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Diversidade e Potencial Biotecnológico de Fungos Endofíticos Isolados do Pantanal

Diversity and Biotechnological Potential of Endophytic Fungi Isolated from the Pantanal

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RESUMO

O Pantanal é um patrimônio natural que abriga diversas espécies, incluindo fungos endofíticos produtores de compostos bioativos. Este estudo buscou isolar e caracterizar fenotipicamente fungos endofíticos de plantas do Pantanal Sul-mato-grossense, avaliando os compostos produzidos. Para o isolamento, amostras foram higienizadas e fragmentos de plantas foram cultivados em meio de Agar Batata, incubados a 30ºC. Após o crescimento, colônias foram repicadas para obter culturas puras. Os isolados foram caracterizados visualmente em placas, tanto na frente quanto no verso. Para verificar a produção de pigmentos, os fungos foram cultivados em meio Batata-Dextrose e incubados a 5ºC após o crescimento a 30ºC. A atividade enzimática foi avaliada pela formação de halos em meios específicos para cada enzima. Ao todo, foram isolados 16 fungos, dos quais 5 apresentaram características fenotípicas distintas, e dois produziram pigmentos nas cores rosa e verde. Os isolados demonstraram atividade enzimática para celulase, indicando potencial biotecnológico. Este estudo destaca a importância de preservar o Pantanal, que funciona como um reservatório natural de recursos com potencial para diversas aplicações.

Palavras-chave: Biotecnologia. Biomoléculas. Preservação ambiental.

ABSTRACT

The Pantanal is a natural heritage site that is home to various species, including endophytic fungi that produce bioactive compounds. This study aimed to isolate and phenotypically characterize endophytic fungi from plants in the Pantanal region of Mato Grosso do Sul, evaluating the compounds produced. For isolation, samples were sanitized and plant fragments were grown on Potato Dextrose Agar medium, incubated at 30ºC. After growth, colonies were picked to obtain pure cultures. The isolates were visually characterized on plates, both on the front and reverse sides. To verify pigment production, the fungi were grown in Potato Dextrose medium and incubated at 5ºC after growth at 30ºC. Enzymatic activity was assessed by the formation of halos in specific media for each enzyme. In total, 16 fungi were isolated, of which 5 presented distinct phenotypic characteristics, and two produced pigments in pink and green colors. The isolates demonstrated enzymatic activity for cellulase, indicating biotechnological potential. This study highlights the importance of preserving the Pantanal, which functions as a natural reservoir of resources with potential for various applications.

Keywords: Biotechnology. Biomolecules. Environmental preservation.

1. INTRODUCTION

The Pantanal is a unique biome on the planet, with an estimated area of approximately 179,330 km². It is located in south-central Latin America and covers parts of three countries: Brazil (78% of its area), Bolivia (18%), and Paraguay (4%). In Brazil, it is distributed between the states of Mato Grosso and Mato Grosso do Sul (TOMAS et al., 2019). The main characteristic of this biome is the alternation between periods of flood and drought, which determines the region's seasonality and directly influences the local fauna and flora. This phenomenon, known as the flood pulse, regulates the ecosystem and affects nutrient cycling, in addition to impacting the biological communities that live there (IVORY et al., 2019).

The Pantanal's biodiversity is one of the richest on the planet. According to the Ministério do Meio Ambiente (MMA, 2022), this biodiversity includes more than 1,600 species of plants, 113 of reptiles, 263 of fish, 41 of amphibians, 463 of birds, and 132 of mammals. Furthermore, the biome is home to a large number of invertebrates and microorganisms, which, despite their ecological importance, are still little studied and cataloged in the scientific literature (TOMAS et al., 2019). The Pantanal is considered the largest floodplain in the world, and around 80% of its native vegetation is still preserved (ALHO et al., 2019). However, the ecological interactions of this region are quite fragile and have been threatened by the increasing expansion of human activities, such as agriculture, livestock, and fires (MARQUES et al., 2021).

