of the reaction (Abs_f). One U of enzyme equals the quantity necessary to form 1 µmol of oxidized phenol red/min.

2.4.7 Determination of lignin peroxidase (LiP) enzyme activity

The quantification of LiP activity was carried out based on the methodology of Kirk and Farrell (1986) with some modifications. 80 µL of sodium tartrate buffer (100 mM, pH 3), 10 µL of veratrylic alcohol (10 mM), 10 µL of freshly prepared H_2O_2 solution (0.1 µmol) and 100 µL of the enzyme extract were added to the PCR plate, which was left for 10 minutes in a water bath at 40 °C.

For the control (Abs_i), the degraded enzyme extract, which had been boiled for 10 minutes, and ultrapure water was added to replace the sample. The readings were performed in a UV spectrophotometer at 310 nm after 10 minutes of reaction (Abs_f). One U equals the quantity necessary to form 1 µmol of veratraldehyde/min.

The calculation of lignolytic activity was carried out according to Aguiar Filho (2008) and Fernandes (2013), as described below:

$$U/L = \left(\frac{\Delta Abs}{\varepsilon x V_a x t_r}\right) x\ Fd\ x\ 10^6$$

Where:

Δ Abs = Abs_f - Abs_i

Ɛ = Molar extinction coefficient of the formed product ($M^{-1}cm^{-1}$)

V_a = Sample volume in L

t_r = Reaction time in minutes

Fd = Sample dilution factor

10^6 = Conversion factor from M to µmol

U = Enzyme Unit (µmol/min)

Molar extinction coefficient:

Laccase (420 nm) = 36,000 $M^{-1}cm^{-1}$

LiP (310 nm) = 9,300 $M^{-1}cm^{-1}$

MnP (610 nm) = 22,000 $M^{-1}cm^{-1}$

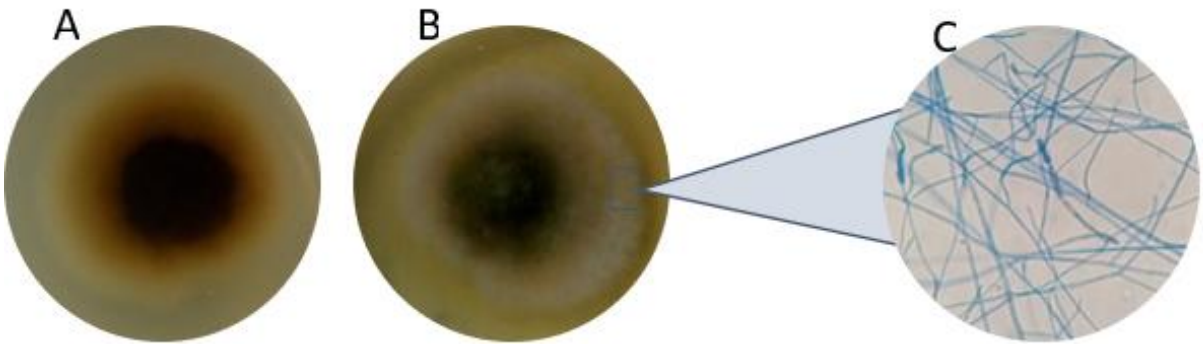
3. Results and discussion

3.1 Molecular identification

After the assembly of the contigs, the strains were identified through the *ITS* regions of the *rDNA* on the NCBI platform. Fungal strain 1291 was classified as *Corynespora torulosa* (NR 145181.1), order Pleosporales, family Corynesporascaceae with 96.76% similarity, which is slightly below our cut-off line, and which means that in future studies more sequenced genes or even revealing of the genome will be necessary. This species. formerly known as *Deightoniella torulosa* is reported in the literature as a phytopathogen that attacks the leaves and causes diseases in more than 70 species of plants, among these cotton plants (Shirsath and Patil, 2018) and banana trees (Almenares and Pérez-Vicente, 2019). It is also known as black spot and Deightoniella's mark. In the northern region of Brazil, it also causes diseases in tomato, cucumber, eggplant and papaya crops (Bentes et al., 2018).

In Figure 1, the front (B) shows the culture with a gradual coloring of gray, beige and white, from the center to the edges and, on the back (A), the culture presented a gradual coloring of dark brown, orange and white. Its conidia are long, straight to slightly curved, cylindrical, tapering at the tips, corroborating the description made by Shirsath and Patil (2018).

