Endophytic fungi of *Brunfelsia uniflora*: Isolation, cryopreservation, and determination of enzymatic and antioxidant activity

Sara Jane Marsola¹ Lais Freitas Jorge¹·Adriano Borges Meniqueti¹ Miria Benetati Delgado Bertéli¹
Thaís Emanuelle Feijó de Lima² José Luiz Bezerra² Ana Daniela Lopes¹ Zilda Cristiani Gazim¹ Juliana
Silveira do Valle¹ Nelson Barros Colauto³ Giani Andrea Linde⁴

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Ana Daniela Lopes

anadanielalopes@prof.unipar.br

ORCID: <https://orcid.org/0000-0003-2027-5741>

¹Universidade Paranaense, Graduate Program of Biotechnology Applied to Agriculture, Umuarama 87502-210, Brazil

²Universidade Federal do Recôncavo da Bahia, Cruz das Almas 44380-00, Brazil

³Federal University of Bahia, Ondina Campus, Graduate Program in Food Science - Faculty of Pharmacy, Salvador 40110-115, Brazil

⁴Federal University of Bahia, Canela Campus, Graduate Program in Food, Nutrition and Health - School of Nutrition, Salvador 40110-907, Brazil

Abstract

Brunfelsia uniflora (Pohl.) D. Don (Solanaceae), commonly known as *manacá-de-cheiro*, is widely distributed in Brazil and used by local indigenous peoples as an antirheumatic, antisyphilitic, depurative, emetic, vermifuge, and purgative agent. Several studies have examined the biological activities and phytochemical profile of *Brunfelsia*; however, few have focused on the diversity of endophytic microorganisms that colonize members of the genus. This study aimed to isolate and cryopreserve endophytic fungi from *B. uniflora* and determine their cellulase, laccase, and antioxidant activities. Endophytic fungi were isolated from *B. uniflora* stems, cultured on wheat grains, immersed in a 150 g·L⁻¹ aqueous sucrose solution, and cryopreserved at -80°C for 1 and 6 months. Cellulase activity was determined by a qualitative test using carboxymethylcellulose medium and laccase activity by a quantitative test based on the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate). Prior to antioxidant activity assays, fungi were grown in malt extract broth for production of mycelial biomass. A methanolic extract was prepared for evaluation of *DPPH*• scavenging activity, FRAP activity, and total phenolic content. A total of 46 endophytic fungal isolates were obtained from *B. uniflora* stems and classified into 24 groups according to morphological similarities. *B. uniflora* was shown to harbor a great diversity of endophytic fungi. Mycelial viability was observed after 1 and 6 months of cryopreservation at -80 °C. Fungi exhibited cellulase and laccase activities. Isolate CE23 had the highest laccase activity after 7 days of cultivation. Twelve isolates were found to have low total phenolic contents and *DPPH*• and FRAP activities.

Keywords: Preservation of microorganisms; medicinal plant; enzyme; diversity; symbiosis.

Introduction

Endophytic fungi are a diverse group of microorganisms that inhabit plant tissues during part of their life cycle (Guo et al., 2008; Hardoim et al., 2008; Saikkonen, 2007; Sun et al., 2008; Xia et al., 2011). Depending on environmental conditions and plant physiological state, these fungi may become pathogenic, although infections are usually asymptomatic. Endophytic fungi may also interact mutualistically with plants (Compant et al., 2010; Davitt et al., 2011; Gundel et al., 2011; Hardoim et al., 2008). In some cases, nematodes can be controlled by endophytes, such as *Fusarium oxysporum*, which produces metabolites that are antagonistic to the nematode *Meloidogyne incognita*. Endophytic fungi can produce compounds with insecticidal and nematicidal activities, protecting host plants from pests and pathogens (Mejía et al., 2008). Furthermore, endophytic fungal metabolites may have antimicrobial, cytotoxic, and anticancer activities (Zhao, 2010). Some endophytic fungi seem to have developed the ability to produce the same or similar compounds as those produced by host plants (Kour et al., 2008). This finding has stimulated research on the relationship between endophytes and their hosts, enabling the development of alternative processes for obtaining bioactive substances (Gunatilaka, 2006; Zhao, 2010).

For instance, paclitaxel, an important anticancer drug isolated from the plant *Taxus brevifolia*, is also produced by the endophytic fungus *Taxomyces andreanae*. Vincristine, another important anticancer agent, is produced by the plant *Catharanthus roseus* and was recently isolated from *Fusarium oxysporum*, an endophyte of *C. roseus* (Jalgaonwala et al., 2011). The pharmacological action of plants seems to be intimately related to plant–endophytic interactions. Therefore, evaluation of the endophytic microbiota of medicinal plants is fundamental for the advancement of herbal medicine use in animals, especially humans, as well as for the production of food and cosmetics.

Brunfelsia uniflora (Pohl.) D. Don (Solanaceae), commonly known as *manacá-de-cheiro*, occurs in several regions of Brazil, Bolivia, Peru, Ecuador, Colombia, and Venezuela. This shrubby plant has simple leaves and purple or white inflorescences or single flowers. Different species of *Brunfelsia* are used by indigenous peoples as, antirheumatic, antisyphilitic, depurative, vermifuge, and purgative agents (Schultes, 2018). Phytochemical studies of the aerial parts of *Brunfelsia* species revealed the presence of

active compounds, such as benzenoids, terpenes, lactones, alkaloids, and lipids (Brunner et al., 2000; Castioni & Kapetanidis, 1996; Mors & Ribeiro, 1957; Ruppelt et al., 1991). Although numerous studies have been carried out on *Brunfelsia* phytochemical compounds, none have investigated the diversity of endophytic microorganisms in these plants.

