An Acad Bras Cienc (2025) 97(4): e20241100 DOI 10.1590/0001-3765202520241100
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

MICROBIOLOGY

Macrofungi with Potential for Bioremediation of the Herbicide Atrazine

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Abstract: Atrazine is used widely corn cultivation, providing as an effective and low-cost control of broadleaf and grassy weeds. However, this herbicide exhibits high persistence in the soil and can potentially interfere with the photosynthesis of non-target plants. Scientific literature has demonstrated that macrofungi offer several advantages for use in the bioremediation of environmental contaminants, mainly due to their ability to withstand stressful conditions and produce extracellular enzymes with low specificity that may be involved in the biodegradation process. This study evaluated the ability of macrofungal species to tolerate the herbicide atrazine and produce laccase, an enzyme capable of degrading xenobiotics. To achieve this, tolerance assays of fungi to atrazine were performed to assess growth rate and mycelial growth inhibition, as well as analysis of laccase enzymatic activity through the oxidation of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) at 420 nm using a spectrophotometer. The results demonstrated that all four analyzed macrofungi were tolerant to the herbicide atrazine. However, *Lentinus crinitus* SA37 stood out, due to its low mycelial growth inhibition rate and laccase production. This research enabled the selection of *L. crinitus* SA37 for future studies on the biodegradation and mineralization of atrazine.

Key words: Basidiomycetes, decontamination, Laccase, tolerance, triazine.

INTRODUCTION

In Brazil, agriculture is the country's most significant economic activity. However, growth of this sectoral has been closely linked to the increased use of agricultural chemicals and, globally Brazil currently leads both in consumption and importation of such products (Mattei & Michellon 2021). Among pesticides, the use of herbicides has substantially contributed to crop-raising success, ensuring that cultivated plants have uninterrupted access to the resources necessary for their development (Salomão et al. 2020, Toller et al. 2021, Ribeiro et al. 2022).

Atrazine (6-chloro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4-diamine) is the second most used herbicide, both in Brazil and worldwide, being both cheap and effective, so making it a highly practical choice. The Brazilian National Health Surveillance Agency (ANVISA) classified this herbicide as class III in terms of toxicity and a class II environmental hazard. Consequently, its intensive use has, justifiably, raised concerns regarding its adverse environmental impacts, as levels above the maximum allowed for extended periods can result in complications in organisms such as mammals, birds, fish, and invertebrates (Usepa 2001, 2018, Embrapa 2011, de Albuquerque et al. 2020).

Due to its solubility in water, atrazine has a high potential for soil contamination, as well as for groundwater. As a result, this herbicide has been banned in a variety countries due to its extended, multi-year, persistence in aqueous

surfaces. It is characterized as a compound with high environmental persistence, with reports of half-life intervals ranging from 10 days to 15 years (Martinazzo 2010, Peruzzo et al. 2020). In the eastern region of Mato Grosso state, Brazil, an area known for high agricultural productivity, there are reports of atrazine residues and its metabolite desethylatrazine in water contamination studies (Dores et al. 2006), highlighting the need for studies on environmental contamination by herbicides in this region.

In such circumstances, bioremediation emerges as a promising solution to reduce the impacts caused by the use of atrazine. This process can occur naturally through the action of bacteria-, fungi-, and plant-produced enzymes, which, through metabolic processes, utilize these contaminants as a source of carbon and energy (Mallmann et al. 2019). Furthermore, unlike physical or chemical approaches, bioremediation is considered more economical and environmentally friendly, as it takes advantage of natural degradation processes (Fernandes & Silva 2021).

Fungi are ideal candidates for the remediation of a wide range of pollutants as they show rapid growth, posesses a vast network of hyphae that gives a high surface area to volume ratio, show resistance to xenobiotics and heavy metals, as well as adaptability to fluctuating pH and temperature, and the ability to produce extracellular and intracellular ligninolytic enzymes, which facilitate the degradation of recalcitrant substances by promoting the cleavage of aromatic rings and destabilizing the chemical bonds present in xenobiotics (Bhattacharya & Das 2011, da Silva Patrício et al. 2021). Among these organisms, macrofungi stand out, as they play a crucial role in the preservation of ecosystems and are fundamental in the production of biomass (Figueiredo et al. 2020, Mendoza et al. 2023). Macrofungi Phyla comprise two phyla, Ascomycota and Basidiomycota characterized by the form of their spore-production structures, known as ascoma or basidioma, respectively.

