



Growth, enzymatic production and morphology of the white-rot fungi *Lentinus crinitus* (L.) Fr. upon 2,4-D herbicide exposition

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Abstract

Dichlorophenoxyacetic acid (2,4-D) is one of the most commonly used weed control herbicides. White-rot fungi (WRF) are recognized as a degrader of a wide range of molecules because due to their enzymatic plasticity, nevertheless, the knowledge of their ability to degrade 2,4 D residues in the environment is still limited. In this study, the tolerance and the mycelium growth kinetics of twelve WRF to 2,4-D (670 g L⁻¹) were evaluated on potato dextrose agar added with 2,4-D using the technique of cup plate. *Agaricus* sp., *Pleurotus pulmonarius*, *Pleurotus djamor* and the EF 58 strain grew in all plates in the presence of 2,4-D. Molecular phylogenetic analysis of the EF 58 strain allowed its determination as a lineage belonging to *Lentinus crinitus* Fr. (Polyporales, Basidiomycota) species-complex. The daily fungal growth rate of *L. crinitus* EF 58 lineage in medium with (5.025 g L⁻¹ and 50.25 g L⁻¹) and without the addition of the herbicide, laccase production and mycelium structure after 6, 10 and 15 days of exposure to 2,4-D was analyzed. There were no significant differences between the control and C1 treatments concerning the growth of *L. crinitus* EF 58 lineage. The enzymatic activity tests showed evidence for the presence of laccases in all essays. Optical microscopy observations did not reveal substantial alterations in its mycelium morphology after exposition to the herbicide. It was the first study showing the potential of *L. crinitus* EF58 lineage in tolerating 2,4-D what represents the beginning of work for the bioremediation process with practical applications.

Keywords Organochlorine pesticides · 2,4-D · White-rot fungi · Cup plate assay

Introduction

Agriculture is a crucial issue in the environmental debate as food production, and other agricultural products absorb 70% of the world's freshwater (UN 2018). In agriculture,

in addition to high water consumption, the generation and disposal of effluents, without proper treatment, constitute a significant challenge in the context of rural sanitation. The negative effects of pesticides on health of humans and in the environment are increasingly worrying (Mahmood et al. 2016; Baumgartner et al. 2017; Budzinski and Couderchet 2018). Despite the international initiatives to discuss and mitigate the problem associated with pesticides, it receives less attention than necessary, such as the contribution of agriculture to water pollution (FAO 2018). The studies to evaluate the presence of agrochemicals in waters used for irrigation and, in turn, the possibilities of treatment of these effluents, aiming at the degradation of these contaminants, are still scarce.

Among the agrochemicals used in agriculture, 2,4-D stands out as the first organic herbicide synthesized in the 1940s (Ahlborg and Thunberg 1980) and is one of the most commonly used products for weed control (Schulz and Segoby 2016; Hiran and Kumar 2017).

On the other hand, this compound forms highly toxic metabolic intermediates (Ditzelmüller et al. 1989; Short

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et al. 1991; Bukowska 2003; Lurquin 2016; Wang et al. 2015). The European Commission (EC) regulates pesticides in drinking water by the Drinking Water Directive (DWD) using a maximum allowable concentration of $0.1 \mu\text{g L}^{-1}$ (DWD 1998). In Brazil, the guideline values for chemicals from agricultural activities of health significance in drinking water (Brazilian Ordinance from Ministry of Health No. 2914/2011) define a concentration of $30 \mu\text{g L}^{-1}$ for 2,4-D as a maximum allowable value (Brazil 2011).

Many microorganisms that act on 2,4-D degradation have been isolated in different environments around the world. However, most studies have emphasized the role of 2,4-D degrading bacteria and not in the corresponding fungi with this ability (Fournier and Catroux 1980; Vroumsia et al. 2005; Singh 2006; Zabaloy et al. 2008; Ferreira-Guedes et al. 2012; Itoh et al. 2013). The screening of white-rot fungi (WRF) for tolerance to single and mixtures of pesticides in vitro is useful to identify possible candidates of active bioremediation applications (Magan et al. 2010). WRF can efficiently degrade many chlorinated organic contaminants, including 2,4-D (Gianfreda and Rao 2004; Pizzul et al. 2009; Gaitan et al. 2011; Jin et al. 2016). Although there are some advances in studies about its degradation, there are still many reports of poisoning cases, histopathological changes, genotoxicity, mutagenicity and carcinogenicity, among other abnormalities including death of humans and animals (Dakhakhni et al. 2016, Hiran and Kumar 2017, Islam et al. 2018).

Many studies related to the potential of Basidiomycota aim at producing compounds of nutritional (Henriques et al. 2008, 2016; Stevanovic et al. 2018), pharmacological and medicinal (Qi et al. 2016; Singdevsachan et al. 2017; Tang and Zhong 2004; Kozarski et al. 2011) utility. Numerous investigations about the biodegradation of xenobiotics by fungi have focused on the discoloration of industrial effluents (Jarosz-Wilkolazka et al. 2002; Machado et al. 2005; Kalmış et al. 2007; Gomes et al. 2009; Manzano-León et al. 2011; Yildirim et al. 2012; dos Santos Bazanella et al. 2013; Al-Ghamdi 2016; Chicatto et al. 2018) and recuperation of heavy metal-contaminated soils (Adenipekun et al. 2011; Dulay et al. 2015) and also persistent organic pollutants (Erguven and Yildirim 2019). For more than 20 years, degradation studies of chlorinated compounds have been carried out with microorganisms from different orders (Erguven 2018). Studies about accumulation and persistence of many chlorinated aromatics in the environment become necessary for the comprehension of biochemical processes for its mineralization (Serbent et al. 2019). Although the WRF potential in bioremediation processes is still far from being fully explored, we have chosen fungi with and without previous studies about their ability to degrade chlorinated compounds.

Agaricales is one of the most diverse orders of the phylum Basidiomycota and has several prominent members, including the genera *Agaricus* L., *Lentinula* Earle, *Macrocybe* Pegler and Lodge, *Pleurotus* (Fr.) P. Kumm. that grow in a wide variety of habitats (Money 2016).

Pleurotus pulmonarius (Fr.) Quél. was the first WRF used in biodegradation studies of chlorinated compounds in liquid culture, more than two decades ago (Masaphy et al. 1993), demonstrating that the fungus ability to dealkylate and hydroxylate atrazine was not dependent on previous incubation with the compound and that the degradation biochemical pathway occurred via the constitutive metabolic activities of natural molecules. A contemporary investigation (Okeke et al. 1993) showed the aerobic metabolism of pentachlorophenol (PCP) by *Lentinula edodes* (Berk.) Pegler in soils and demonstrated the competitive advantage of this fungus to eliminating both PCP and pentachloroanisole when compared to mixed culture with indigenous Funga (Kuhar et al. 2018) of soils contaminated. The ability of the Agaricales *P. pulmonarius*, *L. edodes*, and the Polyporales *Ganoderma lucidum* (Curtis) P. Karst. to remove PCP was demonstrated by Chiu et al. (1998) using a batch cultivation system. In relation to another chlorine compound, 2,4-dichlorophenol (2,4-DCP, primary metabolite from 2,4-D transformation), the rapid oxidation was associated with laccase (60% transformation after 6 h) and versatile peroxidase (100% transformation after 1 h) of *P. pulmonarius* (Rodriguez et al. 2004), while the role of laccase in PCP removal was confirmed by Souza et al. (2011) in submerged cultures of *P. pulmonarius*.

On the other hand, the degradation of 2,4-DCP through *L. edodes* laccase and Mn-peroxidase production was demonstrated by Tsujiyama et al. (2013). In a more recent study, Gąsecka et al. (2015) established the degradation of polychlorinated biphenyls (PCBs) by *L. edodes* and *Agaricus* sp. To a single PCB (28 chlorine atoms), they reported degradations between 7.38 ± 1.06 – $75.30 \pm 1.46\%$ and 31.32 ± 1.52 – $83.91 \pm 1.07\%$, respectively, after 12 weeks of incubation. Complementary applications for *L. edodes* to the removal of heavy metals as cadmium have been reported in recent decades (Chen et al. 2006; Qiao et al. 2014).