An important factor for the study of endophytic fungi, their diversity and quantity, and their ability to produce bioactive molecules is their preservation. There are numerous techniques for preserving fungi. The most common is the periodic subculturing of fungal fragments on growth media. However, this method is laborious, occupies space in culture collections, and is subject to accidental contamination by other microorganisms and loss of biological, genetic, or physiological characteristics. Other short-term preservation methods involve the use of mineral oil and distilled water, but variations in morphology, physiology, and mycelial viability might occur proportionally to the duration of preservation (Humber, 1997). Fungi can also be preserved in the form of spores by freeze-drying; however, this technique has not proven suitable for maintaining mycelial integrity (Homolka et al., 2003). Studies conducted by our research group have shown that freeze-drying of mycelia does not result in efficient preservation of basidiomycetes. Cryopreservation is still considered the safest, most reliable and effective method of mycelium preservation, as it inactivates fungal metabolism and has a low risk of contamination and genetic degeneration (Ito & Nakagiri, 1996).

Endophytic microorganisms are possibly an unexplored source of enzymes such as cellulases and laccases. These enzymes are used in the textile, food (A. Singh et al., 2021), cosmetics (Shin et al., 2019), paper, and pulp industries (G. Singh & Arya, 2019; Yang et al., 2019) as well as in bioremediation (Sharma et al., 2018). The use of enzymes in biotechnological processes has been constantly growing. According to BCC Research, the global demand for enzymes will grow 5% per year, reaching US\$7 billion by 2023.

In addition to producing enzymes, endophytic fungi synthesize antioxidant compounds that protect against free radicals, contributing to disease prevention. There are few reports on the topic (Strobel & Daisy, 2003). Of note, pestacin and isopestacin were obtained from the fungus *Pestalotiopsis microspora*, an endophyte of *Terminalia morobensis* (Harper et al., 2003; Strobel et al., 2002). Both pestacin (Harper et al., 2003) and isopestacin (Strobel & Daisy, 2003) exhibit antioxidant (IC₅₀ of 1.7 mM and 0.185 mM,

respectively) and antibacterial activities (Strobel & Daisy, 2003). In view of the scarcity of studies aimed at the diversity, preservation, and enzymatic and antioxidant activities of endophytic fungi from medicinal plants, this study aimed to isolate, cryopreserve, and determine the biological activity of endophytic fungi isolated from stems of *B. uniflora*.

Materials and Methods

Isolation of endophytic fungi

B. uniflora was cultivated in the medical garden of the Paranaense University, Brazil, and specimens were deposited in the educational herbarium of the Paranaense University (HEUP) under accession no. 2855. Branches were collected and washed twice under running water and distilled water. Endophytic fungi were isolated according to the methods of Pereira et al. (1993) and Petrini (1991), with modifications. Under a laminar flow hood, leaves were removed, and stems were immersed in 1% (v/v) sodium hypochlorite solution for 3 min, washed in distilled water, immersed in 70% (v/v) ethanol for 1 min, immersed in 90% (v/v) ethanol for 30 s, and washed twice in autoclaved ultrapure water.

To assess the effectiveness of sample disinfection, we inoculated 100 µL of ultrapure water from the last washing step on potato dextrose agar (PDA) (39.0 g·L⁻¹, HiMedia) previously autoclaved at 121 °C for 20 min. Water was spread on PDA using a Drigalski spatula, and plates were kept in the dark at 25 °C for 2 days. Lack of microbial growth indicated that the disinfection procedure was effective.

After disinfection, stems were cut into 3–5 mm fragments. Five stem fragments were placed in Petri dishes containing PDA medium (10 replicates) to investigate the effectiveness of stem surface disinfection and five on PDA medium supplemented with ampicillin and streptomycin (20 replicates) to prevent bacterial growth and promote fungal growth. For this, culture medium was autoclaved, cooled at 50 °C, and mixed with 0.2 g·L⁻¹ ampicillin sodium salt and 0.2 g·L⁻¹ streptomycin sulfate. Plates were incubated in the dark at 25 °C, and emerging fungi were plated on PDA containing antibiotics (PDA+) until a pure culture was obtained. After obtaining pure cultures, we maintained fungi by periodic subculturing on PDA medium at 25 °C in the dark. Fungi were examined using a stereoscopic microscope (Motic, SMZ168 series) and grouped according to morphological characteristics, such as mycelial color, branching, and

edge, culture medium color, and spore color. Spores and hyphae were examined by taking photomicrographs of isolates. Samples were obtained from culture media using transparent adhesive tape, transferred to a glass slide, and stained with malachite green.

Identification of endophytic fungal isolates

Fungal isolates were sent to the Laboratory of Microbiology of the Federal University of Recôncavo da Bahia for taxonomic identification. At the laboratory, mycelial fragments were cultured on PDA plates. Then, the fungi were inoculated on different media in an attempt to promote sporulation, such as Sabouraud dextrose agar (10 g·L⁻¹ peptone, 40 g·L⁻¹ dextrose, and 15 g·L⁻¹ agar), 2% malt extract agar (20 g·L⁻¹ malt extract and 15 g·L⁻¹ bacteriological agar), oatmeal agar, yeast extract agar (5 g·L⁻¹ yeast extract, 20 g·L⁻¹ dextrose, and 12 g·L⁻¹ agar), and V8 agar (200 mL·L⁻¹ packaged tomato juice, 3 g·L⁻¹ CaCO₃, 5 g/L dextrose, and 20 g·L⁻¹ agar). When necessary, fungi were cultured in a small block of agar at the center of microscopic slides (Riddell, 1950). Identification at the genus and species levels was based on macrostructural characteristics, such as medium color (surface and reverse) and mycelial growth and texture, as well as on microstructural characteristics, such as mycelium type, conidiophores, presence of spores, spore size, shape, color, and resistance structures (chlamydospores). Identification keys were used as references (Barnett & Hunter, 1986; Domsch et al., 2008; Kirk & Cooper, 2005; Sutton, 1980).

