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Original Article

Isolation and molecular identification of endophytic fungi associated with *Campomanesia adamantium*, a Brazilian Cerrado plant

Identificação molecular e caracterização da diversidade de espécies cultiváveis de fungos endofíticos associados à *Campomanesia Adamantium*

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Abstract

This work presents the isolation of endophytic fungi from the leaves of *Campomanesia adamantium* (Cambess.) O. Berg (Myrtaceae), a native species found in Brazil and popularly known as "guabiroba-do-campo", with abundant distribution in the Brazilian Cerrado. It has been popularly used for its anti-rheumatic, anti-inflammatory, antidiarrheal, blood cholesterol-reducing, urinary antiseptic, and depurative properties. Theese fungi are microorganisms that live inside higher plants, at least for a period of their life cycle, occupying the intercellular spaces of plant tissues such as leaves and stems. These fungi are harmless to the host plant, and their secondary metabolites promote protection, regulate growth, combat bacteria, viruses, and fungi, and promote resistance to abiotic stress, as well as insecticidal effects. Endophytic fungi associated with the leaves of *C. adamantium* were isolated using the culture medium isolation technique. After growth, the fungi were divided into groups based on morphotypes. Fungal genomic DNA was extracted, and a polymerase chain reaction (PCR) was conducted to amplify the ITS1-5.8S-ITS2 regions of rRNA, and the nucleotide sequences obtained were compared with those available in the GenBank database for molecular identification of the isolates. The phylogenetic tree was constructed using MEGA 11 software. The results showed representatives of the Ascomycota phylum, and it was possible to identify at the genus level 18 fungi of the genera *Colletotrichum*, *Diaporthe*, *Epicoccum*, and *Neofusicoccum.*

Keywords: endophytes, DNA barcode, Brazilian Cerrado, Myrtaceae associated fungi.

Resumo

Este trabalho apresenta o isolamento de fungos endofíticos de folhas de *Campomanesia adamantium* (Cambess.) O. Berg (Myrtaceae), espécie nativa encontrada no Brasil, com distribuição abundante no Cerrado brasileiro, popularmente conhecida como "guabiroba-do-campo. Têm sido utilizadas popularmente por suas atividades anti-reumáticas anti-inflamatórias, antidiarreicas, redutoras de colesterol no sangue, antisséptica urinária e depurativas. Fungos endofíticos são microrganismos que vivem no interior de plantas superiores, ao menos por um período de seu ciclo de vida, ocupando os espaços intercelulares dos tecidos vegetais, como folhas e caules. Esses fungos, além de serem inofensivos à planta hospedeira, produzem benefícios a partir de metabólitos secundários que promovem proteção, regulam o crescimento, combatem bactérias, vírus e fungos e promovem resistência a estresse abiótico, além de efeitos inseticidas. Os fungos endófitos associados às folhas de *C. adamantium* foram isolados utilizando-se a técnica de isolamento em meio de cultura. Logo após o crescimento, os fungos foram divididos em grupos baseados em morfotipos. O DNA genômico fúngico foi extraído A reação em cadeia da polimerase (PCR) foi conduzida para amplificação da região ITS-1-5.8S-ITS2 de rRNA e as sequências de nucleotídeos obtidas foram comparados com aqueles disponíveis na base de dados GenBank para a identificação molecular dos isolados. A construção da filogenia da árvore foi realizada utilizando o software MEGA 11. Os resultados apresentaram representantes do filo Ascomycota e em nível de gênero foi possível identificar 18 fungos dos gêneros *Colletotrichum*, *Diaporthe*, *Epicoccum* e *Neofusicoccum*.

Palavras-chave: endófitos, código de barras de DNA, Cerrado Brasileiro, fungos associados a Myrtaceae.

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1. Introduction

Campomanesia adamantium (Cambess.) O. Berg (Myrtaceae) is a native species found in Brazil, with abundant distribution in the Brazilian Cerrado. It belongs to the Myrtaceae family, one of the richest families in the neotropical region (Wilson et al., 2001).