Most published research with *Agaricus brasiliensis* Fr. has focused on their properties as a diet supplement in human nutrition (Henriques et al. 2008, 2016; Stevanovic et al. 2018) and in their antioxidative and immunomodulating activities (Tang and Zhong 2004; Henriques et al. 2008; Kozarski et al. 2011; Henriques et al. 2016; Stevanovic et al. 2018).

Macrocybe titans (H.E. Bigelow and Kimbr.) Pegler, Lodge and Nakasone strains have been used for the biodegradation of food industry waste (Wisniewski et al. 2010; Zilly et al. 2012) as long as *Pleurotus djamor* (Rumph. ex Fr.) Boedijn, another member of Agaricales order, has been studied because of their antioxidant potentials (Sasidhara

and Thirunalasundari 2014) and their decolorizing mechanisms (Kalmış et al. 2007; Manzano-León et al. 2011; Yildirim et al. 2012; Duarte et al. 2014) and, also, as an alternative for the treatment of water containing recalcitrant pharmaceuticals (Mayorga et al. 2017).

Inonotus splitgerberi (Mont.) Ryvarden, another of the species studied in this work, have been mostly studied about ecological aspects as habitat preferences, communities, demographics, diversities, and distributions (Thaug 2007). The antioxidative and immunomodulatory activity, as well as an alternative treatment of cancer, was demonstrated in *Tropicoporus linteus* (Berk. and M.A. Curtis) L.W. Zhou and Y.C. Dai, another member of the Hymenochaetales group (Sliva 2010; Huang et al. 2010, 2011; Kozarski et al. 2011).

As dominant wood-decay fungi, species of Polyporales are of interest to both fungal ecologists and applied scientists (Justo et al. 2017). Although that most studies with *G. lucidum* are related to its medicinal (Kozarski et al. 2011, Tang and Zhong 2004) and pharmacological properties (Boh et al. 2007; Čilerdžić et al. 2017; Barbieri et al. 2017; Chen and Lan 2018; Ghobadi et al. 2018; Zhao and He 2018), recent works have explored the use of this fungus or its enzymes to degrade organic compounds xenobiotics (Gaśecka et al. 2015; Coelho-Moreira et al. 2018). In respect of organochlorine compounds, the efficiency of *G. lucidum* in the bioconversion of diuron (Da Silva Coelho et al. 2010; Coelho-Moreira et al. 2018), bentazon (Da Silva Coelho et al. 2010) and pentachlorophenol (PCP) (Jeon et al. 2008) had been shown.

Ganoderma applanatum (Pers.) Pat. is a widely distributed wood-decaying species whose ligninolytic enzyme system has not been sufficiently studied (Čilerdžić et al. 2016, 2017). There are reports about their capacity for degradation of dyes used in textile industries (Jarosz-Wilkolazka et al. 2002; Al-Ghamdi 2016). Regarding pesticide bioconversion, the organochlorine-degrading capabilities of *G. lucidum* associated with the enzymatic mechanism for diuron (Da Silva Coelho et al. 2010; Coelho-Moreira et al. 2018) and lindane (Kaur et al. 2016) have shown the potential of this species for managing organochlorine bioremediation at contaminated sites.

Medicinal properties of *Polyporus lipsiensis* (Batsch) E.H.L. Krause include anti-tumor (Qi et al. 2016), antioxidant and anti-bacterial activity (Singdevsachan et al. 2017). Nevertheless, the decolorization activity has been attributed to *P. lipsiensis* strains isolated from a tropical ecosystem (Machado et al. 2005).

Previous studies with the Australasian–South American fungus *Tyromyces pulcherrimus* (Rodway) G. Cunn. evaluated their role in the decomposition of indigenous coarse woody debris as well as explored their ecological aspects (Hood et al. 2008; Westphalen and Silveira 2013).

Finally, the genus *Lentinus* Fr. belongs to Polyporaceae and presents several species widely distributed throughout the world (Pegler 1983). Many of them have already been used to produce compounds of nutritional, pharmacological and medical interest (Attarat and Phermthai 2014; Dulay et al. 2015; Sharma et al. 2015; Umeo et al. 2015), industrial enzymatic applications (Kirsch et al. 2011, 2013; Petchluan et al. 2014; Tahir et al. 2013; Valle et al. 2014) including the absorption capacity of heavy metals (Bayramoglu et al. 2002; Yakup Arica and Bayramoglu 2005), the treatment of organic solid (Kabbashi et al. 2014; Adebayo et al. 2015; Nitayapat et al. 2015) and the decolorization capability (Machado et al. 2005; Niebisch et al. 2010). The decolorization efficiency by *L. crinitus* (L.) Fr. isolates was previously reported (Machado et al. 2005; Moreira Neto et al. 2009; Niebisch et al. 2010).

In fungi, the agar-plate culture system has been used in enzymatic activity assays (Adebayo et al. 2015; Cambri et al. 2016) and for the isolation and characterization of strains with potential for the degradation of organochlorine compounds (Silva et al. 2007; Sing et al. 2014; Carascal et al. 2017; Wang et al. 2017; Erguven and Koçak 2019; Erguven and Demir 2019).

In order to deepen the understanding of the degradation of 2,4-D, studies were carried out in which 12 species of WRF were first tested due to their ability to grow in an agar-plate culture system with the herbicide. Afterward, growth, enzymatic activity and morphological structure in response to the 2,4-D of the fungus *L. crinitus* EF 58 lineage, selected in the previous step, was determined. Due to the characteristics of WRF, the purpose of the present study was to evaluate the potential use of Basidiomycota fungi as degradation agents of 2,4-D by assessing their tolerance and growth in an agar-plate culture system with this herbicide.

Materials and methods

Organisms and culture system

Twelve fungi (Table 1) with and without reports in the literature about the ability to degrade organochlorine compounds were tested for their tolerance to 2,4-D by the addition of this pure herbicide in *agar-plate* culture system (PDA—potato dextrose agar with bacteriological peptone 0.1% and malt extract 1%). The EMBRAPA Forests Collection/EF (Brazil) provided the species/strains except for *Ganoderma* sp., acquired by the Collection of Cultures of the São Paulo Botanical Institute/CCIBT (Brazil), used for this study.

Table 1 Descriptions of the studied species/strains

Studied fungi	Species/strains code	Geographic origin
Agaricales		
<i>Agaricus</i> sp.	EF 14	Colombo/PR—Brazil
<i>Agaricus brasilensis</i>	EF 07	Colombo/PR—Brazil
<i>Macrocybe titans</i>	EF 68	Cornélio Procopio/PR—Brazil
<i>Pleurotus djamor</i>	EF 86	Pinhais/PR—Brazil
<i>Pleurotus pulmonarius</i>	EF 88	Araucária/PR—Brazil
Hymenochaetales		
<i>Inonotus splitgerberi</i>	EF 46	Colombo/PR—Brazil
<i>Tropicoporus linteus</i>	EF 80	Colombo/PR—Brazil
Polyporales		
<i>Ganoderma applanatum</i>	CCIBt 2978	Curitiba/PR—Brazil
<i>Ganoderma lucidum</i>	CCIBt 2994	Argentina
<i>Polyporus lipsiensis</i>	CCIBt 2689	Fazenda Intervalos/SP—Brazil
<i>Tyromyces pulcherrimus</i>	EF 99	Colombo/PR—Brazil
<i>Lentinus strigellus</i> Berk. Currently <i>Panus strigellus</i> (Berk.) Overh.	EF 58	Colombo/PR—Brazil

Preliminary screening for 2,4-D herbicide tolerance

Sterile microbiological dishes were prepared with 20 mL of culture medium and kept in a sterile laminar flow chamber under UV light until culture medium solidification. 2,4-D herbicide solution (670 g L⁻¹) was sterilized by filtration through 0.45-µm-pore size Millipore membranes and added on the surface of the medium to a final volume of 100 µL and spread with the help of a sterile Drigalski spatula (Reis et al. 2013). A five-millimeter mycelial disk of each fungus was placed mycelium facedown on the center of the agar plates with and without prior application of 2,4-D (control test). All assays were performed in triplicate.