Cryopreservation

The first step was to prepare the fungal inocula for cryopreservation. For this, wheat 'TPR-136 IAPAR' grains were washed under running water, washed three times with ultrapure water, and cooked at 90 °C for 45 min in CaCO₃ solution (1.5%). After cooking, excess water was removed, the grains were transferred to 15 mL Falcon tubes and autoclaved at 121 °C for 90 min (Mata and Estrada 2005). One tube was used for pH determination; wheat grains were ground with a glass rod, homogenized with ultrapure water, and analyzed using a pH meter. The pH was found to be 6.9. The remaining tubes were inoculated with a PDA disk containing mycelia and kept in the dark at 25 °C for 15 days.

For cryopreservation, 8 mm diameter plastic straws were cut into 6 cm pieces, heat-sealed at one end, and used as cryotubes (Challen & Elliot, 1986) after autoclaving at 121 °C for 15 min (Mantovani, 2008). Under a laminar flow hood, the wheat grain solution was filtered through a 0.22 µm membrane filter (low

protein binding PVDF membrane, Durapore®) and each cryotube received 5 wheat grains or 5 PDA disks colonized by fungi and 1 mL of sucrose solution ($150 \text{ g}\cdot\text{L}^{-1}$). Cryotubes were heat-sealed at the other end and stored in an ultrafreezer at -80°C (Colauto, Cordeiro, et al., 2012).

Recovery of cryopreserved fungi

At 1 and 6 months after cryopreservation, three cryotubes per treatment were removed from the ultrafreezer and evaluated for mycelial viability. Briefly, cryotubes were removed from the ultrafreezer and placed in a water bath at 30°C for 15 min. Under a laminar flow hood, cryotubes were immersed in 70% and 95% ethanol for surface cleaning (Colauto et al., 2011; Colauto, Cordeiro, et al., 2012; Colauto, Da Eira, et al., 2012). One end of the cryotube was cut off and the cryoprotectant removed. Five wheat grains and five PDA discs containing cryopreserved fungi were plated on PDA medium previously autoclaved at 121°C for 20 min. Then, plates were kept in the dark at 25°C , and mycelial growth was monitored daily. Each wheat grain or PDA disk represented one replicate, totaling five replicates per treatment. To evaluate fungal recovery, we assessed whether there was mycelial growth on PDA medium.

Cellulase activity

A qualitative test was used to assess the cellulase activity of mycelial extracts (Sunitha et al. 2013). Petri dishes with carboxymethylcellulose (CMC) medium ($10 \text{ g}\cdot\text{L}^{-1}$ CMC, $1.4 \text{ g}\cdot\text{L}^{-1}$ $(\text{NH}_4)_2\text{SO}_4$, $2.0 \text{ g}\cdot\text{L}^{-1}$ KH_2PO_4 , $0.3 \text{ g}\cdot\text{L}^{-1}$ MgSO_4 , $0.3 \text{ g}\cdot\text{L}^{-1}$ CaCl_2 , $0.25 \text{ g}\cdot\text{L}^{-1}$ yeast extract, 0.75 g/L casein peptone, $17 \text{ g}\cdot\text{L}^{-1}$ agar, and 10 mL/L mineral solution containing $0.5 \text{ g}\cdot\text{L}^{-1}$ CaCl_2 , $0.4 \text{ g}\cdot\text{L}^{-1}$ $\text{MnSO}_4\cdot\text{H}_2\text{O}$, $0.35 \text{ g}\cdot\text{L}^{-1}$ $\text{ZnSO}_4\cdot\text{H}_2\text{O}$, and $0.125 \text{ g}\cdot\text{L}^{-1}$ $\text{FeSO}_4\cdot\text{H}_2\text{O}$) were inoculated with a 6 mm diameter disk of PDA containing fungal mycelia. Plates were incubated at 21°C and monitored for growth during 7 days. After this period, each plate was flooded with 10 mL of Congo red ($2 \text{ g}\cdot\text{L}^{-1}$ Congo red in 1 M Tris-HCl, pH 8.0) and kept for 10 min at room temperature. Then, the dye solution was removed, and plates were washed twice with 1 M NaCl to reveal the cellulolytic halo. Mycelium and halo diameters were measured at three equidistant points using a caliper. The enzymatic index was calculated as the ratio of the mean halo diameter to the mean mycelium diameter (Sunitha et al., 2013). Fungi with an enzymatic index greater than 1 were considered as potential cellulase producers.

Laccase activity

For analysis of laccase activity, fungi were inoculated in 250 mL Erlenmeyer flasks containing 100 mL of culture broth ($1.5 \text{ g}\cdot\text{L}^{-1} \text{ KH}_2\text{PO}_4$, $1.023 \text{ g}\cdot\text{L}^{-1} \text{ MgSO}_4\cdot 7\text{H}_2\text{O}$, $0.5 \text{ g}\cdot\text{L}^{-1} \text{ KCl}$, $0.036 \text{ g}\cdot\text{L}^{-1} \text{ FeSO}_4\cdot 7\text{H}_2\text{O}$, $0.035 \text{ g}\cdot\text{L}^{-1} \text{ ZnSO}_4\cdot 7\text{H}_2\text{O}$, and $10 \text{ g}\cdot\text{L}^{-1}$ glucose; (Pontecorvo et al., 1953) and autoclaved at 121°C for 20 min. Then, urea ($6 \text{ g}\cdot\text{L}^{-1}$) was added and the flasks inoculated with four discs (~6 mm diameter) of fungal mycelia, in triplicate. Samples were incubated at 25°C for 10 days. Enzyme activity was analyzed every 24 h during this period.

Laccase activity was determined by measuring the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), according to Cardoso et al. (2018). Briefly, 200 μL of crude extract, 700 μL of water, 450 μL of sodium acetate buffer (0.1 M, pH 5.0), and 150 μL of ABTS (1 mM) were added to a test tube. Tubes were kept at 30°C for 10 min and the increase in absorbance was monitored at 420 nm ($\epsilon = 36000 \text{ M}^{-1} \text{ cm}^{-1}$). Two analytical controls were used, one without crude extract and the other without ABTS. Experiments were performed in two replications. One unit (U) of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS in 1 min of reaction.