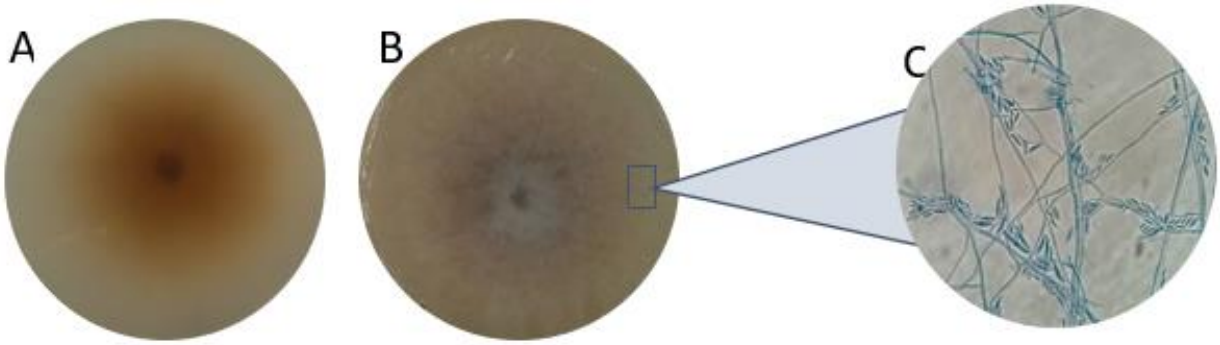
Figure 1. Strain 1291, *Corynespora torulosa* - A: back, B: front, C: Microculture observed under 40x magnification.



Source: Authors.

Strain 1290 was classified as *Fusarium pseudocircinatum* with 99.58% similarity (NR_163683.1) Hypocreales order, Nectriaceae family and belonging to the *F. fujikuroi* species complex (FFSC), which has approximately 50 species (Figure 2).

Figure 2. Strain 1290, *Fusarium pseudocircinatum* - A: back, B: front, C: microculture observed under 40x magnification.



Source: Authors.

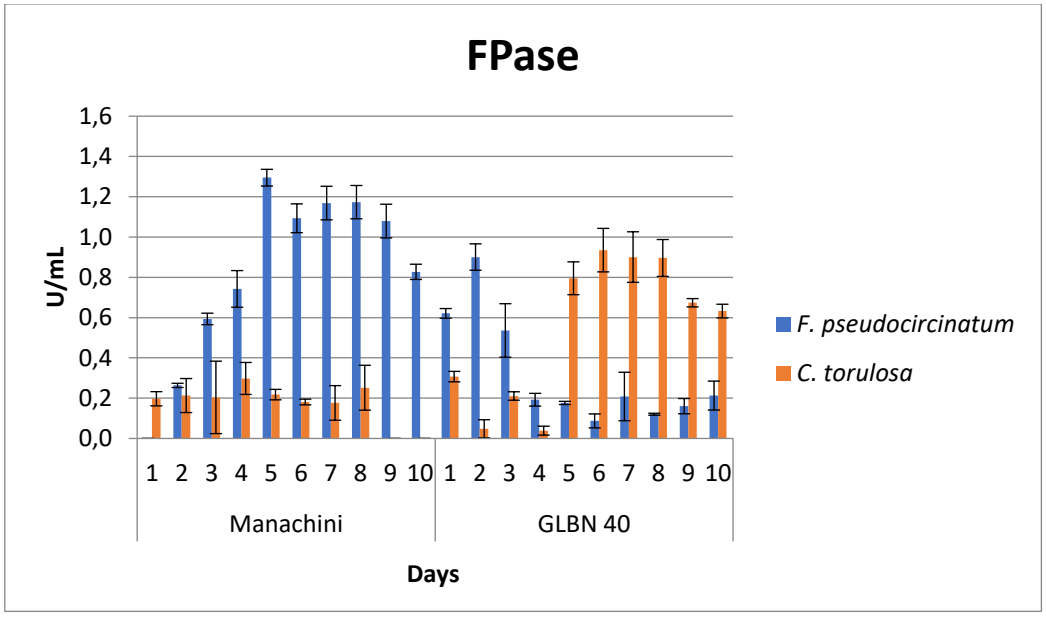
Fungi of the *Fusarium* genus are cosmopolitan and can have a systemic distribution in plants. These species produce mycotoxins, such as fumonisin, fusaric acid, moniliformin, among others, that contaminate foods such as rice and corn, cause chronic and acute toxicity to humans and livestock, and thus generating a risk to food security (Leslie et al., 2005; O'Donnell et al., 2013, Nicollia, 2020, Qiu et al., 2020), in addition to being phytopathogens of *Sansevieria trifasciata*, which is an ornamental plant known as mother-in-law's tongue, and causing leaf scorch (Kee et al., 2020). They are also phytopathogens of the mango (*Mangifera indica*) and have been reported to cause malformation in the fruits and dwarf leaves in Mexico (Freeman et al., 2014). It was confirmed that the production of extracellular enzymes, such as catalase, laccase, cellulase, caseinase, amylase, protease, lipase and pectinases, play an important role in the invasive potential of pathogenic species, as observed by Mezzomo et al. (2019) in yerba mate seedlings contaminated with *F. solani* and *F. oxysporum*, resulting in infection and death of the seedlings. On the front, the colony has an orange coloration in the center and on the back, a pink coloration with hyaline macroconidia, which are straight or slightly sickle-shaped, tapering at the ends, thus corroborating with the description by Farias (2020).