The dishes were incubated in a BOD chamber at 27 °C ± 1 °C for 14 days (approximate time in which the control occupied the entire surface of the culture medium). After that period, measurements of single-position central mycelial diameter were taken with digital pachymeter (Digimess). Daily fungal growth rate (DGR) was calculated for each fungal colony by using Eq. 1 (Venâncio et al. 2017):

$$DGR_{ab} = (D_b - D_a) / (t_b - t_a) \quad (1)$$

and expressed as cm day⁻¹, where D_b is the average of the two measured diameters at the end of the assay, D_a is the diameter of the disk at the beginning of the assay (0.5 cm) and $t_b - t_a$ is the exposure time interval (in days).

Due to its capability of growth on agar plates supplemented with 100 µL of 2,4-D at 670 g L⁻¹ (acid equivalent in the commercial solution), the EF 58 strain was selected for the following assays with the concentration used in the field (C1): 5.025 g L⁻¹—1.5 L of commercial solution and 198.5 L of water—(Cupul et al. 2014) and with 10 times the

concentration used in the field (Sapieha-Waszkiewicz et al. 2003) (C2): 50.25 g L⁻¹. The control plates contain only the fungus without 2,4-D addition. The average diameter of the EF 58 mycelium was daily measured with digital pachymeter (Digimess) until the plate was completely covered and the mycelial diameter growth rate was calculated. For this, two perpendiculars to one another straight lines were marked on the bottom of each microbiology dish. The crossing point matched with the center of the 5-mm initial mycelial pellet, and measurements were recorded daily from the edge of the initial fungi disk until the last area of fungi mycelia development, following the segments formed by the perpendicular lines (Miyashira et al. 2010).

DNA isolation, PCR and sequencing methods

Genomic DNA was isolated from the EF 58 culture using a CTAB protocol (Góes-Neto et al. 2005). Internal transcribed spacer (ITS) DNA region was amplified in this study with primers ITS6R and ITS8F (Dentinger et al. 2010). PCR products were purified according to the protocol with polyethylene glycol (PEG) (Sambrook et al. 1989). The sequencing of markers was by capillary electrophoresis in the ABI3730 apparatus using POP7 polymer and BigDye v3.1 at Myleus Biotechnology (www.myleus.com).

Sequence alignment and phylogenetic analyses

The chromatograms were edited manually in Geneious software version 9.0.5 (Kearse et al. 2012), and subsequently, the sequences obtained were compared with those most similar deposited in GenBank, through the BLASTn tool. The sequences were aligned in MAFFT v.7 (Katoh and Standley

2013) and later edited manually by MEGA software v.7 (Kumar et al. 2016). Maximum likelihood (ML) methods with 1000 pseudoreplications (bootstrap) and Bayesian inference (IB) were used for the construction and analysis of the phylogenetic trees using RAxML version 8.2.10 (Stamatakis 2014) and Mr. Bayes v. 3.2.6 (Ronquist et al. 2012) software, respectively. The most suitable molecular evolution model for the data was obtained through the jModelTest2 v.2.1.6 using the Akaike information criterion (AIC) (Posada and Buckley 2004). The programs were used by the CIPRES platform (<https://www.phylo.org/>) (Miller et al. 2010). The ML analysis involved 100 searches, each from a random step-by-step addition tree, under the GTRGAMMA model, with no proportion of invariant sites and all other parameters estimated by the software. After obtaining the final topologies of ML and IB, their congruences were evaluated visually, being considered incongruent only clades with a different configuration that presented significant support, and in all cases, ML topology was selected, and IB values were recorded. *Polyporus dictyopus* Mont. was chosen as out-group*. The tree was edited in the program FigTree® v1.4.0. Access numbers for generated sequencing and additional sequence information analyzed are listed in Table S1 (Supplementary methods).

Kinetics of Laccase production

After 6, 10 and 15 days, samples from mycelium of the culture medium with and without 2,4-D were re-cultured by introducing a 7-mm Ø disk (of each fungus mycelium in each treatment) in new microbiological dishes (90 mm Ø) with freshly prepared agar-malt medium with 0.5% (m/v) gallic acid described by Davidson et al. (1938). The pH was corrected to 5.5 with 5% NaOH. The assays were performed in duplicate. Laccase production was accompanied by the oxidation of the substrate, resulting in halo formation that was measured daily for 5 days. The tolerance assays and daily growth measures were conducted at Laboratory of Biomass Engineering (LEBIO) in Regional University of Blumenau (FURB).

Morphological analysis

To verify noticeable changes in the morphology when fungi growth in the presence of the herbicide, mycelia of the 2,4-D-tolerant EF 58 strain from the agar medium (control and 2,4-D supplemented agar plates) were examined by optical microscopy. Slides were fixed using the cotton blue and floxin to dye and with distilled water and observed under an optical microscope. Fragments of the aerial and submerged mycelia in both, center and borders, were fixed in 3.0% KOH and examined by light microscopy. Floxin (1% aq.) was used for staining the material. Counting and

characterization of microstructures were made to a comparison of treatments, with and without 2,4-D addition. The measurements ($n = 15$) of the structure's sizes (hyphae and chlamydospores) were taken considering the variations in the type of treatment: without (Control) and with herbicide (C1 and C2); time of exposure (5, 10 and 15 days) and location of the mycelium (horizontal: center/edge and vertical: aerial/submerged). Also, chlamydospores shape was classified based on the Q-range intervals Stalpers (2007). The microscopical observations were conducted at Laboratory of Mycology at Federal University of Santa Catarina (UFSC) and Laboratory of Parasitology at Regional University of Blumenau (FURB).

Statistical analysis

Student's *T* test was used to evaluate significant differences of fungal growth rates and laccase production of EF 58 strain between the different treatments from the daily measurements of the colonies' diameter and the halos of the oxidation reaction of gallic (Atri and Sharma 2011; Sharma and Atri 2013). We used the nonparametric Kruskal–Wallis test to compare the caliber of the generative hyphae and the chlamydospores sizes in different treatments and exposition times (Ellegaard-Jensen et al. 2013; Wang et al. 2017).

Results and discussion

WRF tolerance to 2,4-D

The tolerance of the fungi to the 2,4-D herbicide was evaluated for their ability to grow on solid medium added with 100 µL of 2,4 D herbicide solution. Table 2 shows the diametral mycelial growth (mean value) of the 12 fungi used in the *screening test* after 14 days of cultivation (initial mycelial pellet of 0.5 cm) in the presence and absence of the herbicide. For species *Pleurotus pulmonarius*, *Pleurotus djamor*, EF 58, and *Agaricus* sp. mycelial growth were observed in all microbiological dishes (triplicates) in the presence of 2,4-D showing tolerance to this herbicide.

The growth of *Agaricus* sp. [a, b], *Lentinus strigellus* [c, d], *Pleurotus djamor* [e, f] and *Pleurotus pulmonarius* [g, h] after 14d in medium without and with 2,4-D, respectively, is shown in Fig. 1. Mycelial growth in all plates showing tolerance to this herbicide was observed (Fig. 1b, d, f, h). Mycelial growth was higher (*T* test $p < 0.05$) in the absence of herbicide when compared to the control test (Fig. 1a, c, e, g). *Agaricus brasilensis* [i, j], *Ganoderma applanatum* [k, l], *Polyporus lipsiensis* [m, n], *Ganoderma lucidum* [o, p], *Inonotus splitgerberi* [q, r], *Macrocybes titans* [s, t], *Tropicoporus linteus* [u, v], and *Tyromyces pulcherrimus*

Table 2 Growth of WRF species/strains in medium with agar and 2,4-D herbicide (670 g L⁻¹)

Fungi	Mycelial diameter (cm)—control treatment	Mycelial diameter (cm)—treatment with addition of 2,4-D	DGR in 2,4-D-tolerant WRFs (mm day ⁻¹)
<i>Agaricus</i> sp. EF 14	9.00 ± 0.00	3.20 ± 1.43	0.19
<i>Agaricus brasiliensis</i> EF 07	7.25 ± 0.33	0.50 ± 0.00	–
<i>Ganoderma applanatum</i> CCIBt 2978	9.00 ± 0.00	0.50 ± 0.00	–
<i>Polyporus lipsiensis</i> CCIBt 2689	8.20 ± 0.1	0.50 ± 0.00	–
<i>Ganoderma lucidum</i> CCIBt 2994	6.35 ± 0.5	0.50 ± 0.00	–
<i>Inonotus splitgerberi</i> EF 46	8.80 ± 0.28	0.50 ± 0.00	–
<i>Lentinus strigellus</i> (currently <i>Panus strigellus</i>) EF 58	9.00 ± 0.00	2.80 ± 0.25	0.16
<i>Macrocybe titans</i> EF 68	5.30 ± 0.42	0.50 ± 0.00	–
<i>Tropicoporus linteus</i> EF 80	6.10 ± 0.14	0.50 ± 0.00	–
<i>Pleurotus djamor</i> EF 86	7.73 ± 0.25	1.50 ± 0.81	0.07
<i>Pleurotus pulmonarius</i> EF 88	7.77 ± 0.31	1.30 ± 0.15	0.06
<i>Tyromyces pulcherrimus</i> EF 99	8.75 ± 0.35	0.50 ± 0.00	–

Control: without addition of 2,4-D. All tests were performed in triplicate (mean value). Initial mycelial pellet of 0.5 cm. DGR Daily fungal growth rate

[w, x] did not present mycelial growth, indicating a negative response to the 2,4-D tolerance test (Fig. 1).