Determination of antioxidant activity

The first step was to obtain sufficient mycelial biomass for analysis. Four 6 mm discs containing the mycelium of isolated fungi were transferred to a 250 mL Erlenmeyer flask containing 100 mL of malt extract broth ($20 \text{ g}\cdot\text{L}^{-1}$) and kept under agitation at 85 rpm and 25°C for 3 to 5 days. Fungal biomass was collected by filtration and dried in a forced-air oven at 60°C for 2 days. After drying, the biomass was ground with mortar and pestle and the resulting powder used for extract preparation. Ground dry biomass (1 g) was homogenized with 10 mL of methanol in 50 mL Falcon tubes. The mixture was kept at 60°C for 60 min, then cooled in an ice bath for 10 min, and centrifuged at $4000\times g$ and 5°C for 10 min. The supernatant was collected and used as crude extract.

Total antioxidant activity (DPPH• assay)

Total antioxidant activity was assessed by the *DPPH•* (2,2-diphenyl-1-picrylhydrazyl) assay, according to Brand-Williams et al. (1995), with modifications. The method is based on the color change from purple to yellow as *DPPH•* is reduced by electron transfer.

Crude extracts were first tested at a single concentration. For this, 3.9 mL of a methanolic solution of *DPPH*• (60 µM) was homogenized with 100 µL of crude extract. After 30 min, the absorbance was read at 515 nm on a UV/Vis spectrophotometer. Total antioxidant activity was calculated against a standard solution of quercetin (60 µM), taken as a reference (100% activity) (Molyneux, 2004).

Fungi with antioxidant activity in the screening test were selected for IC₅₀ determination. First, *DPPH*• solutions at 0, 10, 20, 30, 40, and 60 µM were prepared in methanol. Then, 3.9 mL of each *DPPH*• solution was homogenized with 100 µL of methanol. After 30 min, absorbance readings were taken at 515 nm on a UV/Vis spectrophotometer, and the absorbance corresponding to 50% (30 µM) of the initial *DPPH*• concentration (60 µM) was calculated. Subsequently, crude extracts were diluted with methanol to concentrations of 33, 25, 16, 8, 4, and 2 mg·mL⁻¹. For determination of antioxidant activity, 3.9 mL of *DPPH*• methanolic solution (60 µM) was homogenized with 100 µL of crude extracts at different concentrations, samples were left to rest for 30 min, and absorbance was measured at 515 nm. Solutions of quercetin at 0, 10, 20, 30, 40, and 60 µM were used as positive controls. The concentration of extract required to inhibit 50% of free radicals in the sample (IC₅₀) was determined by plotting absorbance versus sample concentration. Experiments were performed in triplicate.

Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed as described by Benzie and Strain (1996), with modifications. The method is based on the reduction of the Fe³⁺/2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) complex to a blue Fe²⁺/TPTZ complex in acidic pH by the action of an electron-donor antioxidant. Briefly, 25 mL of acetate buffer (0.3 M), 2.5 mL of an aqueous solution of TPTZ (10 mM), and 2.5 mL of an aqueous solution of ferric chloride (20 mM) were mixed together to obtain the FRAP reagent. Then, 90 µL of extract was mixed with 270 µL of distilled water and 2.7 mL of FRAP reagent, vigorously homogenized, and kept at 37 °C for 30 min. The change in absorbance was determined spectrophotometrically at 595 nm. Antioxidant activity was calculated against a standard curve of ferrous sulfate (2000 µM). Trolox (800 µM) was used as a positive control (Thaipong et al., 2006). Experiments were performed in triplicate.

Determination of total phenolic content

For determination of total phenolics, crude extracts (300 µL) were mixed with 2.5 mL of Folin–Ciocalteu

reagent (10% in water) and 2.0 mL of Na₂CO₃ (75 g/L), homogenized, and incubated at 50 °C for 15 min.

Total phenolic content was calculated from a standard curve of gallic acid (0, 10, 20, 40, 60, 80, and 100 µg/mL). Absorbance readings were taken at 740 nm. Experiments were conducted in triplicate.

Statistical analysis

Differences between mean values were analyzed by the Scott–Knott test using Assistat version 7.7.

Results

Isolation of endophytic fungi from *B. uniflora*

Figure 1 shows the growth of endophytic fungi obtained from internal tissues of *B. uniflora* stems. Each fungus was isolated until a pure culture was obtained.

A total of 46 fungi were isolated from *B. uniflora* stems. Isolates were classified into 24 morphotypes according to morphological similarities (Table 1). The morphological traits analyzed were mycelium color, spore color, and color changes in the culture medium. Other early growth characteristics of mycelia were considered, such as shape, halo formation, outer edge growth, and specific traits.

Group 2 comprised the largest number of isolates (CE2A, CE2B, CE2C, CE2D, and CE2E), accounting for 11% of the fungi isolated from *B. uniflora* stems. Of the 46 fungi isolated from *B. uniflora*, 9 were classified according to conventional taxonomy as *Nigrospora oryzae* (CE1E and CE25), *Nodulisporium* sp. (CE11A), *Phomopsis archeri* (CE4A, CE8A, CE17, and CE24), and *Penicillium* sp. (CE19 and CE22), all of which are ascomycetes.

Assessment of mycelial viability after cryopreservation

All fungi cryopreserved on PDA or wheat grains with sucrose as cryoprotectant showed 100% mycelial viability after 6 months of cryopreservation at −80 °C (Table 2). Although PDA and wheat grains resulted in the same mycelial viability, there were differences in the time to onset of mycelial growth after thawing. For fungi maintained on PDA and cryopreserved for 1 month, the time to growth onset ranged from 18 to 42 h, with a mean of 23 h, whereas, for fungi maintained on wheat grains, the time to growth onset ranged from 12 to 24 h, with a mean of 18 h. Thus, after 1 month of cryopreservation, the time to onset of growth was shorter for fungi maintained on wheat grains. After 6 months of cryopreservation, the time to growth

onset ranged from 18 to 23 h (mean of 21 h) for fungi maintained on PDA and from 17 to 26 h (mean of 22 h) for fungi maintained on wheat grains. These findings show that cryopreservation for up to 6 months at -80°C is effective in maintaining mycelial viability.