It is a fruit-bearing species, native to the Cerrado region, popularly known as *guavira*, *guabiroba-do-campo*, or *guabiroba-do-cerrado*. The *guavira* fruit is consumed fresh and used as a food and medicinal source in sweets, ice creams, soft drinks, liqueur ingredients, and as a flavoring agent in alcoholic distillates, reflecting its economic value and sociocultural importance in the Midwest region of Brazil (Dousseau et al., 2011; Vieira et al., 2011; Nascimento et al., 2022).

Barks and leaves, prepared by infusion, are popularly used against diarrhea, urinary tract problems, and leukorrhea (Carrara, 1997; Markman, 2002); however, the medicinal potential of *C. adamantium* has been scientifically proven through its bioactive compounds, as a source of anticancer and phytochemical, toxicological and pharmacological agents, as well as digestive, diuretic, antimicrobial, anti-rheumatic and antidiabetic activity (Serafin et al., 2007; Alves et al., 2020).

Endophytic microorganisms are mainly fungi and bacteria that live inside plants without causing damage to them. They play important roles in the vitality of the host plant. The interaction between the endophyte and the plant can be described as a symbiotic relationship, since both benefit from this association. The host plant provides a protective environment for microorganisms (Santos et al., 2018) which, in turn, produce substances that improve nutrient absorption, influencing plant growth and increasing its biomass. These metabolic substances also trigger a process of plant resistance to pathogens, reducing their vulnerability to infections caused by pathogenic agents and functioning as disease control agents.

Among all known producers of natural products, microorganisms represent a rich source of biologically active metabolites that have various biotechnological applications (Gunatilaka, 2006; Matasyoh et al., 2011, Zhai et al., 2017). Producing bioactive compounds is compatible with commercial expansion, especially those compounds that are exclusive to their host plants. Preservation of these species is not only important from an ecological standpoint, but also from a biochemical and molecular perspective, considering as well their social and economic importance. Some of these compounds have already proven useful for the discovery of new drugs (Kusari et al., 2012, Guo et al., 2008; Yan et al., 2011).

Global health problems caused by drug-resistant viruses, parasites, bacteria, and fungi have become increasingly alarming. In addition, cancer has become a growing health problem worldwide. To solve these problems, the discovery of new endophytic metabolites is an important alternative (Yu et al., 2010).

These compounds present different physicochemical characteristics and properties (polarity, solubility, ability to form hydrogen bonds and potential for oxide reduction) and interfere with specific physiological targets that determine their biological function (whether oxidative, inflammatory or mutagenic processes); thus, they can modulate the activity of different enzymes, by interacting with receptors and signal transduction pathways related to various chronic-degenerative diseases and cancers (Oliveira and Bastos, 2011).

The importance of isolating microorganisms from *C. adamantium* is highlighted as a means to broaden our knowledge about its cultivable endophytic diversity and future prospecting of its bioactive compounds and, consequently, potential technological applications in various sectors.

Thus, this research aimed to isolate and identify, through the amplification and sequencing of the ITS1-5.8S-ITS2 region of the rDNA, endophytic fungi from the *C. adamantium* plant cultivated in Campo Grande – MS, Brazil.

2. Material and Methods

2.1. Obtaining and collecting the material

The plant material used in the research (leaves) was collected at CEPAER - Research Center of AGRAER (Agency for Agricultural Development and Rural Extension) of the state of Mato Grosso do Sul (-20.421900, -54.668259), Brazil, registered with SISGEN (National Genetic Heritage and Associated Traditional Knowledge Management System) under the nº AC050DF.

The plant samples were in healthy condition, without stains or any type of lesion caused by pathogens or mechanical damage. After collection, the material was placed in plastic bags and taken to the Entomology laboratory at UCDB, where the research continued.