Several studies have highlighted the capacity of ligninolytic fungi to degrade key xenobiotics, including certain herbicides, due to the action of extracellular oxidative enzymes, such as laccase, lignin peroxidase (LiP), and manganese peroxidase (MnP). Additionally, cytochrome P450 is known to be both an important intracellular enzyme and involved in the biodegradation of xenobiotics (Lemos et al. 2008). Together, these enzymes can contribute to the detoxification of contaminated environments, and the reduction of environmental impacts associated with many environmental contaminants (Rodríguez et al. 2022).

Initial biological assays to evaluate the potential for macrofungal bioremediation can be assessed through a variety of in vitro tests, including: (i) analysis of the Bavendamm reaction by the oxidation of gallic acid or guaiacol (Cavalcante et al. 2023, Pesenti et al. 2023), (ii) tests for the decolorization of anthraquinone dyes such as Remazol Brilliant Blue R (RBBR) (Silva et al. 2017, Vasconcelos 2010), and (iii) tests for tolerance to the environmental contaminant of interest (Colla et al. 2008, Argumedo-Delira et al. 2012, Lee et al. 2020, Toller et al. 2021). It is important to emphasize that an ability to tolerate the presence of herbicides does not mean that the fungus is directly degrading the compound, but rather that it can maintain its vital functions and grow even under adverse conditions. However, this tolerance is a primary indicator of the fungus's potential to excrete extracellular enzymes involved in the degradation of recalcitrant molecules (Schneider et al. 2021, da Silva & Moreira 2024). Studies on the production of ligninolytic enzymes in the presence of the

contaminant of interest can also provide an indication of the fungi's capacity to degrade xenobiotics (Bail 2020, Pacheco & Santos 2022).

Thus, identifying herbicide-tolerant fungi and conducting an analysis of extracellular enzyme activity form vital and important steps for selecting candidate species for bioremediation, as such organisms are more likely to contribute effectively to the degradation and neutralization of these contaminants. In this context, the current study aims to evaluate the ability of macrofungi species to tolerate the herbicide atrazine, as well as to produce laccase, with the goal of selecting strains that demonstrate potential for use in new studies on the bioremediation of soils historically contaminated with herbicides.

MATERIALS AND METHODS

Macrofungal Strains

The macrofungi species used in this study were: *Phanerochaete australis* SA18, *Polyporus* sp. SA23, *Lentinus crinitus* SA37, and *Hypoxylon fendleri* SA41 (Figure 1a, b, c, d, respectively), with their mycelia isolated and purified from material collection in at the Serra das Araras Ecological Station - EESA (15°39' S 57°1' W), a fully protected conservation unit located in the southwestern region of Mato Grosso State, Brazil. These species were pre-selected for their ability to produce phenoloxidases, as indicated by tests for gallic acid oxidation capacity or the ability to decolorize the dye Remazol Brilliant Blue R (RBBR) (following de Souza et al. 2022) (Table I). The mycelia of the macrofungi used in this study are stored in the Mycological Collection of the Microbial Biotechnology Laboratory (LBM) at the Center for Agro-Environmental Research, Studies, and Development (CPEDA) of the Universidade do Estado de Mato Grosso (UNEMAT), Tangará da Serra, Campus "Eugênio Carlos Stieler".