The vast biodiversity of the Pantanal, which includes countless species of animals, plants, and microorganisms, carries with it an incalculable genetic value. Many of these organisms produce unique chemical compounds, which are still little known and have great potential for biotechnological applications, especially in the development of new products and medications (ASSAD et al., 2021). Living organisms that inhabit this biome have developed complex biological mechanisms to survive adverse conditions (SILVA et al., 2023). These mechanisms include the production of chemical substances that may be of great interest to science and industry. In this scenario, endophytic fungi stand out, living inside plant tissues without causing damage to the plants, and are little studied, especially in tropical ecosystems such as the Pantanal (COERTJENS; DO SOCORRO MASCARENHAS; BATISTOTE, 2024).

Recent studies indicate that the endophytic fungal community in regions such as the Cerrado and the Pantanal is highly diverse and has enormous biotechnological value, but is still little explored. The great diversity of endemic plant species in these regions contributes to the richness of the endophytic fungal community. The lack of large-scale research hampers a deeper understanding of its biodiversity, ecological relevance, and high biotechnological potential. (DEVI et al., 2023; NORILER et al., 2018).

Endophytic fungi are highly relevant microorganisms, capable of producing bioactive molecules with potential applications in several areas, such as pharmacology, industry, health, and bioremediation processes, among others. These bioactive molecules result from the unique interaction between fungi and their host plants, where endophytic fungi colonize plant tissues without causing damage; on the contrary, they can offer significant benefits. This symbiosis results in the production of specific chemical compounds that not only help plants defend themselves against pathogens but also increase their resistance to abiotic stress conditions, such as high temperatures and water shortages, which are essential for survival in extreme environments (FONTANA et al., 2021; SANTOS et al., 2021).

The production of secondary metabolites by endophytic fungi is vast and diversified, leading them to be considered true natural “factories” or “biofarms” of new compounds. Among these metabolites are steroids, alkaloids, phenols, flavonoids, isocoumarins, xanthones, quinones, and terpenoids, which present promising pharmacological properties (ADELEKE; BABALOLA, 2021). Studies highlight that these substances have antimicrobial, antifungal, antiparasitic, anticancer, and antiviral activities, expanding their potential for use in medicine to combat various diseases and in the development of new drugs and treatments (MANGANYI; ATEBA, 2020).

Biotechnology has expanded its focus on endophytic fungi, leading to advances in the exploration of highly efficient enzymes, which are of interest to sectors such as food, textiles, pharmaceuticals, and biofuels. The enzymes derived from these fungi present a combination of essential attributes for the industry: they are produced in large quantities and at low cost, have high stability, and are easy to recover since the fungi grow on economical and abundant substrates (BHADRA et al., 2022; EL-GENDI et al., 2021). These characteristics make endophytic fungi a sustainable source for industrial-scale enzyme production, offering alternatives for more efficient processes aligned with the demands for less environmental impact (DINAKARKUMAR et al., 2024).

Endophytic fungi, in addition to producing enzymes and medicines, show promise in bioremediation. The substances they produce can degrade complex pollutants, helping to recover contaminated environments, such as polluted soils and waters (SHARMA; KUMAR, 2021). This alternative is low-cost and ecologically sustainable, as it uses natural biological agents instead of conventional chemical treatments that are often harmful to the environment. Thus, the biodiversity of endophytic fungi is a source of bioactive resources with wide applicability, contributing to sustainable practices in environmental remediation and conservation of natural ecosystems (YADAV et al., 2022).

Exploiting these biotechnological capabilities can significantly boost advances in sectors such as environmental technologies and the bioeconomy, highlighting the need for in-depth studies on the biodiversity and ecological role of these microorganisms. In tropical biomes such as the Pantanal, estimates of species richness and their ecological relevance are still incomplete, which limits a full understanding of the value of these fungi for science and technology (BERTAZZO-SILVA et al., 2022; FEITOSA et al., 2022). This gap reinforces the importance of detailed investigations into these microorganisms and their interactions with the environment. In this sense, the study aims to isolate and phenotypically characterize endophytic fungi from different plants in the Pantanal Sul-Mato-Grossense, as well as to investigate the possible compounds produced by these microorganisms.