3.2 Determination of enzymatic activity

3.2.1 Determination of activity of total cellulases (FPase)

FPase activity was detected from the second day of cultivation for *F. pseudocircinatum* in Manachini solution, with its peak on the fifth day (1.294 U/mL), though there was a slight drop in production on the sixth day, with no sudden changes until the ninth day of cultivation. The fungus *C. torulosa* had its peak production on the sixth day (0.934 U/mL); however, from the fifth to the eighth day, there were no sudden changes in production in the GLBN 40 solution (Figure 3).

Figure 3. FPase production during 10 days in the different solutions for two endophytic fungi isolated from caesarweed leaves (*Urena lobata*) in the State of Amazonas – Brazil.



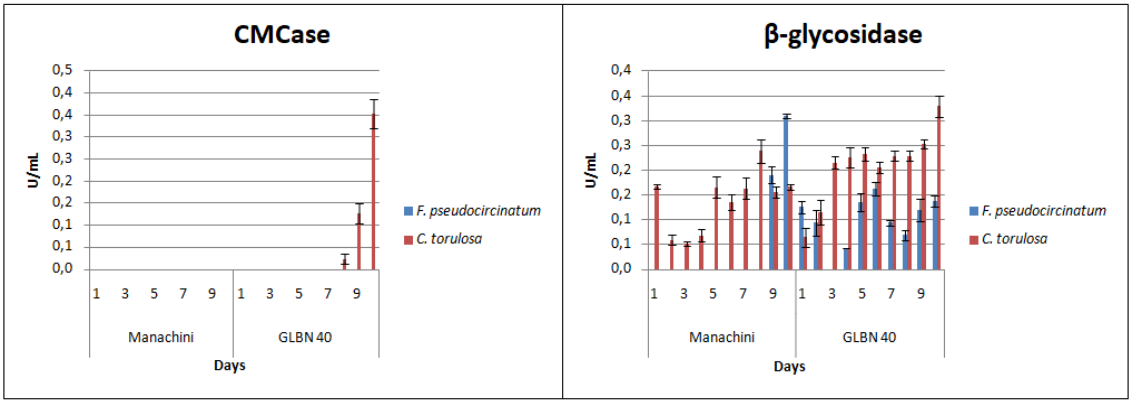
Source: Authors.

While studying FPase produced by *Fusarium* sp., Faheina Junior (2012) obtained a maximum production of 0.006 U/mL in a mineral medium supplemented with microcrystalline cellulose, which occurred over 72 h. Nascimento (2015) evaluated the production of FPase by *Fusarium* sp. in submerged culture, using a cake of macaw palm (*Acrocomia aculeata*) pulp as the substrate and, on the fourth day of cultivation, production was approximately 0.012 U/mL. The current study presented higher values than those previously mentioned. The production of cellulases is influenced by the concentration of cellulose in the composition of the substrate used (Vilela, 2013) and caesarweed has 76% cellulose in its fiber, according to Agopyan (2005).

