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Removal of Diuron by a Laccase Rich Crude Extract from *Ganoderma lucidum*

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RESUMO

Diuron is a phenylurea herbicide commonly used on non-crop areas and on a wide variety of agricultural sites, such as sugar-cane fields. Inappropriate handling of pesticides bears risks of contamination of the environment. The aim of this work was to evaluate the capability of a laccase rich crude extract from the white rot fungus Ganoderma lucidum to catalyze the degradation of diuron. A significant reduction of the diuron (more than 60%) was obtained after in vitro treatment with crude enzymatic extract. The results suggested that laccases played an important role in the biodegradation of diuron by G. lucidum. No known intracellular diuron metabolites such as N´-(3,4-dichlorophenyl)-N-methylurea (DCPMU), 3,4-dichlorophenylurea (DCPU) and 3,4-dichloroaniline (3,4-DCA) were detected in the culture filtrates. The apparent absence of these transformation products might be indicating that the crude laccases do not act through demethylation pathways.

Key words: diuron, laccase, fungus, *Ganoderma lucidum*.

INTRODUÇÃO

Diuron, N-(3,4-dichlorophenyl)-N,N-dimethylurea, is a phenylurea herbicide commonly used on non-crop areas such as home gardens and roads and on a wide variety of agricultural sites, such as sugar-cane fields, an important economic crop in Brazil (Giacomazzi and Cochet 2004). Unfortunately, inappropriate handling of pesticides during activities of this type bears risks of contamination of several environments, such as soil, air and water, and foods, not only by diuron but also by its metabolites, N´-(3,4-dichlorophenyl)-N-methylurea (DCPMU), 3,4-dichlorophenylurea (DCPU) and 3,4-dichloroaniline (3,4-DCA)

In the last years, technologies based on the application of microorganisms to reduce the contamination of the environment have received much attention, mainly because these are highly efficient, ecologically responsible and cost-effective methods (Watanabe 2001). Among the microorganisms that are used, white rot fungi are of special interest due to their complex enzymatic system which confers them a wide metabolic capacity. White rot fungi belong to the Basidiomycete group and possess an extensive extracellular enzymatic system (the ligninolytic system) used in the degradation of their natural substrates. In addition, they are able to degrade abroad range of pollutants, including low-solubility pesticides. *Ganoderma lucidum* is a widely distributed white rot fungi which possess a great ability to produce extracellular enzymes, especially laccases and Mn peroxidases as part of its

ligninolytic system (Mota et al. 2014). Within this context, the aim of this research was to evaluate the diuron degradation by rich laccase crude extracts from *G. lucidum.*

MATERIAL AND METHODS

Diuron (≥98%) was purchased from Sigma Chemical Corp. (St Louis, MO) and its stock solutions were prepared by dissolving diuron in dimethyl sulfoxide **(**DMSO), filtering through a Millipore membrane (0.45 mm) and storing at 4 ºC.

Ganoderma lucidum was obtained from the Culture Collection of the São Paulo Botany Institute and cultured on potato dextrose agar Petri dishes (PDA) for up to 2 weeks at 28 ºC. After the complete growth, mycelial plugs measuring 15 mm in diameter were made and used as inoculum for liquid cultures.

For the production of laccase, *G. lucidum* was cultivated in liquid mineral medium containing 1% glucose as carbon source and 3% corn cob extract rich in phenolic compounds as laccase inducer under static conditions (Souza et al. 2011). At periodic intervals, the cultures were interrupted by filtration, and the culture filtrates used to determine laccase and manganese peroxidase activities.

The laccase activity was determined with 2,2'-azino-di-(3-ethylbenzothialozin-6-sulfonic acid) (ABTS) as the substrate in 0.05 M sodium acetate buffer (pH 4.0) at 40ºC. Oxidation of ABTS was monitored as absorbance increase at 420 nm (ε_{420} = 36,000 $M^{-1}cm^{-1}$). The MnP activity was assayed spectrophotometrically by following the oxidation of $MnSO_4$ in malonate buffer in the presence of H_2O_2. One unit (U) of enzymatic activity was defined as the amount of enzyme required to produce 1 μmol product per min and was expressed as U/L.

The potential of crude laccase to degrade diuron was evaluated using 15-day culture filtrates according to the protocol described in Coelho et al. (2010). The reaction mixture (2.0 mL) contained 3.5 μg/mL of diuron (final concentration) in acetate buffer 50 mM, pH 4.0. The effects of $MnSO_4$, ABTS, Tween 80, and H_2O_2 were tested separately or in combination. After 24 h, the reaction mixtures were filtrated with a membrane filter (0.45 μm) before the HPLC analyses.