2. MATERIALS AND METHODS

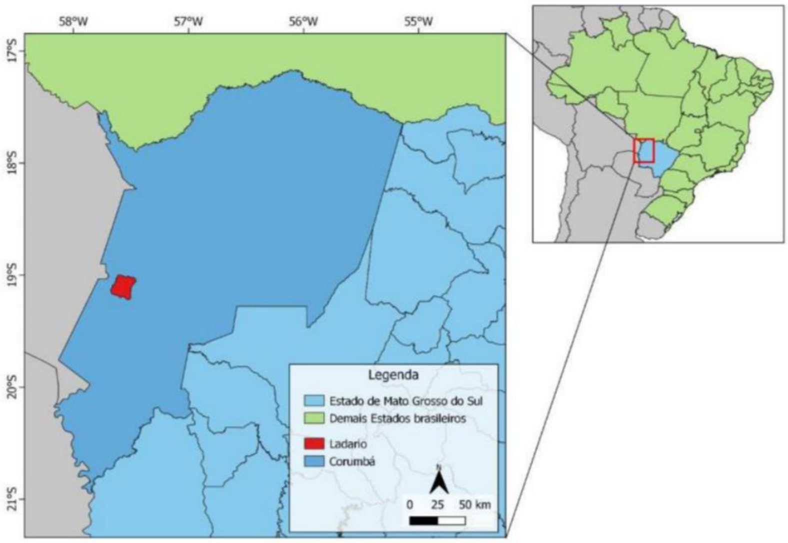
Location of study development

The study was conducted at the Laboratório de Biotecnologia, Bioquímica e Biotransformação, part of the Centro de Estudo em Recursos Naturais - CERNA of the Universidade Estadual de Mato Grosso do Sul, Dourados/MS.

Plant material collection area

The area where the plant material was collected is the Área de Proteção Ambiental Baía Negra (UC APA Baía Negra), located in the municipality of Ladário in Pantanal Sul-Mato-Grossense. The APA Baía Negra covers approximately 6,000 hectares and is situated at the coordinates: 19°01’18.8” S and 57°30’39.3” W (DA SILVA-MELO; DE MELO; GUEDES, 2019), with the region's altitude ranging from 80 to 1060 meters (SOUZA et al., 2019). The Pantanal falls under the Aw climate classification, characterized as tropical, according to the Köppen classification, with an average temperature of approximately 26ºC and annual precipitation of 1,184.3mm, with drier months from April to September and the rainiest from October to March according to Soriano et al. (2020). Figure 1 shows the location map of Área de Preservação Ambiental Baía Negra.

Figure 1. Map of the collection area for plant material in the Área de Preservação Ambiental Baía Negra.



Source: Adapted from Benites et al. (2022).

The sampling points were located in the municipality of Ladário, in Unidade de Conservação Áreas de Proteção Ambiental Baía Negra. The plant material was collected, identified, packed in paper bags, and transported to the Laboratório de Biotecnologia, Bioquímica e Biotransformação. In the laboratory, the plant material was washed under running water to remove excess soil and dust. Then, leaves, flowers, and seeds were separated and disinfected with detergent to eliminate impurities, then rinsed in running distilled water. Subsequently, the samples were immersed in 70% alcohol ($v.v^{-1}$) for 1 minute, in 2.0% sodium hypochlorite for 2 minutes, and finally washed three consecutive times with sterile distilled water for 1 minute each. The samples were then placed on paper towels to remove excess water, following the methodology described by Araújo (2002).

The fragmentation of the vegetable material was performed using a sterile scalpel blade. The fragments were cut to approximately 1 cm² and, using sterile tweezers, three fungal fragments were distributed in Petri dishes containing Potato-Dextrose-Agar culture medium (PDA). The medium was autoclaved for 20 minutes at 120°C and supplemented with the antibiotic chloramphenicol at a concentration of 50 mg/L to inhibit bacterial growth. The Petri dishes were incubated at 30°C and observed daily for up to 10 days. The emerging colonies from the fragments were inoculated again in Petri dishes containing PDA medium and incubated again at 30°C for 7 days. This procedure was repeated until pure colonies were obtained. Pure cultures were placed in slant tubes on PDA solid medium for short-term preservation with 20% glycerol at -20°C. Sixteen fungi were isolated from various parts of plant materials. The selection criteria included those that exhibited striking and differentiated phenotypic characteristics, such as greater spore formation, rapid growth, and the production of more expressive colors in the culture medium during preliminary studies.

