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ORIGINAL ARTICLE

Response of ligninolytic macrofungi to the herbicide atrazine: dose-response bioassays

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Abstract
The effect of atrazine concentrations on mycelial growth and ligninolytic enzyme activities of eight native ligninolytic macrofungi isolated in Veracruz, México, were evaluated in a semi-solid culture medium. Inhibition of mycelial growth and growth rates were significantly affected (p = 0.05) by atrazine concentrations (468, 937, 1875, and 3750 mg/l). In accordance with the median effective concentration (EC_{50}), *Pleurotus* sp. strain 1 proved to be the most tolerant isolate to atrazine (EC_{50} = 2281.0 mg/l), although its enzyme activity was not the highest. *Pycnoporus sanguineus* strain 2, *Daedalea elegans* and *Trametes maxima* showed high laccase activity (62.7, 31.9, 29.3 U mg/protein, respectively) without atrazine (control); however, this activity significantly increased (p < 0.05) (to 191.1, 83.5 and 120.6 U mg/protein, respectively) owing to the effect of atrazine (937 mg/l) in the culture medium. *Pleurotus* sp. strain 2 and *Cymatoderma elegans* significantly increased (p < 0.05) their manganese peroxidase (MnP) activities under atrazine stress at 468 mg/l. The isolates with high EC_{50} (*Pleurotus* sp. strain 1) and high enzymatic activity (*P. sanguineus* strain 2 and *T. maxima*) could be considered for future studies on atrazine mycodegradation. Furthermore, this study confirms that atrazine can increase laccase and MnP activities in ligninolytic macrofungi.

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PALABRAS CLAVE
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Lacasa;
Manganeso peroxidasa;
Estrés oxidativo;
Hongos de la pudrición blanca

Respuesta de macrohongos ligninolíticos al herbicida atrazina: bioensayos dosis-respuesta

Resumen
Se evaluó el efecto de diferentes concentraciones de atrazina sobre el crecimiento micelial y la actividad enzimática de ocho macrohongos ligninolíticos aislados en Veracruz, México. La inhibición del crecimiento micelial y la tasa de crecimiento diaria fueron significativamente ($p < 0,05$) afectadas por todas las dosis de atrazina (468, 937, 1875 y 3750 mg/l) adicionadas al medio de cultivo. De acuerdo con la concentración efectiva media (CE_{50}), *Pleurotus* sp. cepa 1 fue el aislamiento más tolerante a la atrazina (CE_{50} = 2281 mg/l), aunque sus actividades enzimáticas no fueron altas. *Pycnoporus sanguineus* cepa 2, *Daedalea elegans* y *Trametes maxima* mostraron actividades altas de lacasa (62,7, 31,9 y 29,3 U mg/proteína, respectivamente) en ausencia de atrazina (control); estas actividades se incrementaron ($p < 0,05$) significativamente (191,1, 83,5 y 120,6 U mg/proteína, respectivamente) en presencia de atrazina (937 mg/l) en el medio de cultivo. *Pleurotus* sp. cepa 2 y *Cymatoderma elegans* incrementaron significativamente ($p < 0,05$) sus actividades de manganeso peroxidasa (MnP) bajo la concentración de 468 mg/l de atrazina. Los aislamientos con alta CE_{50} (*Pleurotus* sp. cepa 1) y alta actividad enzimática (*P. sanguineus* cepa 2 y *T. maxima*) podrían ser considerados para futuros estudios en la micodegradación de atrazina. Además, el presente estudio confirma que la atrazina puede incrementar las actividades lacasa y MnP en macrohongos ligninolíticos.

Introduction

During the last century, farming and agricultural activities released many persistent and toxic chemical pesticides into the environment including insecticides, fungicides, nematicides, rodenticides and herbicides[21]. They comprise a variety of molecules possessing different properties that confer some degree of environmental persistence and mobility, as well as different toxic, carcinogenic, mutagenic and teratogenic potentials[10,17,21]. Such substances may also affect the endocrine systems of non-targeted organisms, including humans[38].

The use of herbicides in Mexico increased strongly in the last decade, where according to recent studies more than 45% of pesticides marketed were herbicides, trazine being the most frequently used[7]. The use of atrazine is not regulated and is widely used in Mexican agriculture, where the application rates range from 0.1 to 4 kg/ha/year. At these application rates, the annual consumption of atrazine in Mexico has been estimated to be around 1078 tons/year with an annual consumption increase of 10%[18,19].

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is a selective herbicide that belongs to the s-triazine family, which contains an aromatic hexameric and symmetrical ring, constituted by three carbon and three nitrogen atoms in alternate positions[18]. Atrazine is used to control broadleaf and grass weeds in corn, sorghum, sugar cane, coffee crops and conifer reforestation. Atrazine kills susceptible plants by binding to the quinone protein in photosystem II and inhibits photosynthetic electron transport[36].

