Pesticide Biochemistry and Physiology 105 (2013) 84–92

Contents lists available at SciVerse ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Response of *Ganoderma lucidum* and *Trametes* sp. to the herbicide picloram: Tolerance, antioxidants and production of ligninolytic enzymes

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ARTICLE INFO

Article history:
Received 13 September 2012
Accepted 18 December 2012
Available online 27 December 2012

Keywords:
Herbicide
Ligninolytic enzymes
Oxidative stress
White rot fungi

ABSTRACT

In this work the effects caused by 0.1 mM picloram on the white-rot fungi *Ganoderma lucidum* and *Trametes* sp. were studied. At this concentration the herbicide caused an increase in the dry mycelial biomasses as well as in the protein and polyssacharide contents, suggesting an auxin-like action. Picloram also caused oxidative stress which was more accentuated in *Trametes* sp. The latter was indicated by increases in the levels of catalase, superoxide dismutase, reactive oxygen species, and phenolics. Picloram caused an increase in the laccase activity in *Trametes* sp. (from 93.0 ± 12.0 to 180.0 ± 2 3.0 U/g dry biomass), but an unusual reduction in *G. lucidum* (from 70.2 ± 6.2 to 53.2 ± 8.9 U/g dry biomass). Both fungi showed a peculiar mechanism of transitory bioaccumulation of picloram, but none of them was effectively able to degrade the herbicide.

1. Introduction

The steadily advancing increase of production and application of pesticides for agriculture as well as for plant protection and animal health has caused the pollution of soil, ground and surface water. This obviously involves a serious risk to the environment and also to human health due to direct exposure or through residues in food and drinking water [1].

Picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) is a systemic chlorinated herbicide, known as a synthetic auxin. This herbicide is widely used in many countries because of the high degree of efficacy in the control of broadleaf weed and woody plants in pasture, wheat, rice, barley, sugarcane and other crops [2]. Picloram is relatively water soluble (430 μg mL^{-1}), weakly adsorbed in soil and slowly degraded in the environment, becoming quite persistent and highly mobile in soil and water [3]. As a result of agricultural application and improper waste disposal, picloram may remain active in the environment from months to years (average half-life in soil of 90 days) constituting a risk to non-target sites and organisms.

Herbicides are in general designed and applied for the control of specific plants but they may affect non-target organisms by interfering with respiration, cell growth, division and molecular composition [4]. Changes in the structure of microbial communities may occur resulting in important ecological outcomes. Microbial diversity and nutrient cycling may be affected as metabolic processes are enhanced or inhibited. In fact, picloram can exert toxic effects on soil microorganisms [5].

White rot fungi are a fundamental part of forest ecosystems. This group of fungi is essentially composed of basidiomycetes capable of completely degrading lignin by producing ligninolytic enzymes such as laccases, lignin peroxidases, manganese peroxidases, and versatile peroxidases. These enzymes are known to be relatively non-specific and, thus, capable of catalyzing the degradation of a wide variety of recalcitrant pollutants structurally similar to lignin. Fungi can sense and induce appropriate cellular responses to many components of their environment such as nutrients, temperature, pheromones, light, and also xenobiotics [6]. A proper response to various environmental factors is required for fungal survival, particularly under stressful conditions. As all aerobic organisms, the white-rot fungi produce reactive oxygen species (ROS), including superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). However, during their physiological life cycle, the white-rot fungi are in a certain way dependent on relatively high concentrations of ROS. The latter, together with the ligninolytic enzymes, are responsible for the

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http://dx.doi.org/10.1016/j.pestbp.2012.12.002

ability of white-rot fungi to penetrate lignified cell walls in sound wood [7–9].

It is well-known that the organisms have developed efficient antioxidative protection systems consisting of enzymatic and non-enzymatic elements [10]. However, relatively few studies on the cellular responses and defense mechanisms of white rot fungi towards organopollutants have yet been published [6,11–13]. Consequently, knowledge of the interaction and responses of white rot fungi to herbicides is quite fragmentary. Besides, characterization of cell growth and metabolites production is fundamentally important to develop efficient bioremediation strategies for the removal of recalcitrant pollutants and waste treatment and to understand microbial biochemistry, physiology and ecology [14].

Research on strategies for degradation of picloram have focused on the application of microbial (bacteria and yeast) or non-microbial systems such as electro-Fenton process, photocatalytic, and zero-valent iron powder [15–19]. However, there is no published information about the application of filamentous fungi, such as white rot fungi, for the removal of picloram. In view of the above considerations, this paper reports experiments about the ability of two white rot fungi, *Ganoderma lucidum* and *Trametes* sp. to tolerate picloram. It also reports data on picloram removal as well as a comparison of the production of biomass, enzymes and other metabolites in the presence and absence of the herbicide.