3.2.2 Determination of the enzymatic activity of CMCase and β-glycosidase

Only *C. torulosa* produced CMCase from the eighth day of cultivation and, on the tenth day, its peak production was witnessed (0.351 U/mL). No CMCase production was noted for *F. pseudocircinatum* in either of the solutions. There was production of β-glycosidase in the two solutions for *C. torulosa*. which presented a peak on the tenth day (0.328 U/mL) in the GLBN 40 solution; however, for *F. pseudocircinatum*, the production occurred during the 10 days, except on the third day, in the GLBN 40 solution, while, in the Manachini solution, the production occurred only on the ninth and tenth day of cultivation when the peak production was obtained (0.309 U/mL) (Figure 4).

Figure 4. CMCase and β-glycosidase production during 10 days in the different solutions for two endophytic fungi isolated from caesarweed leaves (*Urena lobata*) in the State of Amazonas – Brazil.



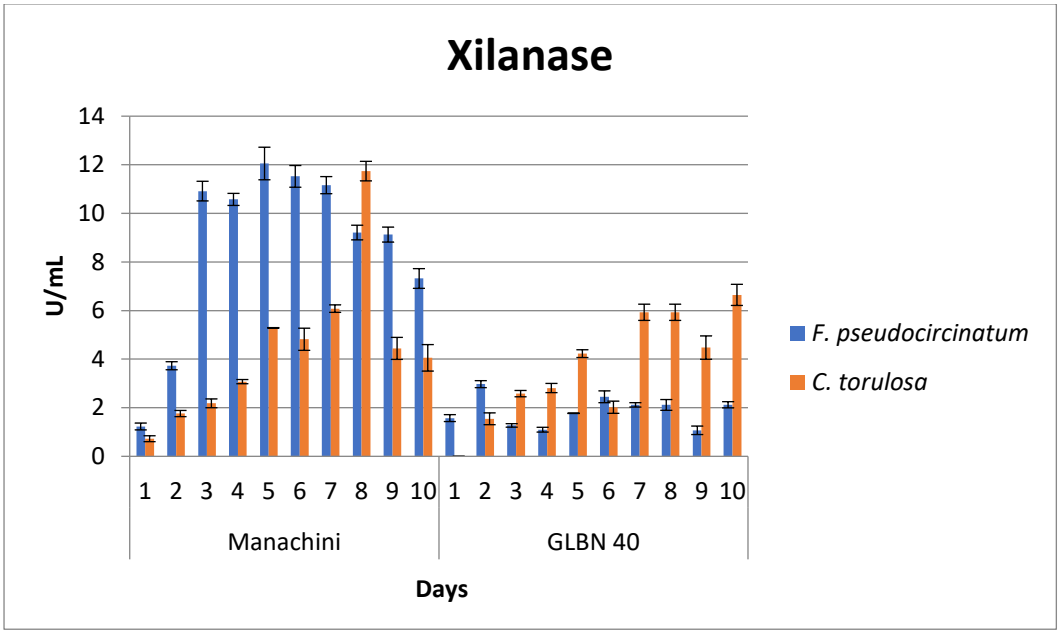
Source: Authors.

Although CMCase and β-glycosidase work in synergy, CMCase is an endoglucanase and acts by randomly hydrolysing the internal β-(1,4) glycosidic bonds of amorphous cellulose, while exoglucanases act at the terminals of crystalline cellulose releasing cellobiose, which in turn is the substrate used by β-glycosidase (Farinas, 2011). Based on the obstacle model (Eriksson, Karlsson and Tjerneld, 2002), the hydrolysis provided by exoglucanase is blocked due to an obstacle, which may be an isolated cellulose chain, masking the chain to be degraded and preventing the action of the exoglucanase. Given this necessity, the production of endoglucanase occurs to remove this obstacle, degrading it and partially reducing its fiber, thus enabling the resumption of exoglucanase action, and reducing the likelihood of it being trapped again by the same obstacle and releasing cellobiose molecules to be degraded by β-glycosidase (Ogeta and Petri, 2010).

3.2.3 Determination of xylanase enzyme activity

Enzymatic production occurred in the two saline solutions; however, xylanase production was superior in the Manachini solution, in which the strains had similar production peaks. The fungus *F. pseudocircinatum* maintained enzymatic production without major changes between the third and seventh day; however, the peak production was on the fifth day (12.052 U/mL). The peak production of *C. torulosa* was on the eighth day (11.738 U/mL) (Figure 5).