Cellulase activity

All isolates showed mycelial growth on CMC medium (Table 3), demonstrating their ability to produce cellulase, as the only carbon source of the medium was CMC. The following isolates produced a noticeable halo outside the mycelial diameter: CE5A, CE7A, CE10A, CE12A, CE12B, CE15B, CE20, and CE23. Despite the morphological similarity of groups 5 (CE5A and CE5B), 10 (CE10A and CE10B), 12 (CE12A, CE12B, CE12C, CE12D, and CE12E), and 15 (CE15A and 15B), these fungi had different enzyme activities. Of the isolates of group 5 (CE5A and CE5B), only CE5A produced a halo; CE5B did not significantly alter medium color, as assessed by the Congo red dye method.

Laccase activity

Laccase was produced by eight fungi (Figure 2). CE23 differed from the other isolates in laccase activity from the fifth to the ninth day of culture. The highest laccase activity was 1.071 U/mL, detected in CE23 on the seventh day of culture. CE5A, CE7A, CE10A, CE12A, CE12B, CE15B, and CE20 did not show significant enzyme activity (Table 4).

Antioxidant activity

After screening for *DPPH*• scavenging activity, 12 isolates (CE1E, CE11A, CE12B, CE14A, CE14B, CE15B, CE18, CE19, CE21, CE22, CE23, and CE24) were selected for determination of IC_{50} , FRAP, and total phenolic content (Table 5).

The lowest *DPPH*• IC_{50} values were observed for CE14A (15.049 ± 0.017 mg/mL), CE19 (15.716 ± 0.393 mg/mL), CE24 (15.818 ± 0.327 mg/mL), CE15B (17.122 ± 1.054 mg/mL), CE18 (18.281 ± 0.386 mg/mL), and CE22 (20.776 ± 1.503 mg/mL). The IC_{50} values of the other isolates ranged from 29.240 ± 1.832 to 43.055 ± 5.830 mg/mL. The *DPPH*• IC_{50} of isolates was 485–670 times higher than that of the positive control quercetin ($\text{IC}_{50} = 0.031 \pm 0.002$ mg/mL), showing that the endophytes had a low *DPPH*• scavenging activity. Ascêncio et al. (2014) observed significant *DPPH*• (IC_{50}) activity in 5 of the 13 endophytic fungi isolated from *Costus spiralis*; *Cochliobolus* spp. had an IC_{50} of 0.189 mg/mL, higher

than that observed here for *B. uniflora* endophytic fungi.

CE14A had the highest FRAP activity ($0.061 \pm 0.001 \mu\text{M Fe}_2\text{SO}_4/\text{mg}$) and total phenolic content ($55.296 \pm 3.536 \mu\text{g}/\text{mg}$). Higher FRAP activities were reported by Ascêncio et al. (2014) for endophytic fungi (133.74 ± 0.29 to $471.15 \pm 2.87 \mu\text{M Fe}_2\text{SO}_4/\text{mg}$). Mahdi et al. (2014) identified a total phenolic content of $8300 \mu\text{g}/\text{mg}$ in *Aspergillus* sp. isolated from stems of *D. stramonium*. Thus, fungi isolated from *B. uniflora* in the current study had lower antioxidant activity and total phenolic content than other endophytes reported in the literature.

Discussion

There are no reports of endophytic fungal diversity in *B. uniflora*. Mahdi et al. (2014) isolated five endophytic fungi from stems of *Datura stramonium* (Solanaceae). According to Nath et al. (2012), endophytic fungal diversity is greatest in plant stems. The authors isolated 11 fungi from different parts of *Embllica officinalis* (Phyllanthaceae), and 5 were obtained from the stem.

The most frequent endophytic fungi of *Tephrosia purpurea* belonged to the genera *Nigrospora* (14.7%) and *Penicillium* (9.8%) (Luo et al., 2015). The genus *Phomopsis* represented 46% of the isolates obtained from *Picea sitchensis* (Carroll & Carroll, 1978). Costa et al. (2012) found that *Nodulisporium* individuals accounted for 4% of the endophytic fungi isolated from *Rhizophora mangle* (Rhizophoraceae) and *Laguncularia racemosa* (Combretaceae).

Nigrospora was found to synthesize the bioactive compound anthraquinone (deoxybostrycin), effective against cancer cells (Xia et al., 2011). *Penicillium*, the widely known producer of penicillin (Demain & Elander, 1999), also produces mycotoxins (Frisvad et al., 2004) and compounds with anticholesterolemic (Chakravarti & Sahai, 2004) and antitumor action (Nicoletti et al., 2008). *Phomopsis* was shown to exert antifungal and antibiotic activities and produce volatile organic compounds such as sabinene (S. K. Singh et al., 2011). The endophytic fungus *Nodulisporium* sp. isolated from *Juniperus cedrus* (Cupressaceae) produced the antibacterial compounds 1-(2,6-dihydroxyphenyl)butan-1-one, nodulisporin D and F, and benzene-1,2,3-triol (Dai et al., 2006, 2009). These findings suggest that the highly diverse endophytic microbiota of *B. uniflora* is a potential source of biologically active compounds.

Mantovani (2008) reported that cryopreservation of *Lentinula edodes* with wheat grain substrate and

sucrose at 50, 100, or 300 g L⁻¹ for one year was effective in preserving 100% of mycelial viability. Cereal grains are rich in starch reserves, providing a nutrient-rich medium. Furthermore, the capillary structure of wheat grain seems to contribute greatly to mycelial recovery after cryopreservation. The capillary network of cereal grains provides little physical space for water molecules, protecting mycelia from ice formation (Tanaka et al., 2013). The large amount of carbohydrates and proteins that bind to water (Belitz & Grosch, 1997) might be responsible for the reduction in free water and ice crystal formation, minimizing the risk of cryoinjury. Thus, whole wheat grains, which have been successfully used for the cryopreservation of basidiomycetes (Basidiomycota), might be better than PDA medium for long-term cryopreservation of endophytic fungi (Ascomycota) isolated from *B. uniflora*. Future studies investigating longer cryopreservation times are needed to better assess the effect of wheat grains on ascomycete cryopreservation.