Despite its apparent biodegradability, atrazine has led to the contamination of terrestrial ecosystems and can be found and measured in ground and surface waters in many countries[7,30]. The removal of atrazine from the environment is an ecological responsibility, and finding a safe and economical method is both a major concern for land management agencies and a challenge to science[13]. Bioremediation with microbial organisms is one approach. In the last two decades, the use of ligninolytic macrofungi and their enzymes in the mycoremediation of environmental contaminants has become a promising solution[40]. This fungal ability to bioremediate is generally attributed to the production of extra-cellular ligninolytic enzymes such as laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP), which are substrate non-specific enzymes and are able to degrade a wide range of recalcitrant compounds including several herbicides that are structurally related to lignin[3-5,9,39].

The first step to establish future studies of atrazine degradation through mycoremediation in contaminated sites is finding tolerant strains to atrazine[28]. The selection through dose-response bioassay is considered a way to identify these strains, which could be used in subsequent studies on soil mycoremediation[8,29]. With this in mind, we proposed the hypothesis that tolerant strains will not show mycelial growth inhibition and reduction in their enzyme activities upon exposure to the herbicide, therefore they will obtain a high mean effective concentration (EC_{50}). To corroborate this hypothesis, the goals of this work were to assess mycelial growth rates and ligninolytic enzyme activities under atrazine stress in eight native ligninolytic macrofungi isolated from Veracruz, Mexico.

Materials and methods

Fungal isolation and identification

Carpophores of ligninolytic macrofungi were collected in the center of Veracruz State, Mexico and registered in the herbarium XAL of the Instituto de Ecologia (INECOL A. C.) situated in Xalapa, Veracruz, Mexico (Table 1). In order to obtain the strains, 0.5–1 cm^2 fragments from the carpophores were washed in ethanol (70%) for 1 min, then in sodium hypochlorite (50%) for 3 min and finally in sterile distilled water for 3 min. The washed and disinfected fragments were placed on potato-dextrose agar plates (PDA, Bioxon, USA), supplemented with chloramphenicol (20 mg/l; Sigma, st. Louis, MO, USA) in order to prevent bacterial contamination, and benomyl (3 mg/l; Biesterfeld Co., USA), which inhibited mold growth. Carpophores that gave rise to the isolates were identified according to their morphologic characteristics following taxonomic keys and specialized literature[14,27,29,33,37].

Dose-response bioassays

"Desyerbal 500™" (ANALJASA, Guadalajara, Mexico), a commercial formulation of atrazine with a concentration of 500 g/l of active ingredient was used. Potato dextrose agar was sterilized at 121 °C for 15 min. Atrazine was added to sterile PDA when it was cooled at 40 ± 2 °C, and then the PDA with atrazine was emptied out to Petri dishes (90 mm Ø). The final concentrations of atrazine in PDA plates were 0 (control), 468, 937, 1875 and 3750 mg/l. The highest concentration of atrazine corresponds to the field application rate (1.5 l of "Desyerbal 500™" dissolved in 200 l of water). One disc (6 mm Ø) from a 7-day-old culture of each strain was inoculated into the center of PDA plates containing different concentrations of atrazine. All plates were incubated in the dark at 25 °C and 75 ± 5% of relative humidity. Four replicates of each atrazine concentrations and a control (without atrazine) were used.

The colonies were photographed daily from the rear of the plates with a Kodak digital camera (Kodak, New York, USA) at 20 cm between the camera and the plates. The area of mycelium growth was calculated using digital photography and the ImageJ software[31] based on the scale 1 cm^2 = 358.6 pixels. The dose-response bioassays finished when the control mycelia filled the Petri dishes or up to 15 days of growth[3].

Growth characteristics

From the colony areas measured, specific growth characteristics were calculated: the percentage of mycelial growth inhibition, as the percentage of Inhibition = $[(C-T)/C]*100$, where C and T are the mycelial area inside the control plates and the mycelial area in the treatment plates, respectively. Average Daily Growth Rate (DGR), was defined as DGR (cm^2/d) = $[\Sigma (R_1-R_0) / (T_1-T_0)] / n$, where n is the number of evaluations, R_1 and R_0 were the colony areas (in cm^2) at time T_1 and T_0 (in days), respectively[2,3]. The effective concentrations inhibiting mycelial growth rate by 50% (EC_{50}) were calculated by Probit analysis[12].

Enzymatic and protein assay

Enzyme activities were determined spectrophotometrically using a Lambda 3A UV/VIS spectrophotometer (Perkin-Elmer, Massachusetts, USA) at room temperature (25-27 °C). The enzyme extracts were performed removing five mycelial discs (6 mm Ø) from fungal colonies established in the dose-response bioassays. Mycelial discs were placed in test tubes containing 5 ml of sodium acetate buffer 100 mM (pH 4.5). Test tubes were shaken for 1 min at 2200 rpm in a minishaker (IKA-MS2 Gemini, Apeldoorn, Netherland), and then were incubated 15 min at 30 °C. Finally, the test tubes were centrifuged for 10 min at 7500 rpm and the supernatant was taken to the determination of the enzymatic activity and total protein content.