The rule shows the size of the petri dish (9 cm).

Among the WRF tested, *Lentinus strigellus* EF 58 was selected for subsequent trials due to its tolerance to 2,4-D (670 g L⁻¹). Atypical characteristics (absence of clamp connection on generative hyphae) of *Lentinus* were observed in the strain in the mycelium of *Lentinus strigellus* EF 58. For that reason, the molecular identification of the strain under study was carried out. A matrix containing in-group sequences of Polyporaceae genera was previously assembled to confirm the positioning of the strains in the family and to confirm the genus *Lentinus*. After the result of the analysis, a second matrix with the in-group species of *Lentinus* was created in order to phylogenetically determine the position of EF58 strains. The topologies of phylogenetic trees based on the dataset generated from Bayes and RAXML analyses are identical. Therefore, only the ML tree with bootstrap values (BS) is shown in Fig. 2, with Bayesian posterior probabilities (BPPs) also indicated. The best evolutionary model estimated using the AIC for ITS was HKY + I + G. Seven clusters belonging to *Lentinus* are represented in this phylogenetic reconstruction.

Phylogenetic analysis showed that the two culture sequences, EF 58a, and EF 58b, were arranged in a branch comprising another specimen of *Lentinus crinitus* (FTF200) with 0.99 BPP. In the phylogeny, it is also possible to observe that *L. crinitus* is polyphyletic, in accordance with previous works such as Grand (2004) and Seelan et al. (2015). Also, the literature indicates that this taxon presents a considerable morphological variation, which suggests that it is a complex of species, deserving a morphological review in order to define the lineage *L. crinitus* s.s. and perform taxonomical

and nomenclatural treatment for those lineages previously named as *L. crinitus* s.l. also based on in-depth additional and more representative molecular analyses (Pegler 1983). In summary, molecular sequence-based identification and phylogenetic analysis of the strain allowed its definition as *Lentinus*, up to now belonging to *L. crinitus* s.l. Then, we choose to call this as *L. crinitus* EF 58 lineage. The species-complex *Lentinus crinitus*, as morphologically assumed, is common in different biomes or regions worldwide (Pegler 1983; Seelan et al. 2015; Drechsler-Santos et al. 2009, 2012).

Responses of *L. crinitus* EF 58 lineage in agar medium with 2,4-D

The same period of acclimatization to the culture medium with and without herbicide was observed (Table 3). The results of the factorial ANOVA and the Tukey's multiple comparison test of the daily colony diameters for the treatments show that from the third day, there were no significant differences between the control and C1 treatments concerning the growth of *L. crinitus*.

Daily fungal growth rate to control and C1 treatment was 13.83 mm day⁻¹. Similar values of growth rate (5, 8, 13, 15 and 18 mm day⁻¹) were reported for *Panus conchatus* (Bull.) Fr., *Lentinus conatus* Berk., *Lentinus sajor—caju* (Fr.) Fr., *Lentinus cladopus* Lév. and *Lentinus squarrosulus* Mont., respectively, on solid medium—Potato Dextrose Agar (Sharma and Atri 2013). *L. crinitus* strains reached similar rates of mycelial extension rate (13.3–14.7 mm day⁻¹) during growth on agar malt (2%) associated with Remazol Brilliant Blue R (RBBR) decolorization (Machado et al. 2005). Our data also agree with those reported by Magalhães et al.

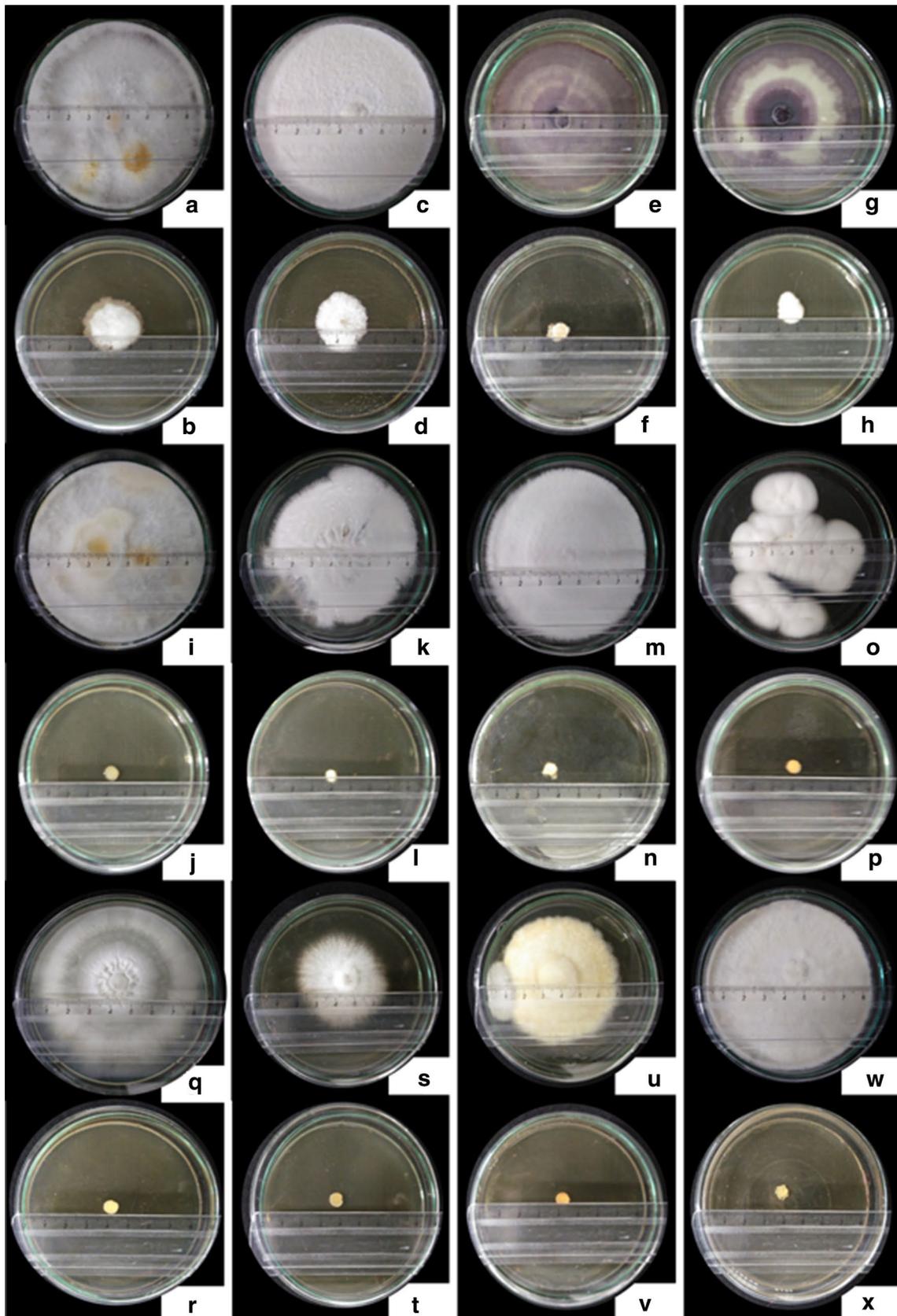


Fig. 1 Growth of white-rot strains in medium without and with 2,4-D herbicide (670 g L^{-1})