Determination of phenotypic characteristics

To determine the macro and micro morphological characteristics of the endophytic isolates, a fungal fragment was inoculated into Petri dishes containing Potato-Dextrose-Agar (PDA) culture medium, previously autoclaved for 20 min at 120ºC. The plates were incubated at 30°C and observed until mycelium development.

The characterization was performed through macroscopic and microscopic analysis of the morphological profile of the isolates. Macroscopically, characteristics such as color, texture, surface, presence of diffuse pigments, and the appearance of the reverse of the colony were evaluated. For microscopic analysis, a fragment of the colony was removed and placed on a slide for evaluation. The structures were observed using an optical microscope, paying attention to the septations of the hyphae according to the methodology of Soares et al., (1987).

Growth and production of bioactive compounds

In the growth profile, the endophytic isolates were cultivated in the Potato Dextrose Agar culture medium, which was diluted in distilled water and sterilized in an autoclave at 120°C for 20 min. Subsequently, the medium was distributed in Petri dishes, and with the aid of tweezers, fragments of each isolate were placed on the plate and incubated at 30ºC. Cell growth was monitored daily by visual observation. For the selection of endophytic fungi with evidence of pigment production, after the growth period of the samples, the plates were incubated at a temperature of 5°C for approximately 10 days. Pigment production occurred through diffusion in the culture medium, being evaluated macroscopically by observing both the upper and reverse surfaces of the colonies according to the methodology adapted by Pandey et al. (2018). Most fungi have great potential for enzyme production, and the use of tests in solid medium facilitates the screening of these enzymatic activities. The fungi were cultured and grown in Petri dishes in Potato Dextrose Agar medium, and with the aid of a scalpel, the fragments were cut and used in the respective enzymatic media. The analyses were visual, with or without halo formation.

Fragments of the fungi were transferred, with the aid of tweezers, to Petri dishes containing sterile Carboxymethylcellulose synthetic medium (CMC) and incubated at 28°C. To evaluate enzyme production, 10 mL of a Congo red developer solution (2.5 $g.L^{-1}$) in Tris-HCl buffer (0.1 $mol.L^{-1}$, pH 8.0) were added, and washed with 5 mL of NaCl solution (0.5 $mol.L^{-1}$). Enzymatic activity was visually identified by the presence of halos around the colonies, following the methodology adapted according to Bortolazzo, 2011.

To detect amylolytic activity, a 2% starch medium was used, composed of $NaNO_3$ (1.0 $g.L^{-1}$), KH_2PO_4 (1.0 $g.L^{-1}$), $MgSO_4$ (0.5 $g.L^{-1}$), $FeSO_4$ (0.01 $g.L^{-1}$), soluble starch (20.0 $g.L^{-1}$), Agar (20.0 $g.L^{-1}$), adjusted to pH 5. Fragments of the fungi were added to the medium with the aid of sterile tweezers and incubated at 28°C for 72 hours. To evaluate the enzyme activity, an iodine solution (1% in 2% potassium iodide) was added to stain for 10 min, then washed with 1M NaCl solution. The formation of a degradation halo was visually observed, appearing as a clear zone around the colony, according to the methodology.