Atrazine herbicide tolerance assay

The experimental design used was completely randomized with a 4x4 factorial scheme in triplicate, with the factors being: fungal species (*P. australis* SA18, *Polyporus* sp. SA23, *L. crinitus* SA37, and *H. fendleri* SA41) and different atrazine concentrations (0 µg/mL, 1000 µg/mL, 3000 µg/mL, and 5000 µg/mL). Initially, the macrofungi species were reactivated in 2% Malt Extract Agar (MEA) (20 g/L Malt Extract; 15 g/L Agar) and incubated in an incubator for 10 days at 28°C. An atrazine solution (Sigma Aldrich, 334343, 98%) was prepared with a concentration of 10 mg/mL and solubilized in ethyl acetate.

Before starting the assay, Petri dishes containing 0.2% MEA medium (2 g/L Malt Extract; 15 g/L Agar) were superficially contaminated with atrazine aliquots at concentrations of 1000

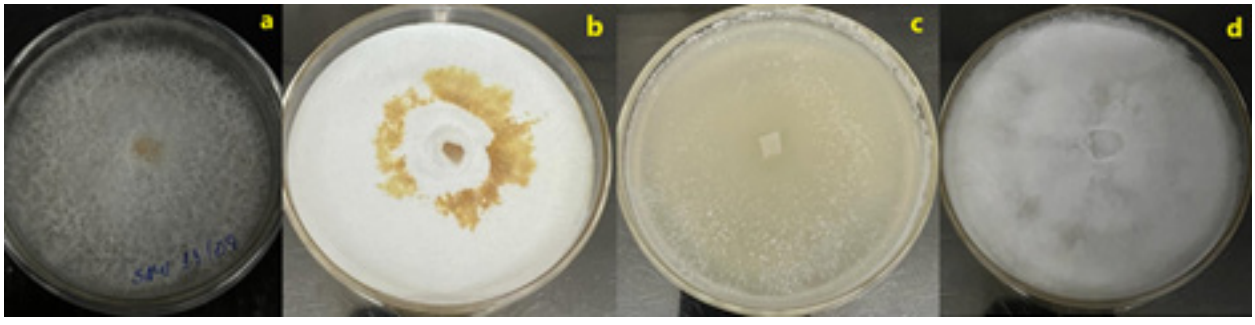

Figure 1. ***Polyporus sp.*** **SA23 (a),** ***Lentinus crinitus*** **SA37 (b),** ***Phanerochaete australis*** **SA18 (c),** ***and Hypoxylon fendleri*** **SA41 (d), grown in MEA 2%.**

µg/mL, 3000 µg/mL, and 5000 µg/mL, using a Drigalski spatula, in triplicate. The concentrations used have values 10 times smaller than that normally used in this type of study (2.5 kg of active ingredient for 150 L/ha) since the various factors that contribute to herbicide loss were considered, from the moment of application until its arrival in the soil and contact with microorganisms. These include: degradation (physical, chemical or biological), leaching, drift and volatilization.

After the incubation period of the fungi, culture discs (8 mm) were taken from the edges of the colonies and transferred to the center of the plates containing the different atrazine concentrations and the control group, which was not contaminated with the herbicide. The assay was conducted with incubation at 28°C for 4 days. Radial mycelial growth was measured daily using a millimeter ruler.

The fungal growth rate (FG) and fungal growth inhibition (IFG) were estimated as follows: FG (%) = (DA / DC) x 100, where the diameter of the mycelial colony exposed to atrazine (DA) was divided by the diameter of the mycelial colony of the control group (DC); and IFG (%) = 100 – FG, calculated from the fungal growth rate (FG) results above (Argumedo-Delira et al. 2012). The data obtained were subjected to 4x4 factorial analysis followed by analysis of variance (ANOVA) and Tukey's test ($p<0.05$) using Assistat Software® Version 7.7.

Laccase enzymatic activity analysis

For the evaluation of laccase enzymatic activity, three 8 mm discs from each macrofungus culture were inoculated in Erlenmeyer flasks containing 50 mL of 2% Malt Extract broth (20 g/L Malt Extract), in triplicate. The flasks were then placed in an incubator with shaking at 28°C and 150 rpm for 48 hours. Immediately following this, the flasks were contaminated with atrazine at concentrations of 1000 µg/mL and 5000 µg/mL, separately and for each fungus, in triplicate. The uncontaminated flasks comprised the control group, and all were maintained under the same incubation conditions mentioned above for an additional three days. After this, vacuum filtration of the flask contents was performed, followed by centrifugation of the enzymatic extract at a speed of 7830 rpm at 4°C for 30 minutes (NT 816 Novatecnica). The supernatant obtained in this step was then used as the enzyme source.