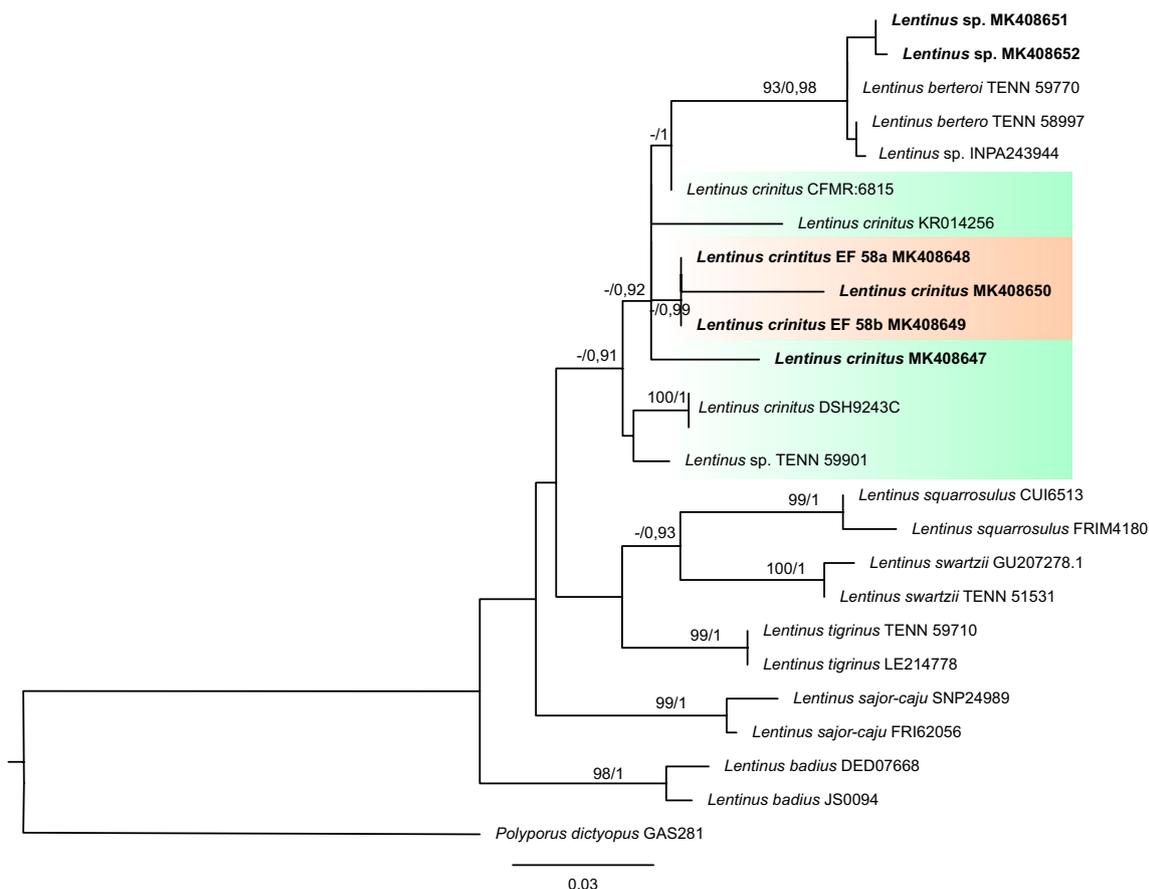


Fig. 2 Maximum likelihood tree of *Lentinus* species based on ITS data and rooted with *Polyporus dictyopus*. The sequences generated in this

work are in bold. Posterior probability (BPP) (above 0.7) and bootstrap (BS) (above 50%) support values are shown above the branches (BPP/BS)

Table 3 Kinetics of mycelial growth (mm) of *L. crinitus* EF 58 lineage with (C1 and C2) and without (control) addition of 2,4-D as a function of time (h)

Time (h)	Control	C1	C2
24	(7.0 ± 0.0)aA	(8.0 ± 0.0)aA	(7.5 ± 0.0)aA
48	(32.5 ± 0.7)bA	(30.2 ± 0.9)bAB	(26.5 ± 1.7)bB
72	(52.0 ± 1.3)cA	(49.7 ± 0.7)cA	(38.7 ± 1.5)cB
96	(67.7 ± 1.5)dA	(64.7 ± 0.9)dA	(51.1 ± 3.2)dB
120	(79.7 ± 0.3)eA	(78.7 ± 0.7)eA	(62.4 ± 3)eB
144	(90.0 ± 0.0)fA	(90.0 ± 0.0)fA	(73.1 ± 1.3)fB
DGR (mm day ⁻¹)	13.83	13.83	11.02

Means followed by different lowercase letters (period) and/or capital letters (treatment) represent significant differences ($p < 0.05$)—Student's *T* test

(2017), who observed the highest growth (18-mm day⁻¹) for *L. crinitus* in PDA culture medium at 35 degrees and 5.0–5.5 of pH.

In fungi, pollutant dose-dependent growth has been described by Nicolas et al. (2016). They demonstrated

that roundup herbicide (s. a. glyphosate) has an inhibitory effect on *Aspergillus nidulans* (Eidam) G at concentrations 100 times lower than recommended. Nevertheless, Kappas (1988) reported that this herbicide is 100 times less toxic than 2,4-D for the same fungus. In a study on the effect of pesticides in the growth of *L. crinitus*, Machado et al. (2006) reported that the fungus was inhibited by about 34% at the systemic fungicide benomyl concentration of 5 mg L⁻¹, whereas that inhibition of about 66% and 92% was detected when 15 and 50 mg L⁻¹ of the fungicide, respectively, were added to solid medium.

A short lag period of one day was observed by Vroumsia et al. (2005) for *Aspergillus penicilloides* Speng. and *Umberlopsis isabellina* (Oudem.) W. Gams, which exhibited 62% (1.40 mg removed) and 54% (1.20 mg removed), respectively, of 2,4-D depletion after 20 days of cultivation. The absence of lag phase in strains cultivated in culture medium with 2,4-D (Cycoń et al. 2011) or isolated from soil regularly treated with this herbicide (Silva et al. 2007) may indicate that the enzymes or enzyme systems involved in 2,4-D degradation by these strains were produced constitutively,

and it is not necessary a prior acclimatization of the organisms to the compound. On the other hand, in bacteria, even though kinetic models have not been suggested, the production and accumulation of 2,4-DCP have an inhibitory effect by prolonging the lag phase and decreasing the rate of 2,4-D removal (Ning et al. 1997).

Tolerance and growth of WRF to xenobiotic compounds could change depending on their structure and concentration, in addition to the nutritional and the environmental characteristics (Magan et al. 2010). From the study on the effect of atrazine concentration on mycelial growth and the enzymatic activities of eight Basidiomycota (*Cymatoderma elegans* Jungh., *Trametes elegans* Spreng, *Pycnoporus sanguineus* (L.) Murrill strain 1, *Pycnoporus sanguineus* strain 2, *Trametes maxima* (Mont.) A. David and Rajchenb, *Pleurotus* sp. strain 1, *Pleurotus* sp. strain 2, *Pleurotus djamor*), Cupul et al. (2014) reported that in all strains the percentage inhibition of mycelial growth increased as the concentrations of the herbicide increased in the medium. Meanwhile, none of the eight strains of basidiomycetes tested were entirely inhibited by atrazine. *Pleurotus* sp. strain 1 showed the highest tolerance to the herbicide (EC₅₀ of 2281.0 mg L⁻¹) followed by *Trametes maxima* (CE₅₀ of 1532.0 mg L⁻¹) and *P. sanguineus* (CE₅₀ of 1311.0 mg L⁻¹). *Pycnoporus sanguineus* strain 1, *Pleurotus* sp. strain 2 and *P. djamor* were the most susceptible to the herbicide, with EC between 447.3 and 706.1 mg L⁻¹ of atrazine. The tolerance of *Diutina rugosa* (H.W. Anderson) Khunnamw., Jindam., Limtong and Lachance, *Lentinula edodes*, *Penicillium simplicissimum* (Oudem.) Thom and *Pleurotus ostreatus* (Jacq.) P. Kumm to the atrazine herbicide at the final concentration of 10 mg L⁻¹ was tested by Pereira (2011). The tolerant fungi were selected for atrazine degradation tests, and according to the authors, only *P. ostreatus* was able to degrade atrazine (71%), demonstrating that tolerance was not strictly related to degradation.