Figure 5. Xylanase production during 10 days in the different solutions for two endophytic fungi isolated from caesarweed leaves (*Urena lobata*) in the State of Amazonas – Brazil.



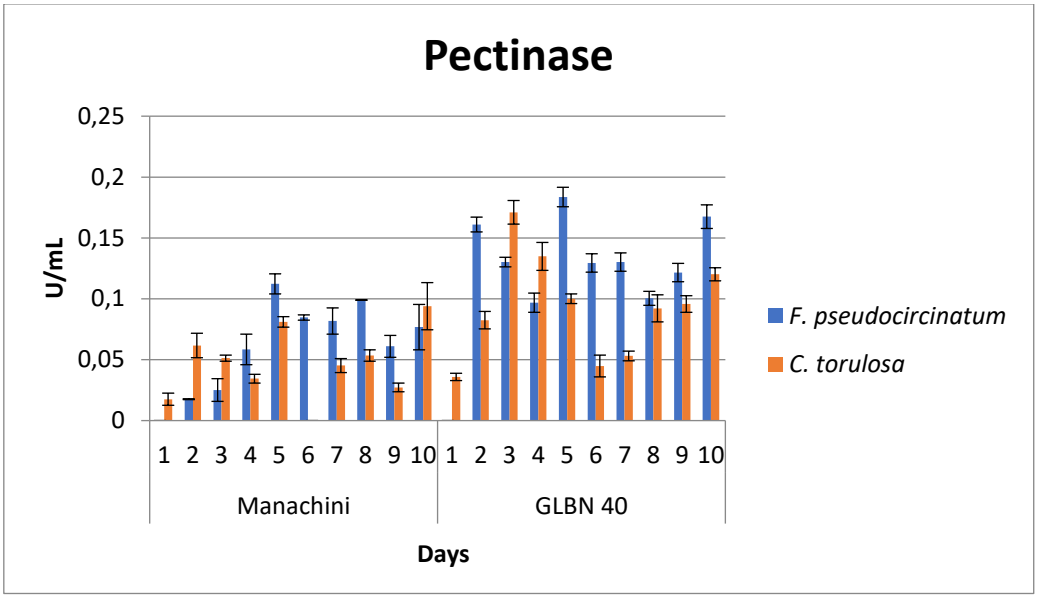
Source: Authors.

Nogueira (2021) used a *Fusarium* sp. to produce xylanase via submerged fermentation with wheat bran, orange peel, cassava peel, pumpkin peel and potato peel, and obtained approximate values of 0.8 U/mL, 0.17 U/mL, 0.08 U/mL, 0.15 U/mL and 0.3 U/mL, respectively, which are higher than the values obtained in this work. Xylanase production is most often induced by the presence of xylan (Fernández-Espinar et al., 1994), which is one of the main constituents of hemicellulose. A study by Bueno and Brienzo (2020) found that, as an inducer of xylanase production, wheat bran has the same efficiency when compared to commercial xylan, due to the concentration of xylan in its composition.

3.2.4 Determination of pectinase activity

Enzymatic production occurred in both saline solutions; however, pectinase production was greater in GLBN 40 solution for both fungi. The fungus *F. pseudocircinatum* had its peak production on the fifth day (0.183 U/mL). For *C. torulosa*, the peak production was on the third day (0.171 U/mL) (Figure 6).

Figure 6. Pectinase production during 10 days in the different solutions for two endophytic fungi isolated from caesarweed leaves (*Urena lobata*) in the State of Amazonas – Brazil.