Ngieng et al. (2013) reported that 65% of the 20 endophytic fungi isolated from *Melastoma malabathricum* (Melastomataceae) exhibited cellulase activity as assessed by a qualitative test. Patil et al. (2015) found that, of the nine fungi isolated from medicinal plants in India, including *Azadirachta indica*, *Citrus limon*, *Gossypium hirsutum*, *Magnolia champaca*, *D. stramonium*, *Piper betle*, and *Phyllanthus emblica*, 44% exhibited qualitative cellulase activity. Similarly, using a qualitative test, Yasser et al. (2019) found that 63% of the 19 endophytic fungi of *Solanum tuberosum* had cellulase activity and 31% showed laccase activity. In our study, only 17% of fungi were positive for cellulase, despite the observed fungal diversity and enzyme and secondary metabolite production.

Urairuj (2003) found that the maximum laccase activity (0.01 U/mL) of *Xylaria* sp. isolated from plants native to Thailand occurred on the seventh day of culture. In the study of Wang et al. (2006), *Monotospora* sp. isolated from the plant *Cynodon dactylon* had a maximum laccase activity of 3.49 U/mL on the eighth day of culture. Under controlled conditions of pH, temperature, and carbon and nitrogen sources, *Monotospora* sp. was found to have an activity of 13.55 U/mL, higher than that observed in the current study for *B. uniflora* endophytes (1.071 U/mL in CE23). The laccase activity of *B. uniflora* endophytic fungi was lower than that of other endophytes reported in the literature. However, it should be noted that enzyme production may be optimized.

It is important to evaluate antioxidant activity using other types of extracts. In a previous study, our research group obtained the oleoresin of leaves and flowers of *B. uniflora* by carbon dioxide (CO₂) extraction at 40, 50, and 60 °C and 150, 175, and 200 bar. Leaf oleoresin obtained at 60 °C and 150 bar showed the highest *DPPH*• (IC₅₀ = 1.90 mg/mL) and FRAP (1.8 µM/mL) activities. These samples also had the highest total phenolic content (66.20–83.33 µg gallic acid equivalents/mg) and oxidation inhibition potential (120.94–124.14%), as assessed by the β-carotene/linoleic acid assay at 40–60 °C and 150–200 bar (Jorge et al., 2017). In the present study, however, the 12 fungal extracts had low total phenolic, *DPPH*•, and FRAP values. It is essential that more than one extractive method be used to assert the biological potential of fungi and that the chosen antioxidant assay be compatible with the isolated active compounds.

Conclusions

B. uniflora stems harbor a great diversity of endophytic fungi. These fungi showed mycelial viability after 1 and 6 months of cryopreservation at –80 °C as well as significant cellulase and laccase activities. Twelve isolates exhibited low *DPPH*• scavenging activities, FRAP activities, and total phenolic contents.

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Figure 1. Growth of fungi from *Brunfelsia uniflora* stem fragments.

Table 1. Endophytic fungi ($n = 46$) isolated from stems of *Brunfelsia uniflora*, grouped by morphological similarity and medium color.

Group	Isolate	Morphology	Morphotype	(%)	Colony characteristics and photomicrographs	Initial identification
1	4	Black and white mycelium, black spores, black growth medium	CE1A, CE1B, CE1C, CE1E	9		CE1E = <i>Nigrospora oryzae</i> (Ascomycota)
2	5	White mycelium, black spores, growth medium with a brown halo	CE2A, CE2B, CE2C, CE2D, CE2E	11		Not identified
3	3	White mycelium, black spores, growth medium with a yellow halo	CE3A, CE3B, CE3C,	7		Not identified
4	4	White and yellow mycelium, brown spores, light yellow growth medium	CE4A, CE4B, CE4C, CE4D	9		CE4A = <i>Phomopsis archeri</i> (Ascomycota)
5	2	White and gray mycelium, brown spores, light yellow growth medium with a dark brown halo	CE5A, CE5B	4		Not identified
6	1	White mycelium with a yellow center, black spores, no color changes in the growth medium	CE6A	2		Not identified
7	2	Yellow and white mycelium, dark yellow spores, yellow growth medium with a brown halo	CE7A, CE7B	4		Not identified
8	2	White and brown mycelium, black spores, brown growth medium	CE8A, CE8B	4		CE8A = <i>Phomopsis archeri</i> (Ascomycota)

Group	Isolate	Morphology	Morphotype	(%)	Colony characteristics and photomicrographs	Initial identification
9	1	White mycelium, brown spores, growth medium with a brown halo	CE9A	2		Not identified
10	2	White and gray mycelium, black spores, yellow growth medium with a brown halo	CE10A, CE10B	4		Not identified
11	1	White and gray mycelium, black spores, dark gray growth medium	CE11A	2		CE11A = <i>Nodulisporium</i> sp. (Ascomycota)
12	3	White and yellow mycelium, yellow spores, yellow growth medium	CE12A, CE12B, CE12C	7		Not identified
13	2	White and brown mycelium, black spores, growth medium with a yellow halo	CE13A, CE13B	4		Not identified
14	3	White mycelium, white spores, growth medium with a yellow halo	CE14A, CE14B, CE14C	7		Not identified
15	2	White and brown mycelium, brown spores, growth medium with a brown halo	CE15A, CE15B	4		Not identified
17	1	Brown and yellow mycelium, brown spores, no color changes in the growth medium	CE17	2		CE17 = <i>Phomopsis archeri</i> (Ascomycota)