2.2. Isolation

The isolation technique in culture medium described by Araújo et al. (2010) was used, which consists of washing the samples in running water, immersion in 70% alcohol solution for 1 min, immersion in 1% sodium hypochlorite solution for 3 to 4 min, followed by immersion in 70% alcohol solution for 30s and rinsing twice in sterile ultrapure water. The samples were fragmented with the aid of a scalpel and the fragments, of approximately five mm in length, were placed on Petri dishes containing Potato dextrose agar (PDA) medium and incubated at 24 ± 2 ºC, with total absence of light, for seven days. For the purification of cultures, fungi grown on the Petri dishes were transferred to new plates with PDA medium.

The isolated fungi were purified to obtain monosporic colonies. The procedure was carried out in a laminar flow hood, in which a small piece of the desired colony was removed and agitated in a microcentrifuge tube containing 1 mL of distilled water with the aid of a vortex agitator. One hundred µL of the supernatant were collected, pipetted onto a PDA culture medium, and then spread on the plate with the aid of a Drigalski spatula. After incubation in a B.O.D. chamber at 24 ± 2 ºC for 24 hours, one of the small

colonies grown was randomly selected and inoculated into other Petri dishes containing PDA culture medium, and allowed to fully grow in a B.O.D. incubator at 24 ± 2 ºC for seven days. After growth, the fungi were divided into groups based on morphotypes.

2.3. Molecular identification of endophytic isolates

2.3.1. DNA extraction and PCR

The fungal cultures were grown on PDA plates at 28 °C for seven days, then scraped with a spatula and transferred to crucibles, macerated in liquid nitrogen, and stored in microtubes at -20 °C. Fungal genomic DNA was extracted using the DNeasy PowerSoil Pro Qiagen kit according to the manufacturer's instructions. The presence of genetic material was observed by gel electrophoresis with the aid of a molecular marker (Ladder Plus 1Kb).

The polymerase chain reaction (PCR) was conducted to amplify the region of interest using the ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') primers. The thermocycler was programmed for initial denaturation conditions at 94 ºC for 5 minutes, f423llowed by 24 cycles of 94 ºC for 30 s, 55 ºC for 30 s, and 72 ºC for 30 s. After the cycles, a final extension at 72 ºC for 7 minutes was performed.

The DNA templates were purified with the ExoSAP-IT™ PCR Product Cleanup reagent (Applied Biosystems) and quantified on a Nanodrop 2000 c instrument (Thermo Scientific). They were then labeled using 2.5 pmol of specific primer and 0.5 µL of BigDye Terminator v3.1 Cycle Sequencing Kit reagent (Applied Biosystems) in a final volume of 10 µL.

2.3.2. Sequencing

The samples were sequenced at ACTGene Análises Moleculares Ltda. (Biotechnology Center, UFRGS, Porto Alegre, RS) using the AB 3500 Genetic Analyzer automatic sequencer equipped with 50 cm capillaries and POP7 polymer (Applied Biosystems). The labeling reactions were performed in an LGC XP Cycler thermocycler with an initial denaturation step at 96 °C for 3 min followed by 25 cycles of 96 °C for 10 s, 55 °C for 5 s, and 60 °C for 4 min. Once labeled, the samples were purified by precipitation with 75% isopropanol and washed with 60% ethanol.

The precipitated products were diluted in 10 µL of Hi-Di™ formamide (Applied Biosystems), denatured at 95 ºC for 5 min, cooled on ice for 5 min, and electroinjected into the automatic sequencer. The sequencing data was collected using the Data Collection 3 program (Applied Biosystems) with the following parameters: Dye Set "Z"; Mobility File "KB_3500_POP7_BDTv3.mob"; BioLIMS Project "3500_Project1"; Run Module 1 "FastSeq50_POP7_50cm_cfv_100"; and Analysis Module 1 "BC-3500SR_Seq_FASTA.saz". The resulting files from Data Collection (.ab1; electropherograms) were converted to FASTA files (.seq; text) by Sequence Analysis Software v. 6 (Applied Biosystems) under standard parameters. The obtained sequences were compared with those deposited in the NCBI database (National Center for Biotechnology Information) using the BLASTn tool.