Table I. Results of tests with gallic acid and decolorization of RBBR by macrofungi isolated from EESA, Mato Grosso, Brazil.

Macrofungi	GenBank Code	Gallic Acid Test		RBBR Dye	
		Results	Amber Halo	Results	Decolorization
Hypoxylon fendleri (SA41)	MN056423.1	-	-	+	***
Lentinus crinitus (SA37)	MH915574.1	+	V	+	*
Phanerochaete australis (SA18)	MH047187.1	+	M	+	***
Polyporus sp (SA23)	AF516541.1	+	V	+	***

(+) positive result; (-) negative result; (V) reddish amber halo; (M) medium amber halo. total decolorization (***); weak decolorization (*). Source: de Souza et al. (2022).

Laccase activity was assayed by the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), using the method described by Buswell et al. (1995). The reaction was performed using: 300 µL of a 0.1 M sodium acetate buffer solution (pH 5.0), 100 µL of the ABTS solution, and 600 µL of the enzyme solution, analyzed in a spectrophotometer (Bel UV-M51 UV-Visible) at 420 nm. After calibrating the equipment, the first absorbance reading was taken at the beginning of the reaction. Then, the cuvette solution was transferred to a water bath at a temperature of 37° C for 10 minutes, followed by a new reading in the spectrophotometer. The initial and final absorbance data were used to measure the ABTS oxidation process by laccase, according to the formula given below, based on Lambert-Beer's Law

$$U.L^{-1} = \Delta A \times V \times 10^6 / \varepsilon \times R \times T$$

Where: $U.L^{-1}$ = enzyme unit, that is, the amount of enzyme necessary to oxidize 1 µmol of substrate per minute/liter; ΔA = Difference between final and initial absorbance; V = Volume of the reaction (0.001 L); 10^6 = Conversion factor (ε moles to µmoles); ε = Extinction coefficient (M^{-1} cm^{-1}); R = Amount of enzyme extract (L); T = Reaction time (min). The data obtained were subjected to analysis of variance (ANOVA) ($p<0.05$) using Assistat Software® Version 7.7.

RESULTS AND DISCUSSION

The four macrofungal species evaluated in this study were tolerant to the herbicide atrazine. In the presence of 1000 µg/mL and 3000 µg/mL of atrazine mycelial radial growth rates did not differ significantly for any of the four tested macrofungal species. However, in assays with 5000 µg/mL of atrazine, growth was greater, and statistically different, for *Hypoxylon fendleri* SA41 compared to the other fungi ($p<0.05$). *Polyporus* sp. SA23 was the least tolerant at higher atrazine concentrations. Generally, radial mycelial growth decreased gradually with increasing herbicide concentration; however, for *Lentinus crinitus* SA37, there was no statistically significant difference in mycelial growth across all evaluated treatments (Table II), indicating similar growth regardless of concentration. For Fungal Growth Rate (FG), *Phanerochaete australis* SA18 showed the highest percentage increase in the presence of 1000 µg/mL of atrazine (90%), followed by *H. fendleri* SA41 (85%) and *L. crinitus* SA37 (82%). However, at concentrations of 3000 µg/mL and 5000 µg/mL of atrazine, *L. crinitus* SA37 maintained its FG (78% and 84%), followed by *H. fendleri* SA41 (70% and 55%), respectively. At atrazine concentrations of 5000 µg/mL *P. australis* SA18 showed a sharp drop in FG (25%) (Figure 1a). Consequently, the Fungal Growth Inhibition Rate (IFG) confirms that the higher the dosage, the

Table II. Radial mycelial growth (mm) of macrofungi on the 4th day of the Atrazine herbicide tolerance assay.