The production of enzymes responsible for cellulose and lignin degradation in a solid medium has been indicated through colorimetric assays for wild fungal species of *Lentinus* (Atri and Sharma 2011). Although the exposure doses do not result in any visual modification in fungi, metabolic

effects can occur due to pesticide residues (Nicolas et al. 2016). In all our assays, the reaction of Bavendamm (gallic acid oxidation) was positive, evidenced by the formation of a dark brown halo around the colony, which indicates the degradation of gallic acid by oxidative enzymes. No significant differences in mycelial growth and halo size were observed considering the condition in which *L. crinitus* had been maintained before inoculation and cultivation in medium with gallic acid, i.e., with/without addition (treatment) of 2,4-D herbicide by 6, 10 and 15 days (exposition time). In all cases, the reaction halo of the oxidation of gallic acid has expanded over time (Table 4).

The spectrum of secreted proteins by *L. crinitus* comprise CAZymes (active carbohydrate enzymes), oxidase/reductases, proteases and lipase/esterases that provides a diverse enzymatic array with promising applications on several branches of industry (Cambri et al. 2016), and as well as the biomass yield, the secreted enzymes differed considerably among growing conditions (Kirsch et al. 2011, 2013).

As regards pollutant biodegradation, *L. crinitus* strains collected in a region contaminated with/without organochlorine industrial residuals exhibited laccase activity (4.3–5.8 U L⁻¹), but they did not display MnP activity during RBBR decolorization (Machado et al. 2005). Moreira Neto et al. (2009) reported a maximum decolorization (100%) of RBBR by the enzymatic extract of *L. crinitus* with an initial pH between 2.5 and 3.5, concluding that the process is associated with the action of laccase-type oxidases (laccase activity of 28 and 35.0 U L⁻¹, respectively, for each pH value). Niebisch et al. (2010) observed a decolorization of 95% for the textile dye reactive blue 220 (RB220) by *L. crinitus* that was related to biodegradation. They suggested the potential use for this organism in the remediation of real dye-containing effluents. Additionally, studies highlight the possible use of *L. citrinus* ligninolytic enzymes (de Araújo Conceição et al. 2017) and proteases (Machado et al. 2016) for application in agro-industrial waste treatment.

The presence of phenolic compounds, which act as laccase inducers, exhibit distinct effects depending on the fungus species or strain (Piscitelli et al. 2011). *L.*

Table 4 Measurements of the halo diameter of the gallic acid oxidation reaction (mm) as a function of exposition time

Time (h)	6d			10d			15d		
	Control	C1	C2	Control	C1	C2	Control	C1	C2
24	7.0±0.0aA	8.0±0.0aA	7.6±0.5aA	9.3±0.4aA	10±0.0aA	8.0±1.4aA	10.8±0.4aA	11.8±0.4aA	12.0±0.7aA
48	27.0±1.4bB	25.0±0.7bB	27.3±1.1bB	18.5±0.0bC	20.5±0.7bBC	20.8±2.5bBC	25.8±1.1bB	26.5±0.0bB	28.3±3.9bB
72	37.8±0.4cC	35.5±0.7cC	36.8±1.1cC	35.0±0.7cC	37±0.7cC	37.8±1.1cC	37.3±0.4cC	37.3±0.4cC	37.8±0.4cC
96	53.5±1.4dD	51.5±0.7dD	50.5±2.8dD	51.0±0.7dD	51.5±0.0dD	54.3±1.8dD	53.8±0.4dD	54.3±1.1dD	54.8±0.4dD
120	66.3±1.8eE	65.0±0.7eE	66.5±1.4eE	63.5±1.4eE	62.5±0.7eE	67.0±4.2eE	66.0±3.5eE	65.0±0.0eE	65.0±0.0eE

Means followed by different lowercase letters (period) and/or capital letters (treatment) represent significant differences ($p < 0.05$)—Student's *T* test

crinitus showed the highest RBBR decolorization (up to 98%) when grown in the presence of organochlorine compounds (Machado et al. 2005). Oxidation of organochlorine compounds has previously been associated with the increase in WRF's laccase activity for PCP (Jeon et al. 2008), dimethylphenol (Piscitelli et al. 2011), atrazine (Pereira et al. 2013; Cupul et al. 2014) and diuron (Coelho-Moreira et al. 2018). The enzymatic diversity observed in *L. crinitus* revealed the capability to the breakdown and consumption of lignocellulose as well as the biotechnological potential of this fungus (Machado et al. 2005; Cambri et al. 2016). Despite some advances in the knowledge of optimal conditions to produce enzymes in *L. crinitus*, there is a lack of information regarding which enzymes are being stimulated or activated and their relation to organochlorine compounds degradation (Machado et al. 2005) and the effect of cultivation media on laccase production (Valle et al. 2014). On the other hand, Marim et al. (2018) reported maximum laccase activity (25.8–50 U mL⁻¹) at pH between 6 and 7, temperature ranges from 28 to 37 °C and nitrogen concentrations higher than 2.8 g L⁻¹ for two isolated of *L. crinitus*, concluding that laccase production is strain dependent for *L. crinitus*.

The morphological characteristics of *L. crinitus* after 6, 10 and 15 days of culture in control (without 2,4-D), C1 and C2 conditions were analyzed by optical microscopy of the aerial and submerged mycelium from the center and edges of the fungal colony. No significant morphological changes were observed in the mycelial samples from media added with the 2,4-D herbicide treatments (C1: 5.025 g L⁻¹ of 2,4-D and C2: 50.25 g L⁻¹ of 2,4-D) compared to the control treatment. The formation and pigmentation intensity (beige) of the mycelial, as well as the branching pattern of hyphae, were similar in all samples.

A lower proportion of structural (skeletal) hyphae was observed in comparison with the generative hyphae. The structuring hyphae had no coloration and were characterized as long (up to 1000 µm), thin (1–2.5 µm caliber), with no transverse septa and thicker wall. Thick-walled skeletal hyphae were found in all the five wild *Lentinus* species from northwest India (Sharma and Atri 2013).

For both C2 and C1 treatments, we observed generative hyphae with a contorted (twisted) lumen (Fig. 3a) that may represent a fungal response to explore, penetrate or grow in less hostile physicochemical regions. On the other hand, for C2, a shorter septation distance in generative hyphae, that is, more numerous septa, was observed approximately every 20 µm in the exposure time of 15 days.

Both types of hyphae had branched portions. Generative hyphae, with thin to slightly thick walls, were colored due to the cytoplasmic dye (Floxin 1%) used in the preparation of the slides and presented simple septation without clamp connections. Specific sites where the hyphae did not grow

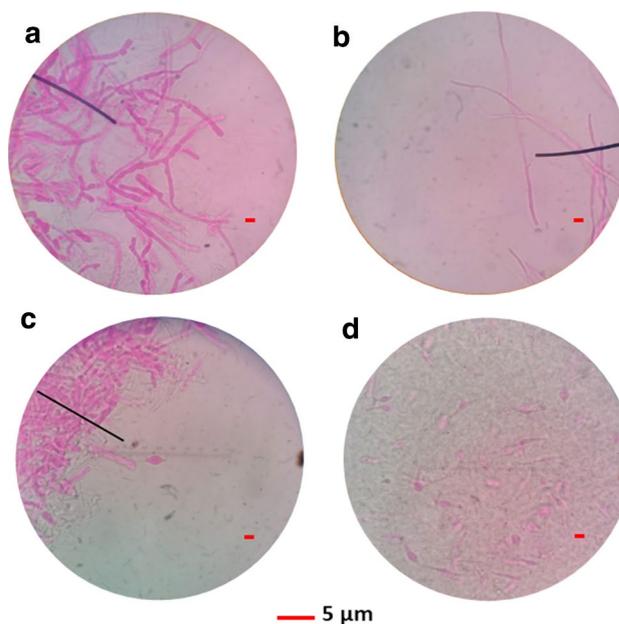


Fig. 3 Optical micrographs of *L. crinitus*. **a** Generative hyphae with a contorted lumen, **b** detail of aborted branches, **c** detail of chlamydospore, **d** chlamydospore densification. Bars = 5 µm

or the process of clamp formation did not occur (aborted branches) were observed (Fig. 3b). This morphological pattern of absence of the clamps was found at all treatments and exposition times, including in control. It would seem to be a condition of the cultivation itself, since it comes from the isolation from the basidioma. Although simple septation is not characteristic of the genus s.l. (Sharma and Atri 2013; Senthilarasu 2015), we observed this behavior to *L. crinitus* (EF58) in culture, and especially regarding this characteristic (loss of clamp connections) do not exist previous records for this species. This pattern in *L. crinitus* EF 58 strain would be associated with the fact of being an isolate of a basidioma collected in 1998 that was replicated several times in culture. The loss of clamp connections has been reported for other genera of Basidiomycetes as *Armillaria* (Fr.) Staude (Larsen et al. 1992), *Lentinellus* P. Karst. (Moreau et al. 2003), and *Sparassis* Fr. (Wang et al. 2004). One possible explanation about this condition is that the clamp connection formation is inhibited by the accumulation of secreted metabolites or an adverse pH shift (Larsen et al. 1992).