Group	Isolate	Morphology	Morphotype	(%)	Colony characteristics and photomicrographs		Initial identification
18	1	Brown mycelium, brown spores, dark brown growth medium	CE18	2			Not identified
19	1	White and yellow mycelium, brown spores, yellow growth medium with a brown halo	CE19	2			CE19 = <i>Penicillium</i> sp. (Ascomycota)
20	1	White mycelium with cottony texture, white spores, no color changes in the growth medium	CE20	2			Not identified
21	1	White mycelium, black spores, yellow growth medium with a light yellow halo	CE21	2			Not identified
22	1	Yellow and white mycelium, yellow dark spores, yellow growth medium with a dark brown halo	CE22	2			CE22 = <i>Penicillium</i> sp. (Ascomycota)
23	1	White and yellow mycelium, black spores, no color changes in the growth medium	CE23	2			Not identified
24	1	White mycelium with a yellow center, black spores, growth medium with a brown halo	CE24	2			CE24 = <i>Phomopsis archeri</i> (Ascomycota)
25	1	White mycelium, black spores, brown growth medium	CE25	2			CE25 = <i>Nigrospora oryzae</i> (Ascomycota)

Table 2. Mycelial viability (MV) and time to onset of growth (TOG) of endophytic fungi isolated from *Brunfelsia uniflora* stems and cryopreserved at -80°C using 1.5 g L^{-1} sucrose as cryoprotectant.

Morphotype	Potato dextrose agar				Whole wheat grain			
	1-month cryopreservation		6-month cryopreservation		1-month cryopreservation		6-month cryopreservation	
	MV (%)	TOG (h)	MV (%)	TOG (h)	MV (%)	TOG (h)	MV (%)	TOG (h)
CE1A	100	22	100	22	100	13	100	24
CE1B	100	25	100	22	100	13	100	23
CE1C	100	22	100	22	100	12	100	23
CE1E	100	22	100	22	100	12	100	23
CE2A	100	17	100	22	100	18	100	18
CE2B	100	22	100	22	100	17	100	17
CE2C	100	22	100	22	100	17	100	17
CE2D	100	31	100	21	100	17	100	17
CE2E	100	16	100	21	100	18	100	18
CE3A	100	16	100	23	100	18	100	18
CE3B	100	18	100	18	100	18	100	18
CE3C	100	27	100	18	100	18	100	18
CE4A	100	27	100	21	100	19	100	20
CE4B	100	22	100	21	100	19	100	20
CE4C	100	22	100	21	100	21	100	20
CE4D	100	22	100	21	100	18	100	20
CE5A	100	22	100	21	100	18	100	20
CE5B	100	18	100	22	100	19	100	20
CE6A	100	18	100	22	100	18	100	20
CE7A	100	29	100	22	100	18	100	20
CE7B	100	24	100	22	100	18	100	20
CE8A	100	28	100	22	100	17	100	24
CE8B	100	28	100	22	100	17	100	24
CE9A	100	21	100	18	100	17	100	24
CE10A	100	22	100	21	100	17	100	24
CE10B	100	21	100	21	100	17	100	24
CE11A	100	42	100	20	100	17	100	23
CE12A	100	31	100	20	100	24	100	24
CE12B	100	20	100	20	100	24	100	24
CE12C	100	20	100	20	100	23	100	24
CE13A	100	31	100	20	100	23	100	23
CE13B	100	20	100	20	100	23	100	23
CE14A	100	20	100	20	100	21	100	23
CE14B	100	20	100	20	100	19	100	23
CE14C	100	20	100	20	100	19	100	23
CE15A	100	18	100	22	100	19	100	23
CE15B	100	18	100	22	100	19	100	23
CE17	100	21	100	21	100	19	100	23
CE18	100	20	100	20	100	12	100	23
CE19	100	21	100	21	100	19	100	23
CE20	80	34	100	20	100	19	100	23
CE21	100	40	100	20	100	18	100	23
CE22	100	39	100	21	100	18	100	23
CE23	100	18	100	22	100	18	100	23

Morphotype	Potato dextrose agar				Whole wheat grain			
	1-month cryopreservation		6-month cryopreservation		1-month cryopreservation		6-month cryopreservation	
	MV (%)	TOG (h)	MV (%)	TOG (h)	MV (%)	TOG (h)	MV (%)	TOG (h)
CE24	100	20	100	20	100	19	100	19
CE25	100	20	100	20	100	22	100	26
Mean	100	23	100	20	100	18	100	22

Table 3. Cellulolytic index of fungi isolated from *Brufelsia uniflora* stems.

Morphotype	Mycelium diameter (cm)	Halo diameter (cm)	Cellulolytic index
CE1A	4.4	0.0	0.00 ^d
CE1B	2.3	0.0	0.00 ^d
CE1C	5.5	0.0	0.00 ^d
CE1E	6.8	0.0	0.00 ^d
CE2A	3.6	3.6	1.00 ^c
CE2B	5.7	5.7	1.00 ^c
CE2C	6.0	6.0	1.00 ^c
CE2D	5.6	5.6	1.00 ^c
CE2E	5.2	5.2	1.00 ^c
CE3A	6.0	6.0	1.00 ^c
CE3B	4.6	4.6	1.00 ^c
CE3C	4.6	4.6	1.00 ^c
CE4A	3.3	3.3	1.00 ^c
CE4B	5.0	5.0	1.00 ^c
CE4C	5.7	5.7	1.00 ^c
CE4D	4.8	4.8	1.00 ^c
CE5A	6.4	6.8	1.06 ^b
CE5B	5.5	5.5	1.00 ^c
CE6A	4.5	0.0	0.00 ^d
CE7A	4.5	4.8	1.07 ^b
CE7B	3.7	0.0	0.00 ^d
CE8A	4.9	4.9	1.00 ^c
CE8B	4.4	4.4	1.00 ^c
CE9A	3.3	3.3	1.00 ^c
CE10A	2.9	3.5	1.21 ^a
CE10B	5.6	0.0	0.00 ^d
CE11A	3.9	0.0	0.00 ^d
CE12A	5.1	5.3	1.04 ^b
CE12B	5.9	6.2	1.05 ^b
CE12C	3.7	3.7	1.00 ^c
CE13A	2.7	0.0	0.00 ^d
CE13B	3.5	0.0	0.00 ^d
CE14A	4.8	4.8	1.00 ^c
CE14B	4.7	4.7	1.00 ^c
CE14C	4.6	4.6	1.00 ^c
CE15A	4.6	0.0	0.00 ^d
CE15B	5.0	5.6	1.12 ^a
CE17	4.2	4.2	1.00 ^c
CE18	3.7	0.0	0.00 ^d
CE19	6.0	6.0	1.00 ^c