2.3.3. Determination of genetic distance of isolates

The obtained sequences were analyzed and edited. For the identification of endophytic fungi, the sequence identity and coverage percentages were compared with the sequences available in GenBank (NCBI) using BLASTn to search for the closest sequences. The sequence data from this study was submitted to GenBank under the accession number OR480083 to OR480100

The phylogenetic tree was elaborated through MEGA 11 software (Tamura et al., 2021). The sequences obtained through sequencing were aligned by ClustalW (Thompson et al., 1994), and the phylogeny was performed by the neighbor-joining method (Saitou and Nei, 1987) with the bootstrap test of 1000 repetitions.

3. Results

3.1. Isolation

After fragmentation isolation, all 250 fragments that were inoculated showed growth, indicating a colonization frequency of 100%. During purification, similar cultures were discarded, and only different samples were kept, resulting in a total of 41 isolates. The endophytes were distributed into 11 subgroups according to their mycelial colony characteristics, size, and coloration.

3.2. Molecular identification of endophytic isolates

The obtained sequences were used to identify the isolates in the NCBI databases (National Center for Biotechnology Information – NCBI) using BLASTn. The identification of fungal species was based on the best similarity value obtained. The determined sequences were aligned and edited using the MEGA 11 program, making it possible to identify 18 isolates of four ascomycetes genera (Table 1).

3.2. Phylogeny

The phylogenetic analysis of endophytes of *C. adamantium* based on rDNA sequencing data revealed that they were composed solely of representatives of the phylum Ascomycota (Figure 1). The first clade is formed by fungi of the class Sordariomycetes and the order Glomerellales, with representatives of the genus *Colletotrichum*. The endophytes CA14, CA09, CA69, CA17, CA08 and CA40 presented identity values between 99.81 and 100%, and phylogenetic similarity between 61 and 99% with other *Colletotrichum* strains, confirming their genus identity.

The second clade is formed by fungi of the class Sordariomycetes and the order Diaporthales, with representatives of the genus *Diaporthe* (*Phomopsis*). The endophyte CA13 (98.17% identity with *Diaporthe* sp. FJ799941.1 by BLAST) was grouped with *Diaporthe* sp. (MT 470639.1) with 73% similarity.

The third clade is formed by fungi of the class Dothideomycetes and the order Pleosporales, as a representative of the genus *Epicoccum*. The endophyte CA28 (100% *Epicoccum* sp. MG976379.1 by BLAST) was