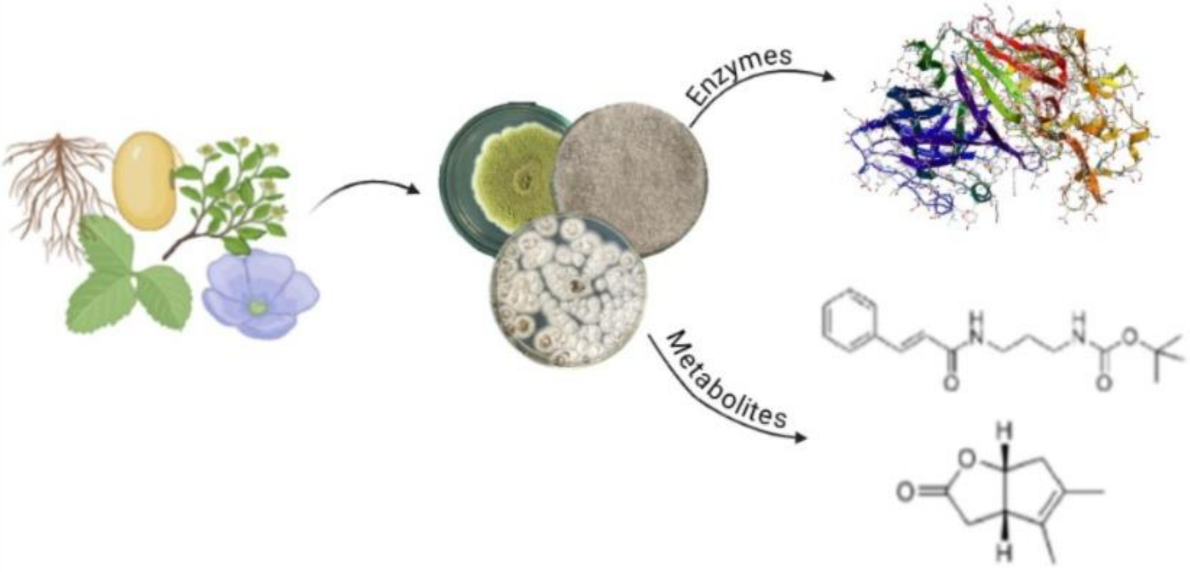
The evaluation of lipolytic activity was performed using the medium containing: $MgSO_4 \cdot 7H_2O$ (0.2 $g \cdot L^{-1}$), K_2HPO_4 (0.7 $g \cdot L^{-1}$), KH_2PO_4 (0.4 $g \cdot L^{-1}$), yeast extract (2.0 $g \cdot L^{-1}$), agar (15 $g \cdot L^{-1}$), Rhodamine B (0.001% $m.v^{-1}$), olive oil (1.0% $v.v^{-1}$) and adjusted pH to 6.5, sterile. The fragments were added to the medium using sterile tweezers, and the samples were incubated at 28°C. The results were considered positive when the presence of a fluorescence halo was detected under ultraviolet irradiation, according to methodology adapted from Kouker and Jaeger (1987).

In the evaluation of protease enzyme activity, fragments of the fungi were transferred with the aid of tweezers to Petri dishes containing the medium composed of powdered milk (5.0 $g \cdot L^{-1}$), gelatin (10 $g \cdot L^{-1}$) and agar (18 $g \cdot L^{-1}$), sterile. The samples were incubated for seven days at 28°C. Enzymatic activity was observed visually by the formation of a transparent zone around the colonies, according to methodology (RODARTE et al. 2011; KRISHNA et al. 2011).

3. RESULTS AND DISCUSSION

Biodiversity is defined as the diversity among living beings, including genetic variation, the number of species, and the differences between the ecosystems that house them. In this study, 16 endophytic fungi were isolated from different parts of the plant tissue, some of which showed the production of interesting compounds. Studies investigating the interaction between endophytes and plants represent an area with great potential to be explored, as these microorganisms inhabit different parts of plants and produce a variety of valuable compounds. Figure 2 illustrates the parts of plants, such as seeds, roots, leaves, branches, flowers, and fruits that harbor a vast universe of microorganisms, including endophytic fungi, and their applications.

Figure 2. Parts of plants that may contain endophytic fungi and produce bioactive compounds.