Laccase activity was determined by measuring the oxidation of ABTS [2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] in a reaction mixture (1 ml) containing 100 µl of ABTS (0.5 mM, Sigma, St. Louis, MO, USA), 800 µl of acetate buffer (100 mM, pH 4.5) and 100 µl of enzyme extract. Absorbance changes in the presence of the enzyme were monitored for 5 min at 420 nm ($\varepsilon = 3.6 \times 10^4$/M/cm). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol ABTS per minute per mg of protein under the assay conditions[24].

Table 1 Overview of strains of ligninolytic macrofungi used

Species	Site of origin	Location
Cymatoderma elegans	Tlalneuayocan	19°30"44.13" N, 96° 56"30.49" O
Daedalea elegans	Xalapa	19°31"25.66" N, 96° 57"35.58" O
Pleurotus djamor[a]	Coatepec	19°28"15.71" N, 96° 57"31.12" O
Pleurotus sp. strain 1	Xalapa	19°30"45.08" N, 96° 56"36.30" O
Pleurotus sp. strain 2	Tlalneuayocan	19°30"45.08" N, 96° 56"36.30" O
Pycnoporus sanguineus strain 1	Xalapa	19°30"45.98" N, 96° 56"30.58" O
Pycnoporus sanguineus strain 2	Actopan	19°30"21.16" N, 96° 36"31.23" O
Trametes maxima	San Andres	18°19"10.83" N, 94°49"55.69" O

[a] The strain was isolated and identified in a previous study by Salmones *et al.*[34].

Manganese peroxidase activity was determined at 610 nm (ε = 4460/M/cm) using the methodology described by Kuwahara *et al.*[22]. The reaction mixture contained: the enzyme extract (700 μl), phenol red 0.2% (50 μl), sodium lactate 0.5 mM (50 μl), egg albumin 0.1% (50 μl), manganese sulfate 2 mM (50 μl) and hydrogen peroxide 2 mM (50 μl). The reaction was carried out in sodium succinate buffer 20 mM at pH 4.5 (50 μl). The reactions occurred for 5 min and were stopped by the addition of NaOH 2N (50 μl). One enzyme unit was defined as 1 μmol of product formed per minute per mg of protein under the assay conditions.

The protein content of the extracts was estimated according to the Bradford method. Bovine serum albumin was used as a standard at known concentrations (0, 0.0062, 0.0125, 0.025, 0.05, 0.1 and 0.2 mg/l). The standard curve was: $y = 0.1615c - 0.0125$ ($y = OD_{595}$, c = protein concentration in mg/l, $R^2 = 0.999$).

Statistical analysis

Assumptions of normality and homoscedasticity were tested on all data. The results of daily growth rate, inhibition and enzymatic activities were analyzed by one way analysis of variance and the least significant difference (LSD) multiple comparison test, using the GraphPad software[15]. The EC_{50} of each strain was obtained by Probit analysis using SAS 8.1 [35].

Results

Mycelial growth inhibition

In all strains the percentage of mycelial growth inhibition increased with an increase in atrazine concentration in the culture medium. However, none of the strains were inhibited at 100% (Fig. 1). The atrazine concentration of 468 mg/l did not inhibit mycelial growth of *Daedalea elegans* (DF = 3/12, F = 975.3, $p < 0.0001$) and *Pleurotus* sp. strain 1 (DF = 3/12, F = 148.4, $p < 0.0001$). Only *Pleurotus* sp. strain 1 (DF = 3/12, F = 148.4, $p < 0.0001$) did not show inhibition in its mycelial growth at the concentration of 937 mg/l of atrazine. With 1875 mg/l of atrazine: *Pleurotus* sp. strain 1, *P. sanguineus* strain 2 and *T. maxima* showed the lowest inhibition percentage (51.8, 53.0 and 55.3%, respectively). *Pleurotus* sp. strain 2 and *C. elegans* achieved the lowest mycelial growth inhibition (DF = 7/24, F = 17.1, $p < 0.0001$) at the highest atrazine concentration (3750 mg/l) with 66.7 and 68.6%, respectively.

Daily growth rate

For most isolates, the daily growth rate (DGR) decreased when the concentration of atrazine was increased in the culture medium, except for *Cymatoderma elegans* (0.6 cm^2 / day; DF = 4/15, $F = 39.4$, $p < 0.0001$) and *Pleurotus* sp. strain 1 (1.6 cm^2/day; DF = 4/15, F = 56.1, $p < 0.0001$). These strains showed a higher DGR when compared with the control in the presence of 468 mg/l of atrazine (Fig. 2). Only *T. maxima* maintained the same DGR in the control (6.9 cm^2/day; DF = 4/15, F = 458.4, $p < 0.0001$) and under 468 mg/l of atrazine (6.8 cm^2/day). Among the tested isolates, significant differences were found in DGR in the control treatments (without atrazine); *T. maxima* (DF = 7/24, F = 377.3, $p < 0.0001$) achieved the highest DGR with 6.9 cm^2/day, and *Pleurotus* sp. strain 1 and *C. elegans* showed the lowest DGR with 0.4 and 0.3 cm^2/day, respectively.