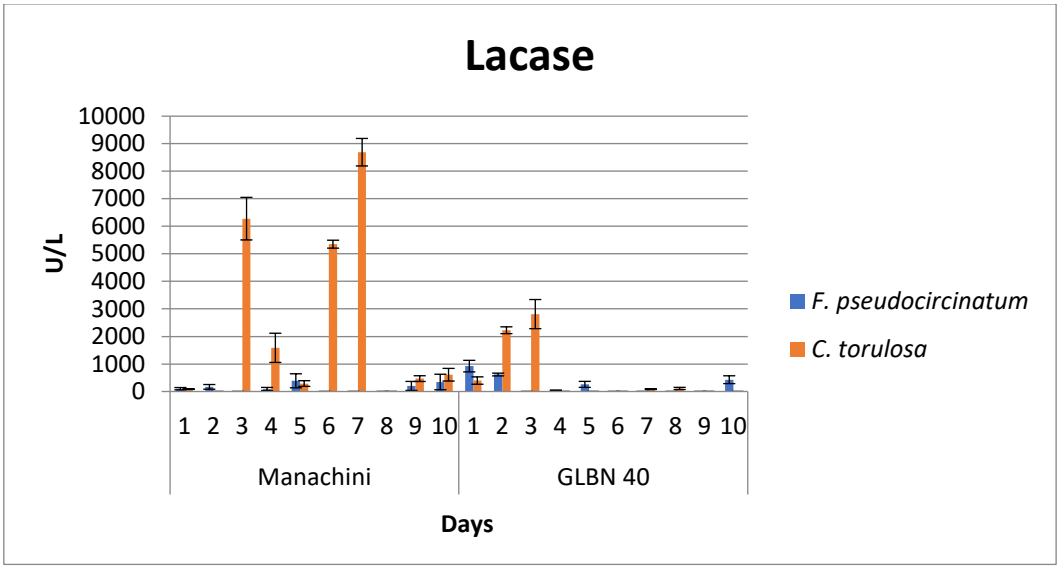
Source: Authors.

According to Ahmed et al. (2016), various fungal species, such as *Aspergillus*, *Rhizopus*, *Trichoderma*, *Penicillium* and *Fusarium*, are used in the production of pectinase. The low production of pectinase by *F. pseudocircinatum* can be explained by the substrate used in the submerged culture. Caesarweed is a shrub plant that has amounts of pectin that are close to 0% (Oliveira and Agopyan, 1993) and, according to Sandhya and Kurup (2013), low fructose indices affect the production of pectinase. Fructose is found in large quantities in fruits and honey; it is a simple sugar and the pectinase-producing microorganism uses simple sugars more efficiently when compared to more complex sugars such as starch and galacturonic acid (Abdullah et al., 2018).

3.2.5 Determination of enzymatic activity of laccase

In the production of laccase, the fungus *C. torulosa* was the best producer on the seventh day of cultivation in the Manachini solution (8,691 U/L). The fungus *F. pseudocircinatum* did not show good production of laccase and production remained low or non-existent in a few days in both of the two solutions (Figure 7).

Figure 7. Laccase production during 10 days in the different solutions for two endophytic fungi isolated from caesarweed leaves (*Urena lobata*) in the State of Amazonas – Brazil.



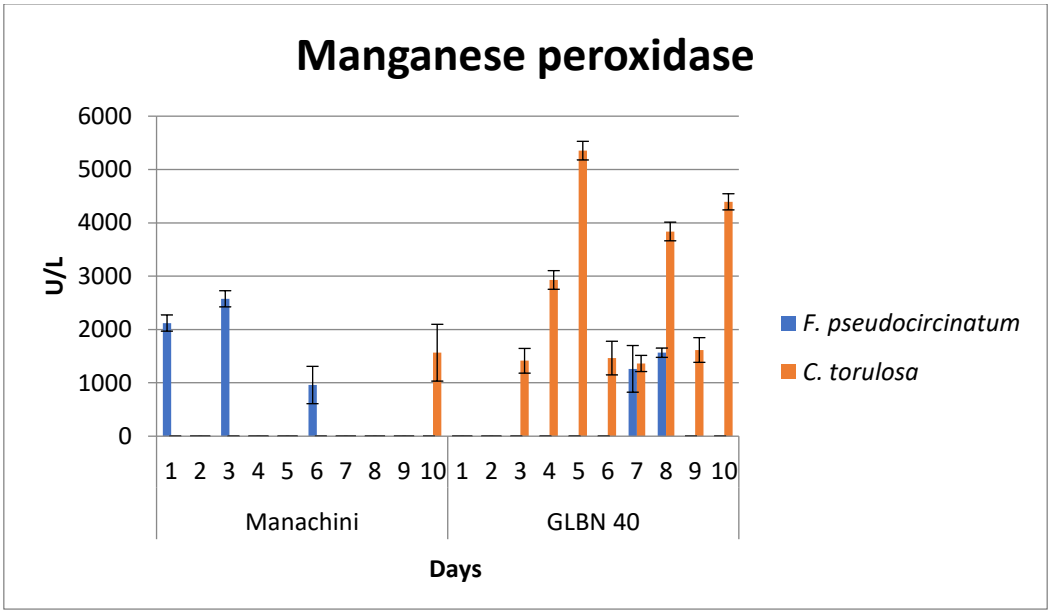
Source: Authors.