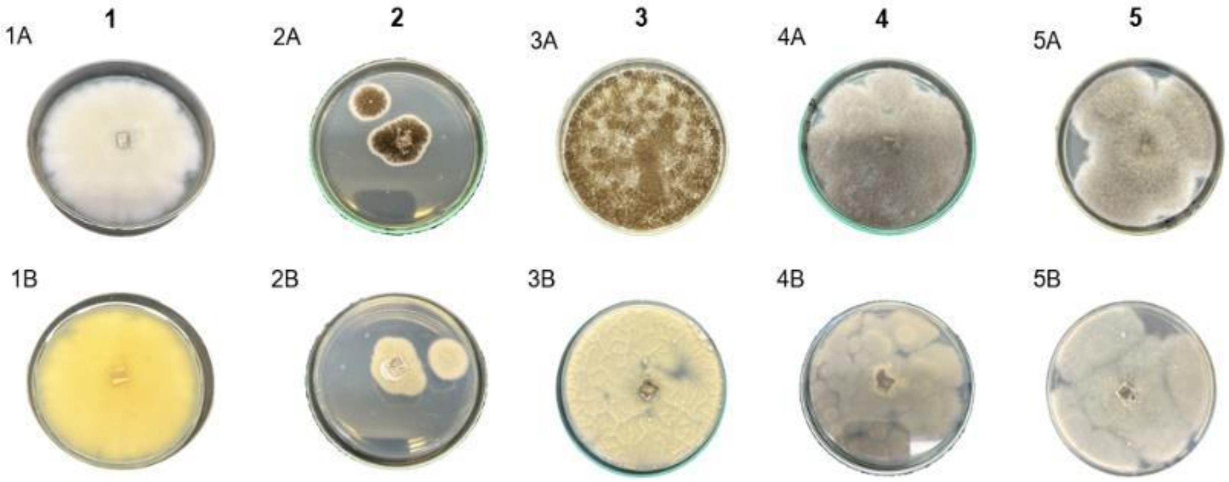


Source: Prepared by the authors.

Figure 3 shows the phenotype of pure colonies of endophytic fungi that were isolated from the fragmentation of plant tissue from leaves, flowers, and fruits. The process of purifying isolates is essential for species identification. Endophytic fungi were cultivated in Potato Dextrose Agar (PDA) culture medium at a temperature of 30°C. The images show the macroscopic morphology of the colonies (1A-5A) and the appearance of the reverse side of the colonies (1B-5B).

Studies show that after the development of fungal colonies, the isolates are purified and identified through morphological or molecular analyses, as described by Pietro-Souza et al. (2017) and Yao et al. (2017). Isolation and cultivation methodologies, although they do not fully represent the real diversity of species, are essential for obtaining new isolates (NORILER et al. 2018). Studies such as the one carried out with Catharanthus roseus, a medicinal plant from the Apocynaceae family, demonstrate the great potential of plants as hosts for endophytic fungi. In these studies, 20 endophytic fungi were isolated in Potato-Dextrose Agar medium, and the isolates were grouped based on their macro and micro morphological characteristics (DHAYANITHY; SUBBAN; CHELLIAH, 2019).

Figure 3. Phenotypic characteristics front (A) and reverse side (B) of endophytic fungal isolates purified and cultivated in Petri dishes containing Potato Dextrose Agar medium.



Source: Prepared by the authors.

Endophytes can be found in all parts of the plant, such as stems, petioles, leaves, fruits, buds, and occasionally, in the inflorescences. A large number of fungal endophytes inhabit these areas, playing a significant role in the plant's life cycle (RANA et al. 2019). These endophytes have also been isolated from plants growing under extreme conditions. Their enormous diversity may be related to the beneficial role they play in the ecosystem and its processes (SAMPANGI-RAMAIAH et al. 2020).

The isolation of endophytic fungi from different parts of plants is a widely known practice, and all plants harbor an immense universe of endophytes. However, this microbiota varies significantly between different plant species and between different parts of the plant itself (OMOMOWO and BABALOLA, 2019). Despite the estimate of approximately one million endophytic species associated with different plants worldwide, only a small percentage of these endophytes have been studied so far (GRABKA et al., 2022). During plant stress conditions, endophytes secrete numerous secondary metabolites into plant cells, which are incorporated into the stressed pathways (KOZA et al., 2022). This highlights the vast unexplored potential of these microorganisms, especially in terms of their metabolic capabilities and biotechnological applications.

The Pantanal has significant potential in terms of biodiversity of microorganisms, which is still little known and explored, including endophytes, which are microorganisms that are associated inside plants without causing harm. These Pantanal endophytes are rarely studied; they constitute important repertoires for the development of strategies aimed at the search for new drugs and bioproducts for the most diverse areas (ASSAD et al., 2022).