In all samples, the presence of chlamydospores (Fig. 3e)—asexual reproductive structures—spherical and cylindrical with equal proportions regarding the location of the same, intercalary, and terminal was detected. These asexual structures grow out on submerged or aerial hyphae (Pöldmaa 2011) and serve as survival structures to overcome periods of unfavorable conditions (Son et al. 2012). It is suggested that both abiotic (temperature and growth media) and biotic (antagonistic microorganisms) determine the

formation of chlamydo spores (Goh et al. 2009). Areas with chlamydo spore densification, especially in samples of mycelium from the edges of the culture plates, were observed (Fig. 3d). The shape and size of the chlamydo spores (length: 7.0–22 μm and width: 3.0–9.0 μm) were similar in all treatments (Table 5).

The type and role of the factors that affect fungal morphology are not yet widely elucidated (Papagianni 2014). The branching density and network characteristics depend on the nutritional and environmental conditions (Islam et al. 2017). The abnormal growth of hyphae characterized by changes in morphology, such as the production of low-branching or unbranched long hyphae, is considered a strategy of fungi to deal with toxic conditions of the environment (Fomina et al. 2003), in which fungi grow by expanding toward less toxic sites and thus responding to environmental stress with minimal expenditure of resources (Ellegaard-Jensen et al. 2013). In this sense, studies on the effects of herbicides on specific microbial species are scarce (Nicolas et al. 2016). Among the results reported, besides growth retardation, glyphosate altered the mycelial organization in *Aspergillus nidulans* (Eidam) G. Winter (Nicolas et al. 2016), while the abnormal mycelial morphology in *Arthrobotrys Corda* fungal isolates caused by the herbicides paraquat, glyphosate, and oxyfluorfen was directly related to their concentration (Mensin et al. 2013). This type of alteration was not observed in the growth pattern of *L. crinitus* in treatments with the addition of 2,4-D at both levels (C1 and C2). In all cases, the mycelium was characterized by a relatively fast growth, with thin and frequently branching hyphae.

Also, the exposure time of *L. crinitus* to 2,4-D had a different effect for the caliber of generative fungal hyphae as well as the chlamydo spore width and length in the treatments without and with 2,4-D addition (Fig. 4) for a total of $n = 15$ measures.

Typically, hyphae have diameters of 1–30 μm , depending on the species and growth environment (Islam et al. 2017). In this study, the caliber of hyphae ranged 2.5–3.5 μm , and the mean value after 6 days did not differ between the treatments compared ($H = 0.87$, $p = 0.63$) (Fig. 4a). After 10 days of contact with both herbicide solutions, C1 and C2, the diameters of these hyphae were significantly higher when compared to the control ($H = 40.97$, $p < 0.001$). However, as the exposure time to the herbicide increases, the mean hyphae size of treatment C1 was not significantly different for the value resulting from the control. As contact time increases, the fungus seemed to acclimatize itself to the culture medium, without presenting quantitative variations in the size of this variable. The average value of generative hyphae is significantly higher when compared to C1 and control ($H = 12.31$, $p = 0.002$) at the highest herbicide concentration (C2), even after 15 days (Fig. 4a).

Table 5 Chlamydo spore shape classification based on length-to-width ratio (Q)

Local-ization	Treat-ment	Exposi-tion time	Ratio Q	SD	Classification	H	p
AB	C1	6	2.17	1.26	Subcylindrical	26.56	0.0008
		10	2.97	0.64	Subcylindrical		
		15	3.21	1.29	Narrowly cylindrical		
	C2	6	2.73	1.54	Subcylindrical		
		10	3.14	0.82	Narrowly cylindrical		
		15	2.45	0.99	Subcylindrical		
	Con-trol	6	2.47	0.78	Subcylindrical		
		10	2.4	0.72	Subcylindrical		
		15	2.17	0.73	Subcylindrical		
AC	C1	6	2.57	0.92	Subcylindrical	24.17	0.0021
		10	2.02	0.52	Subcylindrical		
		15	2.33	0.83	Subcylindrical		
	C2	6	2.44	0.59	Subcylindrical		
		10	2.63	0.62	Subcylindrical		
		15	2.47	1.03	Subcylindrical		
	Con-trol	6	1.89	0.55	Narrowly ellipsoid		
		10	1.72	0.23	Narrowly ellipsoid		
		15	2.21	0.9	Subcylindrical		
SB	C1	6	2.17	0.86	Subcylindrical	18.59	0.0167
		10	2.35	0.8	Subcylindrical		
		15	2.27	0.77	Subcylindrical		
	C2	6	3.05	1.13	Narrowly cylindrical		
		10	2.28	0.46	Subcylindrical		
		15	2.53	0.58	Subcylindrical		
	Con-trol	6	2.65	0.9	Subcylindrical		
		10	2.26	0.63	Subcylindrical		
		15	1.91	0.81	Narrowly ellipsoid		
SC	C1	6	2.39	1	Subcylindrical	20.87	0.0074
		10	2.32	0.77	Subcylindrical		
		15	2.47	0.71	Subcylindrical		
	C2	6	2.33	0.85	Subcylindrical		
		10	2.47	0.71	Subcylindrical		
		15	4.16	5.05	Bacilliform, filiform		
	Con-trol	6	1.87	0.35	Narrowly ellipsoid		
		10	3.02	1.05	Narrowly cylindrical		
		15	3.05	1.25	Narrowly cylindrical		

Localization of mycelium: AC aerial center, AB aerial edge, SC submerged center, SB submerged edge)

Control: without addition of herbicide, C1: addition of 5.025 g L⁻¹ of 2,4-D, C2: addition of 50.25 g L⁻¹ of 2,4-D

In a study on diuron degradation potential, greater hyphae length was observed in five *Mortierella* sp. strains isolated from herbicide treatments (Ellegaard-Jensen et al. 2013).



According to them, the toxicity of this herbicide or its metabolites in relation to *Mortierella* sp. strains cannot be excluded and co-metabolic degradation may represent a detoxification mechanism. When grown in medium containing penconazole, *Aspergillus flavus* Lynk. hyphae generated intermediate and terminal blisters and vesicle, while when grown in cypermethrin medium, their hyphae shrink, deformed and became swollen (Abboud 2014). On the other hand, the growth of the colony and its morphological characteristics (hyaline to pale brown hyphae, septate, branched and anastomosed) of diuron-degrading *Neurospora intermedia* F. L. Tai isolated on diuron-treated soil paired with *N. intermedia* without previous exposition to herbicide (Wang et al. 2017).