Median effective concentration (EC_{50})

The EC_{50} ranged from 447.3 to 2281.0 mg/l of atrazine in the studied strains (Table 2). In accordance with the overlapping of confidence intervals, *Pleurotus* sp. strain 1 ($p < 0.0001$) showed the highest EC_{50} with 2281.0 mg/l of atrazine, therefore it was the most tolerant fungus of all studied strains. *Trametes maxima* and *P. sanguineus* achieved a CE_{50} of 1532.0 and 1311.0 mg/l of atrazine, both strains showed considerable tolerance to atrazine. On the other hand, the strains with the lowest EC_{50} ($p < 0.0001$) were *P. sanguineus* strain 1, *Pleurotus* sp. strain 2 and *P. djamor* with 706.1, 624.1 and 447.3 mg/l of atrazine, respectively. These isolates were the most susceptible to the herbicide.

Table 2 Median effective concentration (EC_{50}) of atrazine in eight ligninolytic macrofungi

Isolates	EC_{50} (mg/l)	Confidence intervals (mg/l)	Slope ± SEM	Probit Equation	χ^2	*Pr*> χ^2
Cymatoderma elegans	1189.0 c	1123.0-1256.0	3.06±0.12	Y=−9.43+3.06X	559.53	<0.0001
Daedalea elegans	1088.0 c	880.3-1315.0	0.75±0.09	Y=−2.17+0.75X	67.32	<0.0001
Pleurotus djamor	447.3 d	367.8-522.7	1.45±0.10	Y=−3.85+1.45X	177.68	<0.0001
Pleurotus sp. strain 1	2281.0 a	2177.0-2393.0	4.15±0.19	Y=−13.95+4.15X	455.48	<0.0001
Pleurotus sp. strain 2	624.1 d	476.5-762.3	0.87±0.09	Y=−2.44+0.87X	82.96	<0.0001
Pycnoporus sanguineus strain 2	1311.0 bc	1198.0-1430.0	2.09±0.11	Y=−6.54+2.09X	341.77	<0.0001
Pycnoporus sanguineus strain 1	706.1 d	659.1-752.6	2.98±0.13	Y=−8.49+2.98X	456.20	<0.0001
Trametes maxima	1532.0 b	1417.0-1660.0	1.96±0.10	Y=−6.27+1.96X	343.62	<0.0001

In accordance with the overlap of the confidence intervals the means with the same letter are not significantly different from each other (α = 0.05).