Macrofungal Species	Control	1000 µg.mL^{-1}	3000 µg.mL^{-1}	5000 µg.mL^{-1}
Hypoxylon fendleri SA41	44,44 ± 0,37 aA	39,66 ± 1,56 aB	30,33 ± 1,33 aC	25,33 ± 3,33 aD
Lentinus crinitus SA37	23,89 ± 1,48 bA	20,22 ± 0,82 bAB	18,11 ± 0,74 bB	20,66 ± 2,22 bAB
Phanerochaete australis SA18	44,44 ± 0,74 aA	42,44 ± 1,41 aA	34,33 ± 0,43 aB	12,11 ± 0,29 cC
Polyporus sp. SA23	27,94 ± 0,85 bA	22,55 ± 0,59 bB	15,00 ± 2,00 bC	13,22 ± 1,26 cC

***Means followed by the same letter, lowercase in the column and uppercase in the row, do not differ from each other according to Tukey's test (p ≤ 0.05). Source: Authors.**

greater the contaminant's interference. In the presence of 5000 µg/mL, there were high IFG levels in *P. australis* SA18 (70%), *Polyporus* sp. SA23 (50%), and *H. fendleri* SA41 (40%), with *L. crinitus* SA37 having the lowest IFG (10%) (Figure 2).

All the macrofungi studied here were tolerant to different concentrations of the herbicide atrazine. This ability to grow in the presence of toxic compounds forms a key part of studies on a species' potential for bioremediation, and is often related to detoxification capability (Bononi 1999, Rodríguez et al. 2022). Unlike *H. fendleri* SA41, the basidiomycete *L. crinitus* SA37 showed similar growth across all treatments, both in the absence and presence of the herbicide, revealing notable resistance to atrazine toxicity without substantial modifications in growth rate.

The genus *Hypoxylon* is cosmopolitan and is generally found colonizing decaying wood debris. In the literature, there are reports of the capacity of *Hypoxylon* spp. to tolerate phenanthrene and pyrene, exhibiting low mycelial growth inhibition indices and greater mycelial growth compared to other evaluated fungi (Souza et al. 2017). *H. fendleri* SA41 has already been shown to be tolerant to the herbicide glyphosate, with high mycelial growth rates (above 57%) in environments with different concentrations (30 µg/mL and 50 µg/mL) of this herbicide (de Souza et al. 2022). Accordingly, the current study corroborates previous reports in the scientific literature, confirming the capacity of fungi from the genus *Hypoxylon* for herbicide tolerance. However, the discovery here that *H. fendleri* SA41 has high radial growth in the presence of atrazine (5000 µg/mL) is the first report in the literature.

The basidiomycete *L. crinitus* is also known to tolerate such xenobiotics as glyphosate and phenanthrene (Schneider et al. 2021, de Souza et al. 2022). The tolerance of *L. crinitus* to the presence of the herbicide 2,4-D has also been reported, with similar mycelial growth observed both in the presence and absence of this herbicide (Serbent et al. 2020). It is worth noting that tolerance demonstrates the organisms' ability to withstand the toxicity of the xenobiotic, but we can't say that such a recalcitrant compound is being degraded by the fungus.

However, when a given macrofungal species tolerates high concentrations of a particular xenobiotic, and produces ligninolytic enzymes, such as laccase, lignin peroxidase, and manganese peroxidase, it is likely that such an organism has the ability to promote the biodegradation of the contaminant under study as a survival alert response due to the presence of a possible limiting factor for its development.

It is important to mention that other enzymes may be involved in the process of xenobiotic degradation, such as monooxygenases, which enable the hydroxylation of carbon and the oxidation of heteroatoms, and arylamine N-acetyltransferase homologous enzymes, which support aromatic amines found in environmental contaminants, in addition to the intracellular enzyme cytochrome P450. This explains the capacity of species of fungi from the Phylum Ascomycota to withstand the toxicity of atrazine. Therefore, it is likely that *H. fendleri* SA41 expresses some of the enzymes mentioned or others that assist the species in surviving the atrazine's high toxicity (Cocaign et al. 2013, Xin et al. 2014, Karagianni et al. 2015, Tan et al. 2015).