The concentrations of diuron and metabolites were determined using a HPLC system (Shimadzu, Tokyo) with a LC-20AT Shimadzu system controller, Shimadzu SPD-20 A UV–VIS detector, equipped with a reversed Shimpack C18 column (4.6 x 150 mm), maintained at 40 ºC (Coelho-Moreira et al. 2013). The concentrations of diuron and its metabolites were determined using calibration curves constructed with peak areas of authentic standards (diuron, DCPMU, DCPU, and DCA). The identification of the compounds was based on their respective retention times and on the fortification of the samples with standards. Under the conditions employed, diuron was eluted at 11.6 min, DCPMU at 10.6 min, DCPU at 9.5 min, and 3,4-DCA at 12.1 min.

RESULTS AND DISCUSSION

Ganoderma lucidum was able to grow in glucose liquid state cultures using corn cob as inducer of lignolytic enzymes. In these cultures, the maximal laccase activities were 1,400 U/L. On the other hand, the very low Mn peroxidase activities that were found never exceeded 2 U/L (Figure 1).

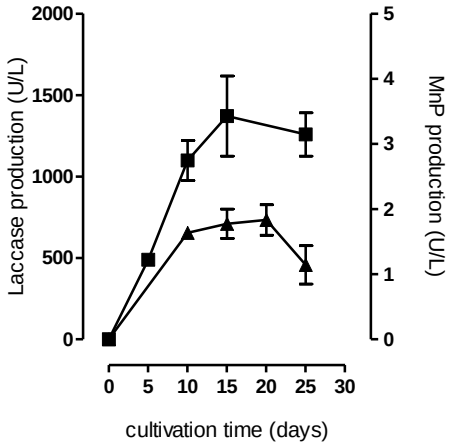

Figure 1. Time course of laccase and Mn peroxidase productions by *Ganoderma lucidum*.

The *in vitro* experiment using the crude filtrate containing high laccase activity demonstrates that all the treatments presented a significant reduction in the diuron concentration as compared to control (boiled crude filtrate) and that there was a synergistic effect of ABTS, Mn^{2+}, H_2O_2, and Tween 80 (Table 1). However, the crude filtrate alone was also able to promote diuron removal. This fact can indicate that diuron is a substrate for laccase, since the process occurs in the absence of mediators. Furthermore, it is important to observe that the presence of tween 80 in association with $MnSO_4$ resulted in an improvement of the diuron degradation, even without H_2O_2. It is well documented that laccase and MnP are able to promote lipid peroxidation generating lipid peroxyl or alkoxyl radicals, which are highly reactive and able to promote the oxidation of other molecules, including pesticides (Pizzul et al 2009; Castillo et al. 2000).

Table 1. Degradation of diuron by the enzymatic crude filtrate from *G. lucidum* after 24 h.

Treatment	ABTS (1mM)	$MnSO_4$ (1 mM)	H_2O_2 (0.05 mM)	Tween 80 (1% v/v)	Crude filtrate (*)	Recovered diuron (µg/mL)**
1	+	+	+	+	-	3.43 ± 0.25 [(a)]
2	-	-	-	-	+	1.39 ± 0.07 [(b)]
3	+	-	-	-	+	2.35 ± 0.33 [(b)]
4	-	+	-	+	+	1.41 ± 0.02 [(b)]
5	-	+	+	-	+	2.37 ± 0.80 [(b)]
6	-	-	+	-	+	2.23 ± 0.04 [(b)]
7	+	-	+	-	+	2.41 ± 0.26 [(b)]
8	+	+	+	+	+	1.87 ± 0.37 [(b)]
9 (#)	+	+	+	+	+	3.87 ± 0.31 [(a)]

(*) 1,500 U/L of laccase. All treatments contained diuron (3.5 µg/mL) and sodium acetate buffer 50 mM, pH 4.0; (#)Treatment 9 was performed using boiled crude enzyme. Degradation values are means ± SD (n = 3); ** different letters indicated significance statistic ($p \leq 0.05$).

Under *in vivo* conditions, two main reactions are responsible for the aerobic degradation of diuron. The first one consists in successive N-demethylations, which generally produce two metabolites, DCPMU and DCPU. The second one is the hydrolysis of the amide bond generating the metabolite 3,4-DCA (Giacomazzi and Cochet 2004). Although the crude laccase was able to reduce the diuron concentration in the *in vitro* experiments, no transformation product of any kind could be detected with the analytical methods that were employed. The apparent absence of these transformation products might be indicating that the extracellular enzymes act by pathways that are different from those of the intracellular enzymes (Coelho-Moreira et al. 2013). This raises the possibility that the filtrate may contain other enzymes that were not the object of the present study.

CONCLUSION

The results obtained in this research show that the laccase rich crude extract of *G. lucidum* cultures can be useful in the control of environmental pollution caused by diuron. Further studies, using purified enzymes from *G. lucidum*, are necessary to elucidate the types and toxicity of the reaction products produced under these conditions.

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