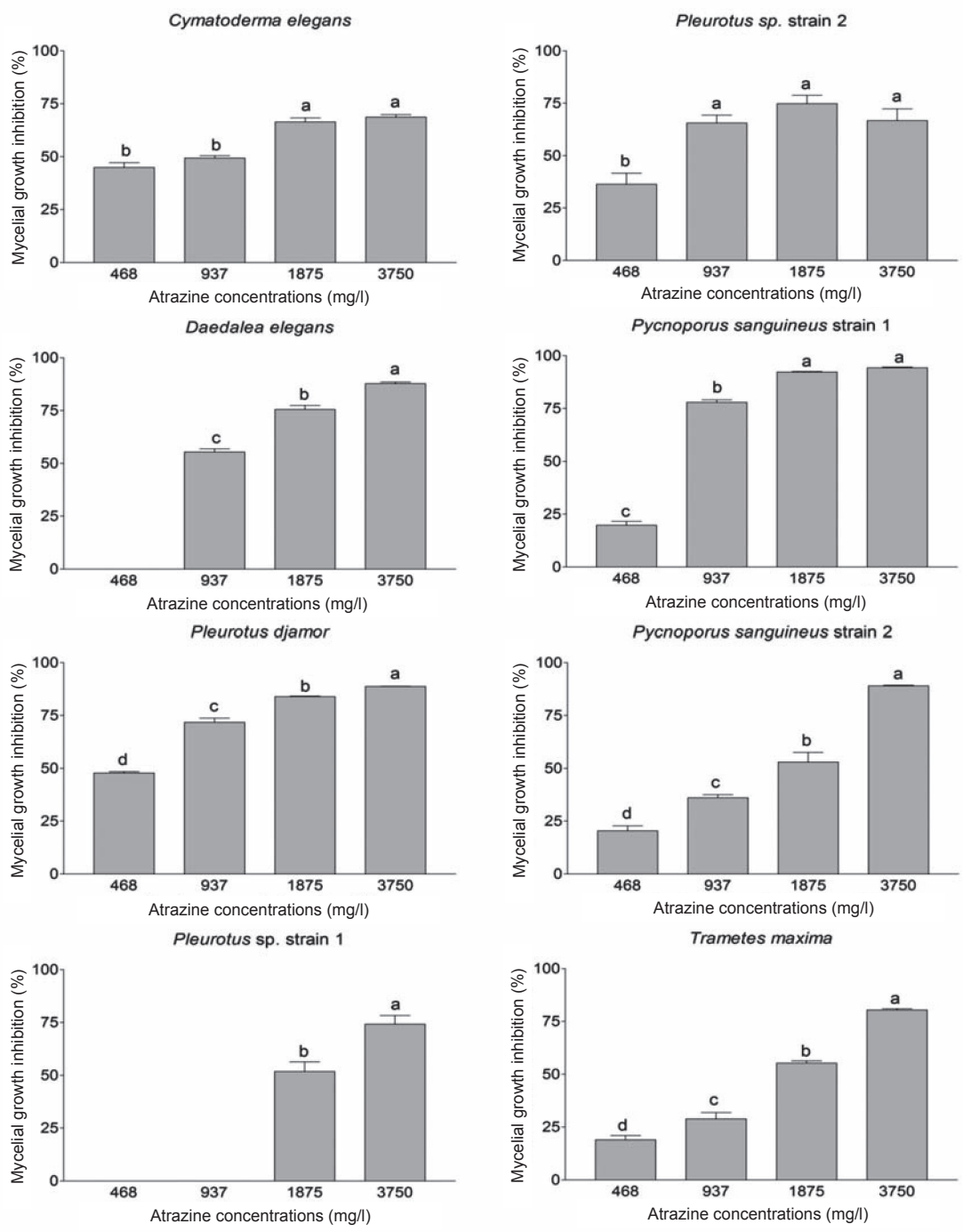

Figure 1 Effect of atrazine concentrations on mycelial growth inhibition (%) of eight ligninolytic macrofungi cultivated on potato-dextrose agar. Means with the same letter are not significantly different from each other (n = 4, mean ± SEM, LDS test, $\alpha < 0.05$).

Effect of atrazine concentrations on laccase and MnP activities

Laccase activity varied owing to the effect of atrazine concentration in all studied strains. *Pycnoporus sanguineus* strain 2 showed higher (DF = 7/16, F = 21.9, $p < 0.0001$) laccase activity in the control (62.4 U mg/protein) compared with the remaining isolates. However, this activity increased significantly (DF = 4/10, F = 14.0, $p < 0.0004$) from 62.4 to 191.9 U mg/protein (205.8%) by the effect of atrazine (937 mg/l) in the culture medium (Fig. 3). Also, when *T. maxima*, *D. elegans* and *C. eleg*ans were grown under 937 mg/l of atrazine, their laccase activities increased significantly ($p < 0.0001$) by 311% (29.3 to 120.6 U mg/protein), 161% (31.9 to 83.5 U mg/protein) and 153% (8.2 to 20.8 U mg/protein), respectively. In the remaining strains: *Pleurotus djamor*, *Pleurotus* sp. strain 2, *P. sanguineus* strain 1 and *Pleurotus* sp. strain 1 showed the highest