Morphotype	Mycelium diameter (cm)	Halo diameter (cm)	Cellulolytic index
CE20	2.3	2.4	1.04 ^b
CE21	4.0	0.0	0.00 ^d
CE22	5.3	0.0	0.00 ^d
CE23	3.5	3.9	1.11 ^a
CE24	3.5	3.5	1.00 ^c
CE25	3.3	0.0	0.00 ^d

Figure 2. Laccase activity of endophytic fungi isolated from *Brunfelsia uniflora* stems.

Table 4. Laccase activity of endophytic fungi isolated from *Brunfelsia uniflora* stems.

Morphotype	Laccase activity (U/mL)
CE5A	0.046 ^b
CE7A	0.131 ^b
CE10A	-
CE12A	0.007 ^b
CE12B	-
CE15B	-
CE20	-
CE23	1.071 ^a

Table 5. Antioxidant activity of endophytic fungi isolated from *Brunfelsia uniflora* stems.

Morphotype	DPPH• scavenging activity (μg QE/mg)	DPPH• IC ₅₀ (mg/mL)	FRAP activity (μM Fe ₂ SO ₄ /mg)	Total phenolics (μg/mg)
CE1A	0.747 ± 0.008	nt	nt	nt
CE1B	0.784 ± 0.030	nt	nt	nt
CE1C	0.643 ± 0.014	nt	nt	nt
CE1E	1.016 ± 0.009	43.055 ± 5.830	0.047 ± 0.001	42.473 ± 1.320
CE2A	0.271 ± 0.005	nt	nt	nt
CE2B	0.569 ± 0.016	nt	nt	nt
CE2C	0.345 ± 0.005	nt	nt	nt
CE2D	0.383 ± 0.023	nt	nt	nt

Morphotype	<i>DPPH</i> • scavenging activity ($\mu\text{g QE/mg}$)	<i>DPPH</i> • IC_{50} (mg/mL)	FRAP activity ($\mu\text{M Fe}_2\text{SO}_4/\text{mg}$)	Total phenolics ($\mu\text{g/mg}$)
CE2E	0.312 ± 0.002	nt	nt	nt
CE3A	0.554 ± 0.027	nt	nt	nt
CE3B	0.329 ± 0.026	nt	nt	nt
CE3C	0.547 ± 0.021	nt	nt	nt
CE4A	0.530 ± 0.021	nt	nt	nt
CE4B	0.474 ± 0.021	nt	nt	nt
CE4C	0.516 ± 0.071	nt	nt	nt
CE4D	0.366 ± 0.027	nt	nt	nt
CE5A	0.563 ± 0.024	nt	nt	nt
CE5B	0.701 ± 0.119	nt	nt	nt
CE6A	ns	nt	nt	nt
CE7A	0.753 ± 0.024	nt	nt	nt
CE7B	0.724 ± 0.051	nt	nt	nt
CE8A	0.309 ± 0.034	nt	nt	nt
CE8B	0.404 ± 0.137	nt	nt	nt
CE9A	0.575 ± 0.011	nt	nt	nt
CE10A	0.296 ± 0.063	nt	nt	nt
CE10B	0.882 ± 0.053	nt	nt	nt
CE11A	1.336 ± 0.005	29.240 ± 1.832	0.028 ± 0.0007	31.473 ± 0.099
CE12A	0.662 ± 0.010	nt	nt	nt
CE12B	1.259 ± 0.034	50.547 ± 3.349	0.027 ± 0.005	40.868 ± 2.019
CE12C	0.293 ± 0.034	nt	nt	nt
CE13A	ns	nt	nt	nt
CE13B	0.185 ± 0.026	nt	nt	nt
CE14A	1.341 ± 0.003	15.049 ± 0.017	0.061 ± 0.001	55.296 ± 3.536
CE14B	1.229 ± 0.045	66.974 ± 6.185	0.040 ± 0.014	30.960 ± 0.296
CE14C	0.998 ± 0.012	nt	nt	nt
CE15A	0.985 ± 0.040	nt	nt	nt
CE15B	5.849 ± 0.006^a	17.122 ± 1.054	0.030 ± 0.001	25.710 ± 0.975
CE17	0.685 ± 0.027	nt	nt	nt
CE18	1.315 ± 0.003	18.281 ± 0.386	0.027 ± 0.017	40.822 ± 3.341
CE19	1.295 ± 0.041	15.716 ± 0.393	0.015 ± 0.001	16.039 ± 1.314
CE20	0.749 ± 0.045	nt	nt	nt
CE21	1.342 ± 0.009	70.138 ± 2.553	0.021 ± 0.015	20.776 ± 1.641
CE22	1.354 ± 0.002	20.776 ± 1.503	0.030 ± 0.002	28.697 ± 2.408
CE23	1.344 ± 0.001	40.205 ± 1.094	0.024 ± 0.011	22.118 ± 1.729
CE24	1.193 ± 0.05	15.818 ± 0.327	0.037 ± 0.001	67.598 ± 3.040
CE25	ns	nt	nt	nt
Trolox	-	-	9.325 ± 0.670	-
Quercetin	0.060 ± 0.003	0.031 ± 0.002	-	-