2. Materials and methods

2.1. Organisms

The fungal strain *G. lucidum* was obtained from the culture collection of the Brazilian Agricultural Research Corporation (EMBRAPA), Colombo, Paraná, Brazil. This fungus was selected due to its capacity to degrade the model compound Poly R-478 in agar plates and also tested positive for the Bavendamm's reaction. The other white rot fungus used in this work was collected from decaying wood in a rural area with intensive agriculture (mainly soybean and corn crops) and history of herbicides application located in the region of Mamborê city (Paraná, Brazil). The strain denominated M3 was isolated at the Laboratory of Biochemistry of Microorganisms in the Maringa State University (UEM, Maringá, Brazil) by plating basidiome sections of the inner tissues previously washed with sterilized water on potato dextrose agar (PDA) plates containing 0.1% tetracyclin solution (sterilized in 0.45 μm pore size membrane-Millipore). The plates were incubated at 28 °C. The identification of the isolate was performed by molecular methods at the Division of Microbial Resources of the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA) at the Campinas State University (UNICAMP, Campinas, Brazil). Genomic DNA was isolated according to the method of Raeder and Broda [20]. The D1–D2 regions (DNAr 28S) from the strains were amplified by PCR using the NL1 and NL4 primers. Sequencing of the purified PCR products (GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) was performed in a MegaBACE 1000 automated sequencer (GE Healthcare) with NL1, NL2, NL3 and NL4 primers. Partial sequences of D1-D2 region obtained with different primers were used to assemble a contig sequence which was compared to sequences of reference strains from GenBank and CBS (Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre). The sequences were aligned with CLUSTAL X [21] and phylogenetic analyses were performed with the MEGA version 4.0 computer program [22]. Estimation of evolutionary distance was carried out using the Kimura two-parameter distance model [23] and the phylogenetic tree was constructed by the neighbor-joining method [24]. Bootstrap analysis was performed with 1,000 replicates. According to the result of phylogenetic tree analysis, the strain M3 is on the 100%-bootstrap-supported branch with *T. versicolor* and *T. suaveolens* and was identified as *Trametes* sp. The strains were maintained on 2% malt extract agar (MEA) plates grown at 28 °C and stored at 4 °C.

2.2. Effect of picloram concentration on surface fungal growth

Inoculum (8 mm disk) from a 14-day-old culture of both fungi was inoculated onto the center of MEA plates containing different concentrations (0–1.0 mM) of picloram. All plates were incubated at 28 °C for up to 14 days. Colony diameter was measured every day during the incubation period.

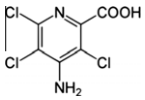
2.3. Culture conditions and degradation experiment

Cultivation was carried out in Erlenmeyer flasks of 125 mL containing 25 mL of medium composed of 1% glucose and mineral salt solution [25]. Each flask was loosely plugged with cotton wool for passive aeration. The experiments with picloram (Table 1) were conducted by adding 0.1 mM of the herbicide to the 25 ml Erlenmeyer cultures. Picloram stock solutions were prepared in distilled water and sterilized in 45 μm Millipore membranes. Flasks were inoculated with 3 agar plugs (9 mm diameter) cut out from the margin of the mycelia grown for seven days in malt extract agar (MEA) plates. Incubation was performed in the dark at 28 °C under stationary conditions for a maximum period of 10 days. Un-inoculated and heat-killed controls were also included. Percent degradation at a specified interval was calculated by comparing concentration in the un-inoculated controls with those in the experimental flasks. Dry weight of the mycelia was determined after harvesting the cultures at different incubation periods, vacuum filtering through pre-weighted filter paper using a Büchner funnel and drying at 60 °C in an oven. Biomass dry weight was expressed as mg per 25 mL of liquid medium. Culture filtrates were used as enzyme sources and for HPLC, exopolysaccharide and total phenolics analyses. All the experiments were carried out with at least duplicate parallel cultures. Data represent the mean values ± standard deviation values.

2.4. Preparation of mycelial extracts

The mycelia grown in picloram and control cultures were harvested by filtration, weighted and washed with cold 50 mM potassium phosphate buffer (pH 7.5) and then homogenized in the same buffer by a glass, motor-driven Potter's homogenizer (TE-099,

Table 1
General properties of Picloram.

Common name	Chemical name (IUPAC)	Molecular weight	Chemical structure	Water solubility at 25 °C	pK_a	Mode of action
Picloram	4-amino-3,5,6-trichloropyridine-2-carboxylic acid	241.5		430 mg/L	2.3 (acid)	Auxin mimic

Data source: [62,63]