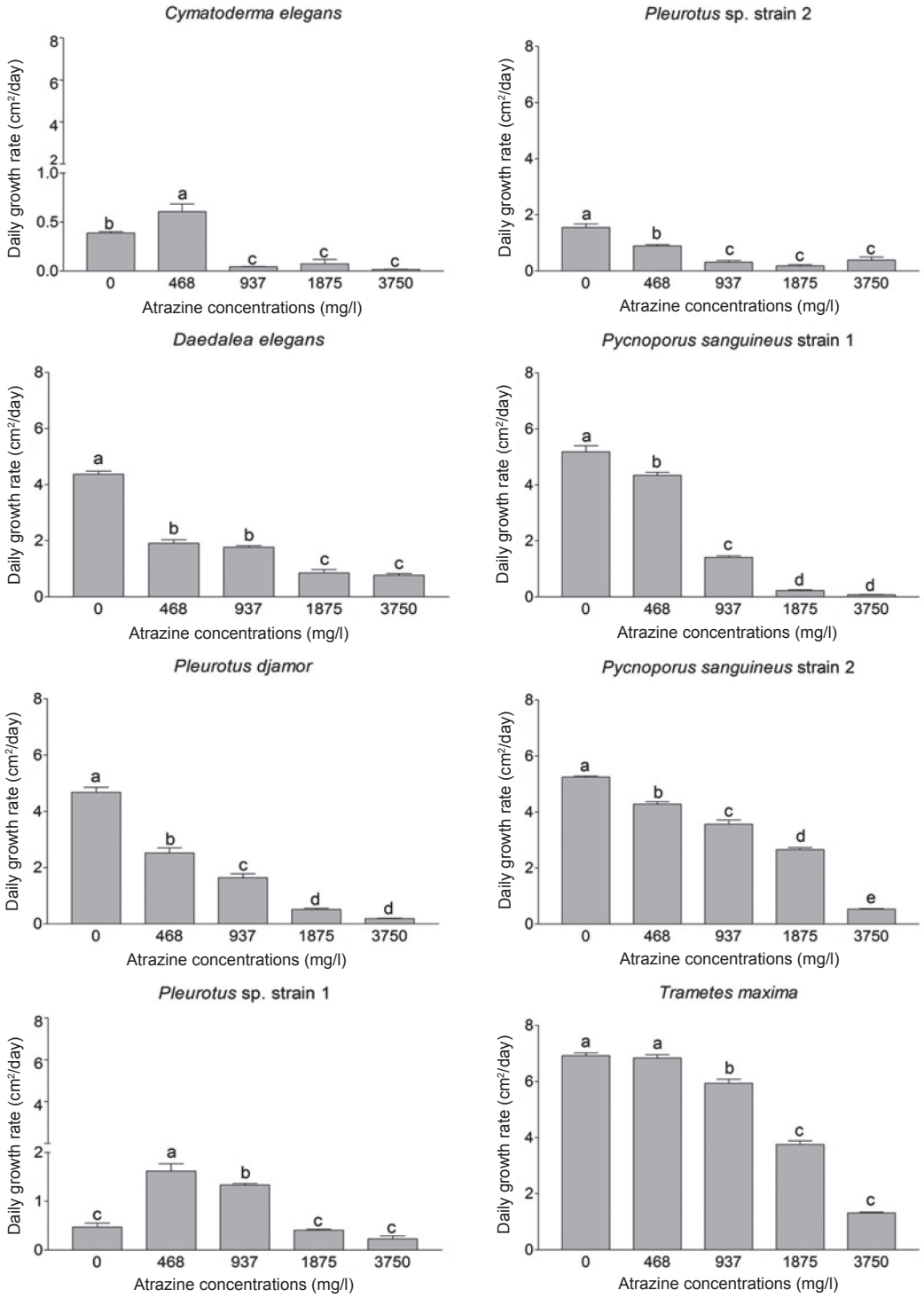

Figure 2 Daily growth rate (cm²/day) of ligninolytic macrofungi in potato-dextrose agar contaminated with different concentrations of atrazine. Means with the same letter are not significantly different from each other (n = 4, mean ± SEM, LDS test, $\alpha < 0.05$).

($p < 0.0001$) laccase activity at the highest concentration of atrazine, with increases of 726% (1.5 to 12.8 U mg/protein), 238% (2.8 to 9.5 U mg/protein), 3886% (0.15 to 6.1 U mg/protein) and 51% (1.4 to 2.1 U mg/protein), respectively.

Only *Pleurotus* sp. strain 2, *C. elegans* and *P. sanguineus* strain 1 showed MnP activity (Fig. 4). In *Pleurotus* sp. strain 2, MnP activity was significantly stimulated by the presence of atrazine in the culture medium, MnP activity was not detected in the control (without atrazine), however, a significantly higher activity (DF = 4/14, F = 41.4, $p < 0.0001$) was found at 937 mg/l of atrazine with 4.3 U mg/protein. *Cymatoderma elegans* achieved high MnP activity due to the effect of atrazine concentrations and showed significant increases, above 130% (0.6 to 1.4 U mg/protein) in the four

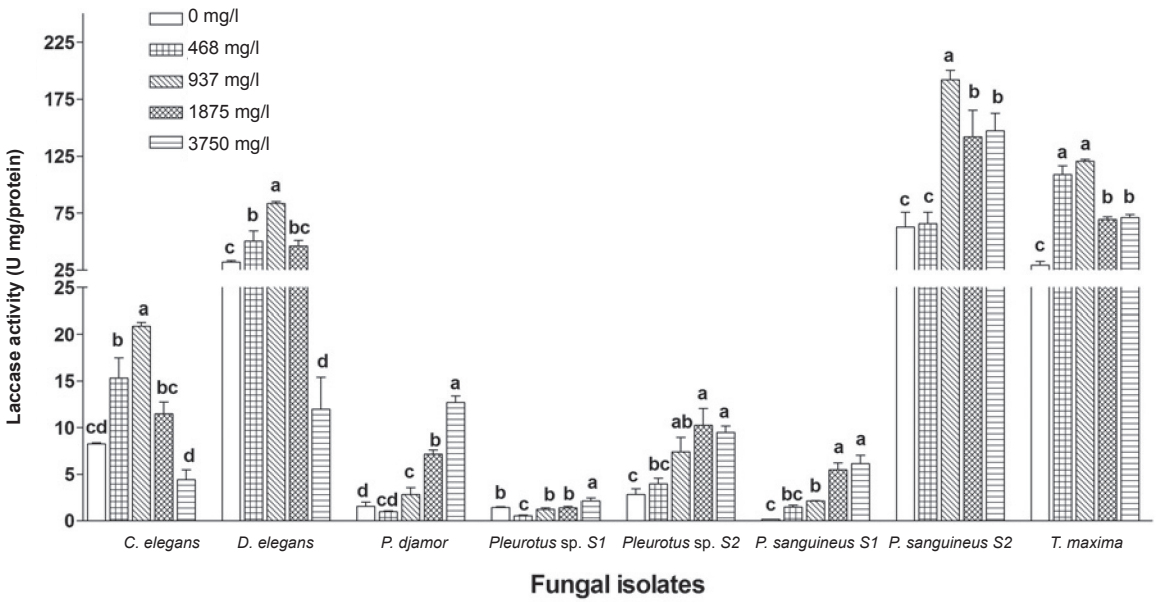

Figure 3 Laccase activity of ligninolytic macrofungi under atrazine concentrations. Means with the same letter in each isolate are not significantly different from each other (n = 4, mean ± SEM, LDS test, $\alpha < 0.05$). The terms S1 and S2 correspond to strain 1 and strain 2, respectively.