In this scenario, macrofungi belonging to the phylum Basidiomycota stand out when it comes to the biodegradation of environmental contaminants, as they produce extracellular enzymes with low specificity, which gives them the ability to decompose persistent organic pollutants (POPs), chlorinated pesticides (DDT), dioxins, polychlorinated biphenyls, polycyclic

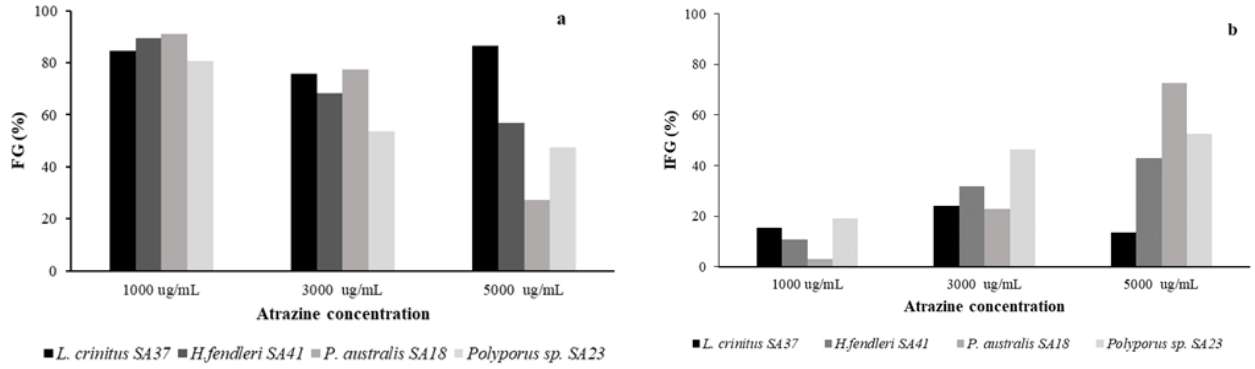

Figure 2. **Fungal growth rate (FG) (a) and Fungal growth inhibition rate (IFG) (b) on the 5th day of incubation.**

aromatic hydrocarbons, pentachlorophenol, hexachlorobenzene, among other pesticides (Harms et al. 2011, Kamida et al. 2007). The scientific literature has shown efficacy in biodegradation and bioremediation with some species, such as *Hygrocybe* sp., *Lentinus crinitus*, *Peniophora cinerea*, *Phellinus gilvus*, *Pleurotus sajor-caju*, *Psilocybe castanella*, *Pycnoporus sanguineus*, *Trametes villosa*, and *Phanerochaete chrysosporium*, the latter of which can mineralize some xenobiotics (Gugliotta 2001, Matheus 2003, Machado et al. 2005, Kamida et al. 2007).

For the laccase enzyme activity, only *L. crinitus* SA37 proved to be effective, both in the control group (0,83 $U.L^{-1}$) and in the other treatments. Furthermore, its enzyme production capacity was higher at greater herbicide concentrations, specifically at a concentration of 5000 µg/mL (4,21 $U.L^{-1}$) (Table III).

Several studies in the literature have demonstrated the capacity of *L. crinitus* to produce ligninolytic enzymes under different cultivation conditions. The relationship of this marked capacities to degrade hexachlorobenzene, decolorize the textile dye reactive blue 220 (RB220), and RBBR dye among other environmental pollutants, abilities that are strongly associated with biodegradation. Research has also indicated the possibility of using these organisms in the purification of dye-containing wastewater (Machado et al. 2005, Neto 2006, Niebisch et al. 2010, Valle et al. 2014, Cambri et al. 2016, Santana et al. 2018, Marim et al. 2018, Almeida et al. 2018).