Carvalho, Fonseca and Silva (2017) obtained a peak production of laccase of 38 U/L on the ninth day of cultivation using Brazil nut shells as the substrate and *Trametes elegans* as the producer, and the results obtained in this work were more than 228 times higher than theirs. Souza (2018) used tucumã (*Astrocaryum aculeatum*) peels to produce laccase by *Pleurotus ostreatus* and, under optimized conditions, the author obtained a peak of 4,000 U/L on the tenth day, which is lower than that found in our study. These values can be improved, since simpler carbon sources, such as glucose, mannitol and cellobiose, can favor a greater production of laccase when compared to more complex sources such as cellulose and lignocellulosic material (Majeau et al., 2010).

3.2.6 Determination of enzymatic activity of MnP

In the production of MnP, the fungus C. torulosa was the best producer (5,353 U/L) on the fifth day of cultivation in the solution GLBN 40, and maintained its production between the third and tenth day of cultivation. The fungus *F. pseudocircinatum* did not present a constant production in either solution; however, the peak in Manachini solution was obtained on the third day (2,575 U/L) (Figure 8).

Figure 8. Manganese peroxidase production during 10 days in the different solutions for two endophytic fungi isolated from caesarweed leaves (*Urena lobata*) in the State of Amazonas – Brazil.



Source: Authors.

Carvalho et al. (2016) evaluated the MnP activity produced by *P. sajor-caju* with stillage (58.295 U/L). Santos and Kamida (2017) obtained 151.12 U/L on the twenty-first day of cultivation using as a substrate the residue of bracts of *Syagrus coronata* and *P. sajor-caju* as the producer. Both values were much lower than those found in our work. On the tenth day, Silva (2014) obtained 2,417 U/L MnP by *P. ostreatus* using empty palm clusters as substrate, approximately half the value found in our work. Studies indicate that the production of MnP is controlled by the concentration of Mn^{2+} and its higher concentration favors the production of MnP, and lower concentrations favor the production of laccase (Perez and Jeffries, 1992; Swamy and Ramsay, 1999), similar to the results obtained in our work.

3.2.7 Determination of LiP enzymatic activity

LiP activity was not detected in the two strains tested. For the production of this enzyme, the conditions vary according to the species of fungi, in addition to the carbon source and nitrogen availability. Studies indicate that some fungi, such as *Ceriporiopsis subvermispora* and *P. ostreatus*, operate producing MnP and laccase without expressing LiP activity (Rajakumar et al., 1996; Santoyo et al., 2008), thus indicating synergism between such enzymes (Silva, 2014). Another possibility is that LiP can be produced, though detection is hindered either by being in very low concentrations or by substances that interfere with detection, particularly those in woody substrates (Rajakumar et al., 1996).

4. Conclusion

The enzymatic production by *C. torulosa* and *F. pseudocircinatum* were different in the two solutions of mineral salts and between themselves. The strain *C. torulosa* was better at producing CMCase, β-glycosidase, laccase, and MnP when using caesarweed as substrate when compared to *F. pseudocircinatum*, which was a better producer of FPase, xylanase, and pectinase. Both strains had no detected LiP activity. The results presented demonstrate that the strains used in this study have potential for enzymatic production. As a result, enzymes with the ability to depolymerize the components of the cell wall of plants are of great interest to the biotechnology sector, since their performance and their products can favor several industrial sectors such as those of biofuels, food, and paper, among others. Further studies should be conducted to evaluate other conditions and substrates.

Acknowledgments

We would like to thank the Graduate Program in Biodiversity and Biotechnology – PPG-BIONORTE, for Sarah R. S. S. Santiago's thesis opportunity, as also the Embrapa Western Amazon/EMBRAPA, Master's Degree in Biotechnology and Amazon Natural Resources - MBT/UEA and LabMicrA laboratory of the Central Analítica – CAM/ UFAM, for the necessary spaces and equipment to the development of the work.

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brazil (CAPES) – Finance Code 001, by the project Pró-Amazônia: Biodiversidade e Sustentabilidade (process number 23038.009442/2013-12) and FAPEAM. This study is part of the doctoral thesis of Sarah R. S. S. Santiago (Universidade Federal do Amazonas).

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