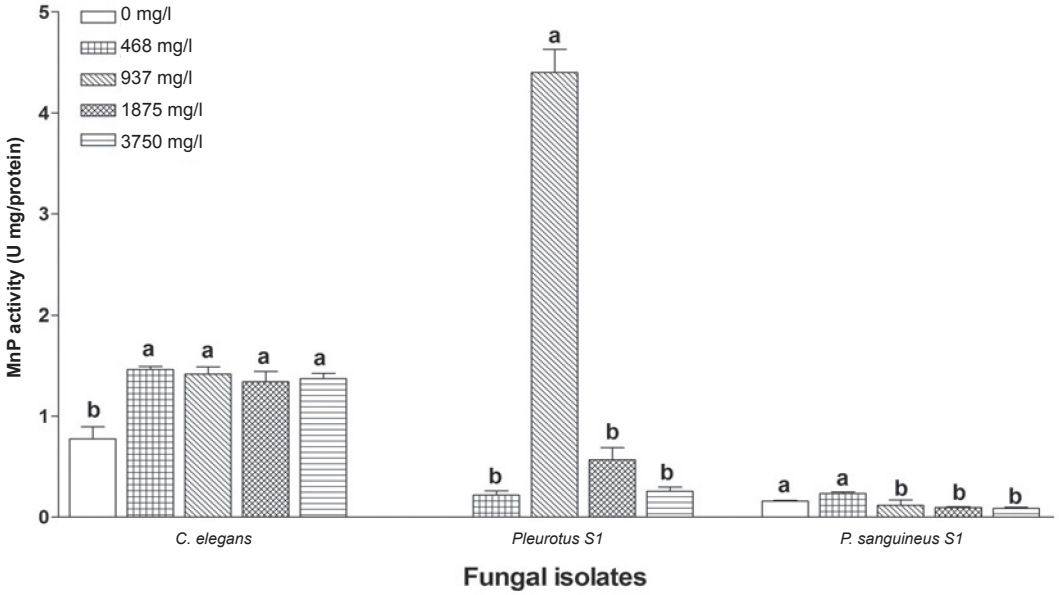

Figure 4 MnP activity of ligninolytic macrofungi under atrazine concentrations. Means with the same letter in each isolate are not significantly different from each other (n = 4, mean ± SEM, LDS test, $\alpha = 0.05$). The terms S1 and S2 correspond to strain 1 and strain 2, respectively.

tested concentrations; meanwhile the control obtained the lowest MnP activity (0.6 U mg/protein). *Pycnoporus sanguineus* strain 1 showed less MnP activity than *Pleurotus* sp. strain 2 and *C. elegans*; however, the atrazine concentrations had negative effects on MnP activity by *Pycnoporus sanguineus* strain 1.

Discussion

Mycelial growth was inhibited by the atrazine concentrations at different rates in the studied strains; however, none reached 100% inhibition under all tested doses. The lowest dose of atrazine (468 mg/l) did not

cause inhibition in *D. elegans* and *Pleurotus* sp. strain 1. The concentration of 937 mg/l of atrazine did not inhibit the growth of *Pleurotus* sp. strain 1. These results suggest that moderate concentrations of atrazine are not toxic to these strains. In a previous study Nwachukwu and Osuji[28] showed 8, 11 and 14% inhibition in the mycelial growth of *Lentinus subnudus* in soil extract agar supplemented with 5, 10 and 20 mg/l of atrazine, respectively. These inhibition percentages were low, which could be due to the low atrazine concentrations used in Nwachukwu and Osuji´s study. In another study, Ribas *et al.*[32] assessed the susceptibility of *Agaricus subrufescens* (basidiomycete) to atrazine and found high inhibition rates of mycelial growth in PDA supplemented with 10 (18.8%), 20 (31.3%), 30 (43.8%), 40 (50%) and 50 (48%) mg/l of atrazine. The concentrations used by Ribas *et al.*[32] and Nwachukwu and Osuji[28] were lower compared with the concentrations used in the present study (from 468 to 3750 mg/l), which corresponded to field application rates.

The study of growth rates of ligninolytic macrofungi is important in order to understand their capacity for colonization and their potential to settle on a particular substrate. However, several pollutants have a negative effect on the growth rate of ligninolytic macrofungi[27]. In previous works, Casale and Hart[5] reported a 69% reduction in the rate of growth for ascomycete *Sclerotinia sclerotiorum* when exposed to 500 mg/l atrazine in the culture medium. Furthermore, the daily growth rate of filamentous microfungi was negatively affected by exposure to atrazine; some susceptible genera are *Trichoderma*, *Penicillium* and *Aspergillus*[7].