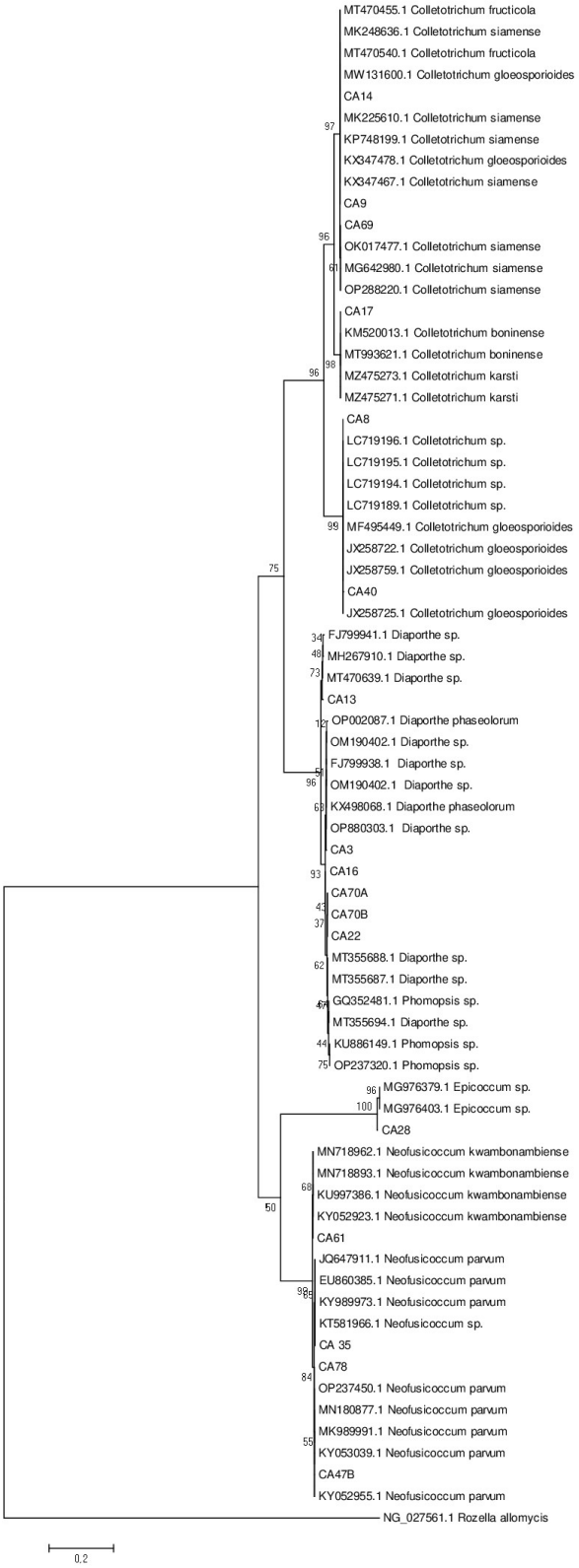

Figure 1. Phylogenetic tree constructed with endophytic sequences from *C. adamantium* and GenBank sequences (indicated by the database code), using the neighbor-joining method and the p-distance matrix for nucleotides, with pairwise gap deletion option. The numbers above and below each node indicate the frequency (%) of each branching in bootstrap analyses of 10,000 repetitions. All clades comprise fungi of the phylum Ascomycota.

Table 1. Molecular identification of endophytes isolated from *Campomanesia adamantium.*

Endophytes	genbank accession number	Closely related fungal sequences	Identity
CA 3	OR480083	*Diaporthe* sp. OP880303.1	99.63%
CA 8	OR480084	*Colletotrichum* sp. LC719196.1	100%
CA 9	OR480085	*Colletotrichum siamense* KX347467.1	99.81%
CA13	OR480086	*Diaporthe* sp. FJ799941.1	98.17%
CA14	OR480087	*Colletotrichum gloeosporioides* MW131600.1	100%
CA16	OR480088	*Diaporthe phaseolorum* OP002087.1	98.89%
CA17	OR480089	*Colletotrichum boninense* KM520013.1	99.82%
CA22	OR480090	*Phomopsis* sp. GQ352481.1	99.08%
CA28	OR480091	*Epicoccum* sp. MG976379.1	100%
CA35	OR480092	*Neofusicoccum* sp. KT581966.1	99.44%
CA40	OR480093	*Colletotrichum gloeosporioides* MF495449.1	99.81%
CA47B	OR480094	*Neofusicoccum parvum* OP237450.1	100%
CA61	OR480095	*Neofusicoccum kwambonambiense* KY052923.1	99.25%
CA69	OR480096	*Colletotrichum siamense* OK017477.1	100%
CA70A	OR480097	*Diaporthe* sp. MT355694.1	99.40%
CA70B	OR480098	*Diaporthe* sp. MT355694.1	99.45%
CA 77	OR480099	*Colletotrichum* sp. MN62668 0.1	99.82%
CA 78	OR480100	*Neofusicoccum parvum* OP237450.1	98.70%

grouped with *Epicoccum* sp. (MG976403.1) with 100% similarity, confirming its genus identity.

The fourth clade is formed by fungi of the class Dothideomycetes and the order Botryosphaeriales, as representatives of the genus *Neofusicoccum*. The endophyte CA61 (99.25% *Neofusicoccum kwambonambiense* KY052923.1 by BLAST) was grouped with *N. kwambonambiense* KY052923.1 with 68% similarity. The endophyte CA35 (99.34% *Neofusicoccum* sp. KT581966.1 by BLAST) was grouped with *Neofusicoccum* sp. (KT581966.1) with 85% similarity, confirming its genus identity. The endophyte CA78 (98.70% *Neofusicoccum parvum* OP237450.1 by BLAST) and the endophyte CA47B (100% *N. parvum* OP237450.1) were grouped with *Neofusicoccum* sp. with 99% similarity.