The analysis of endophytic fungal isolates from Pantanal plants in relation to the production of diffuse pigments demonstrated that of a total of five fungal isolates obtained, only two presented colonies with green and pink coloration when cultivated in Petri dishes containing Potato Dextrose Agar medium, subjected to different growth times (Table 1). Endophytic fungi have aroused interest in the biotechnology field due to their extraordinary ability to produce secondary metabolites with interesting properties. Among the natural bioresources available for pigment production, endophytic fungi are suitable sources for the production of numerous bioactive molecules.

Table 1. Observation of pigment production and absence in a Petri dish

Isolates	Pigment production	Color observed in the Petri dish	Growth Time
1	-	-	6 days
2	-	-	4 days
3	+	Green	3 days
4	+	Pink	3 days
5	-	-	3 days

Source: Prepared with research data.

The production of pigments from fungi is of industrial interest due to its easy cultivation, simple extraction process, and high yield (VALENZUELA-GLORIA et al., 2021). Fungi survive in a variety of conditions, including extreme ones, such as temperatures that can vary from 5 to 3ºC, exposure to high levels of light, such as ultraviolet rays, and low nutrient availability. These microorganisms adapt to these environmental challenges by seeking new sophisticated survival strategies, such as secreting bioactive pigments (MARGESIN and COLLINS, 2019; PANDEY et al., 2019; SAJJAD et al., 2020). Among these adverse conditions, the Pantanal biome presents ideal conditions with its unique characteristics of droughts and floods. In this sense, the Pantanal microbiota is a valuable natural resource of bioactive molecules to be explored.

The production of compounds and regulation of fungal secondary metabolism is a complex process that depends on an intricate network of cellular determinants. Unexplored habitats may contain new endophyte isolates as a promising source of yet-to-be-discovered metabolites, as well as the potential for enzyme production. Analysis of the results reveals that only cellulase activity was significant, while lipase, amylase, and protease activities were not detected. The diameter of the enzymatic degradation halo measures the ability of isolates to secrete enzymes capable of degrading specific substrates, and larger values suggest more efficient enzyme production. Isolate 3 showed the largest degradation halo for cellulase (55.00 ± 4.36 mm), followed by isolates 5 (52.77 ± 2.54 mm) and 4 (48.33 ± 2.89 mm). These values suggest a relatively stable enzymatic efficiency among the samples analyzed.

Table 2. Evaluation of the diameter of the enzymatic degradation halo of the studied isolates.

Isolates	Halo diameter (mm)			
	Cellulase	Lipase	Amylase	Protease
1	31.00 ± 1.00	0	0	0
2	30.00 ± 0.00.	0	0	0
3	55.00 ± 4.36	0	0	0
4	48.33 ± 2.89	0	0	0
5	52.77 ± 2.54	0	0	0

Source: Prepared with research data.

Natural bioactive compounds with therapeutic properties are promising candidates for drug development and can contribute to the treatment and effective management of various diseases. Endophytic fungi have shown promising potential for the production of metabolites with therapeutic properties (HASHEM et al., 2022), and these microorganisms present a promising source for the production of numerous compounds yet to be explored. Amylase, cellulase, lipase, and laccase are important enzymes that have significant industrial applications, and endophytes play a role in their synthesis (SHARMA; DHAR; KAUL, 2023).

The therapeutic potential of endophytes and their metabolites as biotherapeutic agents has aroused much interest today (XIAO et al. 2022). Good health and well-being are included in target 17 of the United Nations Sustainable Development Goals (SDGs), which is intended to be achieved by 2030 (ZORZO et al., 2022).

4. FINAL CONSIDERATIONS

In the present study, 16 endophytic fungi were isolated from different parts of plant material, such as leaves, fruits, and seeds. However, only five pure isolates with diverse phenotypic characteristics were obtained.

Screening of pigment production by endophytic fungi showed the potential for pigment production in two isolates, revealing that the Pantanal could be a promising source of bioactive molecules to be explored.

The evaluation of the enzymatic activity of the endophytic isolates revealed their capacity to degrade cellulose, indicating the production of highly stable extracellular cellulolytic enzymes with great potential for applications in the bioconversion industry.

This study highlighted the Pantanal as a natural reservoir of microorganisms that have not yet been explored, and its preservation is vital for the conservation of biodiversity and for obtaining bioactive compounds. Therefore, it is essential to carry out further investigations on these fungal isolates to take advantage of their biotechnological potential.

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