On the other hand, the daily growth rate of *Pleurotus* sp. strain 1 and *C. elegans* was stimulated by low concentrations of atrazine (468 mg/l). This physiological response has been previously reported in *Microsporum fulvum* (hyphomycete)[1], when cultivated in Sabouraud agar supplemented with atrazine concentrations ranging from 5 to 50 mg/l. However, the increase in the daily growth rate by atrazine in basidiomycetes has not been previously reported. In an earlier study, Maciel *et al.*[23] reported an increase in the mycelial growth rate of *Trametes* sp. in malt extract agar supplemented with 0.1 mM (24.1 mg/l) of picloram (an herbicide). In this case, the increase in mycelial growth rate in fungi by herbicides could be related to the auxin-like action[16,23].

The parameter used to determine fungi sensitivity or tolerance to atrazine was the median (EC_{50}) effective concentration[42]. Based on this concept, *Pleurotus* sp. strain 1 was the most tolerant fungus to atrazine, presenting the highest EC_{50}. There is little information about the EC_{50} of atrazine in ligninolytic macrofungi; therefore it is difficult to compare our results to other references. Only Nwachukwu and Osuji[28] calculated the EC_{50} of atrazine in *L. subnudus* (EC_{50} at 15 °C = 20 mg/l and EC_{50} at 25 °C = 11.9 mg/l), which was smaller than that achieved in this work in all tested strains (from 706.1 to 2281.0 mg/l). The available information only mentions that atrazine exposure in fungal organisms results in the growth inhibition of some ligninolytic macro- and filamentous micro-fungi[6].

In addition, xenobiotics affect the synthesis and activity of fungal enzymes[11,25]. Laccase is one of the main enzymes produced by ligninolytic macrofungi, which plays an important role in fungal morphogenesis, lignin degradation and fungal plant-pathogen/host interaction[3]. Under the culture conditions used in this study all fungal strains produced laccase as the main ligninolytic enzyme. However, there was an increase in laccase activity for all tested strains due to the effect of the atrazine concentrations. In earlier works, Mougin *et al.*[25] added several xenobiotics (among them atrazine), in a *Trametes versicolor* culture. Atrazine increased the laccase activity of *T. versicolor* by 369% (0.61 U/ml) and 443% (0.87 U/ml) when compared to the control (0.13 Ug/g). Bastos and Magan[5] found high laccase activity (19.9 U/g soil) in *T. versicolor* growing in soil contaminated with atrazine at 0.9 and 2 μg/g, in comparison to soil without atrazine (9.0 U/g soil).

With regard to MnP activity, only *C. elegans* and *P. sanguineus* strain 1 showed MnP activity under the culture conditions. Atrazine concentrations in PDA increased MnP activity in *C. elegans*; however, in *P. sanguineus* strain 1 cultures resulted in a reduction. However, a particular physiological response was observed for *Pleurotus* sp. strain 2, which did not show MnP activity in the control treatment (without atrazine), but produced MnP activity in the presence of atrazine. The increase in laccase and MnP activities could be linked to oxidative stress on ligninolytic fungi generated by atrazine. This response is considered a defense mechanism for ligninolytic fungi[26,40-42]. Nevertheless, this response is not a general rule. Recently, Maciel *et al.*[23] found a reduction in laccase activity of *Ganoderma lucidum* due to the effect of picloram. The latter is unusual because, in general, the addition of xenobiotics to the culture of ligninolytic fungi enhances laccase activity[5,23,25]. However, we must not overlook that individual species could respond dissimilarly to different pesticides.

Under natural conditions, fungi are usually not limited in growth by low atrazine concentrations in the environment[30]. However, in locations with an extremely high atrazine content an obvious effect on fungal growth has been observed. It should be noted that the impact of atrazine on fungal physiology is not only limited to inhibition or alteration of mycelial growth. In particular, the activities of extracellular enzymes, such as laccase and MnP, were enhanced with atrazine concentrations. For this reason, more research in the field of fungal ecology for atrazine-polluted habitats could prove helpful.

Based on the results, we conclude that *Pleurotus* sp. strain 1 was the most tolerant fungus to atrazine, as it did not show mycelial growth inhibition due to atrazine at either 468 or 937 mg/l concentrations, thus achieving the highest EC_{50}; however, it did not show high laccase activity and did not enhance to a great extent its enzyme activity by atrazine stress. Nevertheless, there were other fungal isolates such as *D. elegans*, *P. sanguineus* strain 2 and *T. maxima*, which obtained a moderate EC_{50} and high enzyme activity, principally laccase; therefore, we reject the proposed hypothesis. On the other hand, the most susceptible fungus was *Pleurotus djamor*. Atrazine stress increased laccase activity for all the strains. The increase in MnP activity caused by the effect of atrazine occurred to a lesser extent than increases in laccase activity. This study

demonstrated, for the first time, the ability of *C. elegans* to produce laccase and MnP, including its ability to increase enzyme activity by atrazine stress. Overall, we propose the use of native macrofungi such as *D. elegans*, *P. sanguineus* strain 2 and *T. maxima* for future studies in atrazine mycodegradation.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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