4. Discussion

The biodiversity of the Cerrado, one of the richest biomes in chemical and biological diversity, plays a crucial role in the interaction between endophytic fungi and their host plants. This interaction has been studied by various researchers over the years, with a special focus on endemic plant species that inhabit this unique biome (Esposito and Azevedo, 2010). The results from the present study highlight the different genera of endophytic fungi present in the leaves of *C. adamantium*, among which the most frequent are *Colletotrichum*, *Diaporthe, Epicoccum*, and *Neofusicoccum*.

The studies of Carvalho et al. (2012) and Noriler et al. (2018) also support the richness of endophytic fungi diversity in the Cerrado region, with an emphasis on genera such as *Diaporthe*, which are associated with medicinally relevant plants such as *Campomanesia xanthocarpa* and *Stryphnodendron adstringens.* These fungi not only contribute to the biodiversity of the region but also demonstrate significant potential in the production of bioactive compounds, as observed in studies that evaluated antimicrobial, anti-inflammatory, and cytotoxic activities.

The ability of endophytic fungi to produce secondary metabolites has important implications for both the Cerrado ecosystem and biotechnology and the search for new drugs. The interaction of these fungi with their host plants influences the profile of metabolites produced, which can result in the synthesis of plant-specific compounds. This phenomenon not only contributes to the rich chemical diversity of the region but also offers exciting prospects in the discovery of new substances with potential antibacterial, anti-inflammatory, and anticancer properties.

The diversity of genera of endophytic fungi found, such as *Colletotrichum*, *Diaporthe*, *Epicoccum*, and *Neofusicoccum*, suggests that these fungi play an important role in the Cerrado. They occupy niches similar to those of phytopathogens, which may make them effective in biological control through competition for resources, production of antagonistic substances, mycoparasitism, and induction of resistance in host plants. In addition, these fungi can improve plant growth, nutrient absorption, and the ability to resist environmental and biotic stresses (Banerjee, 2011; Canuto et al., 2012; Orlandelli et al., 2012; Gouda et al., 2016; Baron and Rigobelo, 2021).

Regarding specific genera of endophytic fungi in the Cerrado, *Diaporthe* and *Epicoccum* show remarkable potential in the production of bioactive substances. The compounds isolated from these fungi, such as Diaporthein, Phomosines, Orthosporin, Emodin, Mycoepoxydiene, β-glucans, Dicerandrol, Benzene ethanol, and Cordisinine, illustrate the diversity of secondary metabolites that can be obtained from these fungi. These substances have been associated with antitubercular, antibacterial, antioxidant, and antifungal activities, demonstrating the therapeutic potential of these fungi and their ability to contribute to the development of new drugs (Medeiros et al., 2018; Bharti et al., 2023; Elkhateeb and Daba, 2019).

The ability of fungi such as *Epicoccum* sp. to inhibit the growth of various phytopathogenic fungi, such as *Botrytis cinerea*, *Claviceps africana*, *Pythium* spp., *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*, highlights their potential in biological control of agricultural pests, which is particularly relevant for agriculture in the Cerrado region.

Records of *Neofusicoccum* as endophytes in Cerrado plants were found by Carvalho et al. (2012) and Noriler et al. (2018) in association with *S. adstringens*. Extracts of *Neofusicoccum brasiliense* have demonstrated activity against methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Candida albicans* (Carvalho et al., 2012).

5. Conclusion

In summary, endophytic fungi are crucial elements of the biodiversity of the Cerrado, contributing to the chemical and biological richness of this region. Their interactions with host plants, the production of bioactive metabolites, and the potential for pathogen control offer opportunities for research and diverse applications. Therefore, the study of these fungi in the Cerrado is essential for understanding and preserving this biome, while also opening up new perspectives for biotechnology, as the genera found have been described as producers of substances with bactericidal, fungicidal, and antitumoral potential. The knowledge generated by these studies not only expands our understanding of biodiversity but also points to practical applications that can benefit both the environment and society as a whole.

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