The induction of laccase production by the presence of recalcitrant compounds and its relationship with degradation has been known to researchers for several decades. Previous studies have already indicated that oxidation of organochlorine, dimethylphenol, atrazine and diuron compounds are directly related to increased laccase activity (Jeon et al. 2008, Piscitelli et al. 2011, Silva et al. 2013, Cupul et al. 2014, da Silva Coelho-Moreira et al. 2018). Reports in the literature indicate that herbicide atrazine can stimulate the production of the enzyme laccase under adverse conditions (Mougin et al. 2002, Monteiro 2013) and corroborates with the result found in the current study regarding the induction of enzymes that degrade these molecules. Similar responses have been induced by pesticides atrazine and tricyclazole including the textile dyes Neutral Red, Indigo Carmine, Naphthol Base Bordeaux, and Ruby Sulfate, whose presence resulted in a two to fourfold increase in enzymatic activity (Singh et al. 2018). In his experiments, Henn (2009) found that some fungi only exhibited laccase enzyme production when culture extracts contained pesticide,

Table III. Laccase activity of macrofungi in the absence and presence of different concentrations of the herbicide atrazine.

Macrofungi	Laccase ($U.L^{-1}$)		
	Control	1000 $\mu g.mL^{-1}$	5000 $\mu g.mL^{-1}$
Hypoxylon fendleri (SA41)	-	-	-
Lentinus crinitus (SA37)	0,83 ± 0,91a	3,03 ± 0,07b	4,21 ± 1,5b
Phanerochaete australis (SA18)	-	-	-
Polyporus sp. (SA23)	-	-	-

concluding that the enzyme was activated in the presence of the contaminant. Rezende et al. (2005) discovered a significant increase in laccase production by *Pleurotus ostreatus* in cultures with the herbicide imazaquin at concentrations above 1.5%. Additionally, toxic substances such as atrazine, xylidine, nonylphenol, and aniline also stimulated laccase production in *Trametes versicolor* (Mougin et al. 2002). Other fungal species, such as *T. hirsuta*, and *Lentinus edodes*, also demonstrated activation of the lignin and laccase enzymatic system in the presence of atrazine (Gorbatova et al. 2006).

The presence of laccase in *L. crinitus* SA37 reinforces the importance of expanding studies of the potential for biodegradation and mineralization of the herbicide atrazine, a process still little explored using fungi, with the catabolic action of soil bacteria being better known and the associated biochemical degradation pathways being more fully understood. Furthermore, laccase is an enzyme known for its low specificity and capacity to catalyze xenobiotic, needing only a substrate and oxygen. It can also withstand high temperatures without losing activity (Datta et al. 2017), an important attribute on an industrial scale. Laccase can also be used freely or in an immobilized form, making it an interesting option for applications in bioremediation programs (Majeau et al. 2010).

It is important to conduct further investigations with *L. crinitus* SA37 about produce other types of enzymes that work together to facilitate remediation of soils contaminated by herbicides, as well as in vitro assays on biodegradation, metabolite production, and mineralization (Marinho et al. 2017, Mallerman et al. 2019, Kurami et al. 2023). Research conducted in Landfarm may also be the next step toward elucidating the efficiency of the selected species in bioremediation programs (Pereira et al. 2012, Francisco & Queiroz 2018). Furthermore, there are reports in the literature on pesticide biodegradation in soils and biomixes using microbial consortia of fungi and bacteria (Ellegaard-Jensen et al. 2014, Pinto et al. 2016, Crecca et al. 2023, Kang et al. 2024). Studies like this are scientifically important as they contribute to the development of environmental technologies aimed at preserving ecosystem integrity and promoting environmental detoxification.

Acknowledgments

We thank the Fundação de Amparo à Pesquisa do Mato Grosso (FAPEMAT) for granting the scientific initiation scholarship to the first author and Adrian Barnett, for English language revision.

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How to cite

ELIAS RV, INOUE MH & SOUZA HML. 2025. Macrofungi with Potential for Bioremediation of the Herbicide Atrazine. An Acad Bras Cienc 97: e20241100. DOI 10.1590/0001-3765202520241100.

Manuscript received on September 24, 2024;
accepted for publication on April 17, 2025

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