Regarding chlamydospore width, there were no significant differences in the mean value of this variable after 6, 10 and 15 days of in vitro cultivation of *L. crinitus* within each treatment, C1 and C2 (Fig. 4b). This means that exposure time would not affect this variable under the same culture conditions with the addition of the 2,4-D herbicide. After 6 days, the width of the chlamydospores corresponding to the C2 treatment was smaller compared to the other treatments ($H=6.44$, $p=0.0330$). However, after 10 and 15 days, the mean value for this variable was not different between the treatments studied ($H=2.44$, $p=0.2708$ and $H=1.67$, $p=0.4033$) (Fig. 4b). Regarding chlamydospore length (Fig. 4c), in each treatment, there were no significant differences during the exposure to 2,4-D ($H=3.43$, $p=0.1778$ for 6 days, $H=5.58$, $p=0.0599$ for 10 days and $H=1.83$, $p=0.3949$ for 15 days). When comparing the different treatments for 6 days, no significant differences were observed between the average values of chlamydospore length ($H=0.31$, $p=0.8568$). Already for 10 days, the values follow an increasing order: $C2 > C1 > \text{Control}$ ($H=17.22$, $p=0.0002$). After 15 days of exposure, the average values of chlamydospore length for C1 and C2 were higher than the value of the control treatment ($H=6.80$, $p=0.0315$). Previous studies with fungi showed different results on the effect of pesticides on the morphological characteristics of the studied microorganisms. Abboud (2014) reported that *A. flavus* failed to form reproduction structures (conidiophores) and sporulate when growing in penconazole medium, while the diameter of conidiophores decreased in size with cypermethrin. On the other hand, it has been reported that *N. intermedia* strains could degrade organochlorine compounds without changing their internal mycelial structure (Wang et al. 2017). According to the authors, *N. intermedia* conidiophores isolated from diuron-treated soil did not differ with the shape and size characteristics of *N. intermedia* cultivated without previous exposition to the herbicide. In addition to the structure sizes, the shape of chlamydospores was described. When considered the length/width ratio (Q) in each condition, the predominant morphology of chlamydospores was subcylindrical (Table 5).

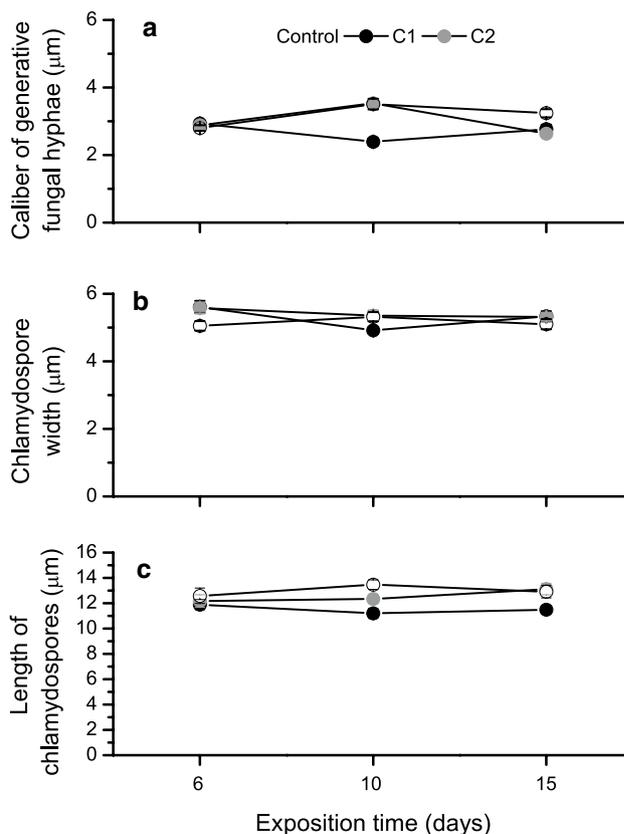


Fig. 4 a Size of the generative hyphae (caliber) over the duration of the treatments, b chlamydospore width over the duration of treatment, c chlamydospore length over the duration of treatment. Control (without addition of herbicide), C1 (5.025 g L⁻¹ of 2,4-D), C2 (50.25 g L⁻¹ of 2,4-D). $N=15$

The bacilliform—filiform category in the C2 treatment (submersed—center) corresponded to the contribution of one (of $n=15$) measure ($22 \times 6 \mu\text{m}$). The production of chlamydospores predominantly subcylindrical, in nature as well as in culture, has been described for the Ascomycota *Cladobotryum* sp. and *Hypomyces* sp. (Pöldmaa 2011). On the other hand, the quotient Q for basidiospores has also been used for micro-morphological characterization of the Basidiomycota *Deconica* sp. ($Q=1.2\text{--}1.6$), *Mycena* sp. ($Q=1.4\text{--}1.8$), *Phaeomarasmius* sp. ($Q=1.8\text{--}2.3$), *Phlebia* sp. ($Q=2.1\text{--}2.4$), *Pluteus* sp. ($Q=1.1\text{--}1.3$), *Postia* sp. ($Q=2.6\text{--}3.1$), *Resinicium* sp. ($Q=2.0\text{--}3.0$), *Scytinostroma* sp. ($Q=1.0$), *Tricholomopsis* sp. ($Q=1.5\text{--}1.8$), and *Tyromyces* sp. ($Q=1.5\text{--}2.0$) (Park et al. 2017).

Despite representing a basis for exploiting fungi for application in environmental cleanup or bioremediation (Carascal et al. 2017), the biological tolerance to a specific chemical pollutant not necessarily is associated with the possibility of performing metabolic detoxification of the compound (Pereira 2011). In a study over the ability of WRF to remove pentachlorophenol (PCP), Chiu et al. (1998) showed that

tolerance level of certain fungi toward PCP did not correlate with its degradative capacity. Although *Armillaria mellea* (Vahl) P. Kumm. was characterized as slow growers due to taking 36–54 days to cover a 9-cm plate fully, this strain showed a great capacity for PCP degradation. Meanwhile, *Polyporus* sp. was considered a fast grower (4–10 days) that showed a great ability for biosorption.

As regards 2,4-D, tolerance and degradation capabilities have been described for many species of basidiomycetes as *L. edodes* (Tsujiyama et al. 2013), *Phanerochaete chrysosporium* (Burds.) Hjortstam and Ryvarden (Valli and Gold 1991; Donnelly et al. 1993; Yadav and Reddy 1993), *Gautieria crista* E. L. Stewart and Trappe, *Gautieria othii* Trog, *Schenella simplex* T. Macbr., *Rhizopogon vinicolor* A. H. Sm., *Sclerogaster pacificus* Zeller and C. W. Dodge, *Trappea darkeri* (Zeller) Castellano (Donnelly et al. 1993), *Dichomitus squalens* (P. Karst.) D. A. Reid (Reddy et al. 1997), and *Trametes versicolor* (L.) Lloyd (Hernández Mendieta et al. 2013).

Herbicide tolerance is generally regarded as the absence of general toxic effects at the macroscopic level, ignoring potential cellular effects if they do not result into visible consequences (growth, morphology, yield) (Nicolas et al. 2016). Analysis of the ultrastructure in PCP-tolerant fungi showed that at concentrations of 100 mg L⁻¹ this compound produced the collapse of fungal structures, hyphae and spores, in *Trichoderma* Pers. and *Cunninghamella* Matr. strains (Sing et al. 2014). Despite these morphological alterations, *Cunninghamella* sp. UMAS SD12 has shown the highest growth rate and tolerance to PCP and removed up to 51.7% of this organochlorine after 15 days. The fact that an organism tolerates or degrades an organochlorinated compound does not mean that it does not suffer morphological or physiological alterations due to exposure to it. A recent study has postulated that pesticides can affect the resistance of a human pathogen fungus (genus *Cryptococcus*) by altering the fungal morphology and causing tolerance to the clinical drug fluconazole (Bastos et al. 2018).

Conclusion

The agar-plate culture system was efficient in evaluating the tolerance of fungi to the herbicide 2,4-D. To the best of our knowledge, this is the first study that has demonstrated the potential of *L. crinitus* to tolerate this pesticide in a wide range (5.025–670 g L⁻¹) when added in medium with agar what represents the starting point of bioremediation process with practical applications.

Previous work on organochlorine removal by fungi focused primarily on its potential as a degradation agent. In contrast, less is known about the morphological and physiological effect of these chemical compounds on the ultrastructure of microorganisms.

We did not find significant morphological alterations of the fungal structure sizes (hyphae and chlamydo-spores) during the experiment—the similar morphological responses of *L. crinitus* after cultivation with or without 2,4-D, suggesting that there was not deleterious effect on the ultrastructure of this fungi due to the presence of 2,4-D in the culture medium. The absence of clamps in *L. crinitus* culture is not a consequence of 2,4-D exposition, and this characteristic was also observed in control assays. To the best of our knowledge, this is the first record of loss of clamp connections for *Lentinus* species in cultures. Few studies investigate the mycelium characteristics after herbicide exposition regarding reproductive structures, hyphae feature and exposition time–structure property relationships, which are relevant to a variety of applications of mycelium as is the case of a bio-reactor for the treatment of contaminated effluents.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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