Enhanced mineralization of lindane in soils supplemented with the white rot basidiomycete Phanerochaete chrysosporium

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ENHANCED MINERALIZATION OF LINDANE IN SOILS SUPPLEMENTED WITH THE WHITE ROT BASIDIOMYCETE *PHANEROCHAETE CHRYSOSPORIUM*

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Summary

Biotransformation of the insecticide lindane was investigated in sterilized and non-sterilized soils, each supplemented or not by the white rot basidiomycete *Phanerochaete chrysosporium.* After spore inoculation of the sterilized soil, the fungal biomass increased rapidly during the first week, then proceeded at a lower rate during the following 8-wk. Conversely, only limited fungal growth was detected in non-sterile soils. Lindane mineralization, as a result of indigenous microflora activity, was increased following fungal inoculation. Then, extractable amounts of lindane were quite similar in soils, with or without fungal addition. Mass-balance and SPME analysis revealed the volatilization of degradation intermediates only from non-sterilized and non-inoculated soil. *P. chrysosporium* seemed to modify lindane degradation pathway by increasing the conversion of volatile intermediates to CO_2.

Introduction

Because of an extensive use of lindane worldwide and in France, residual pesticide is commonly detected in food, living organisms, soils and surface waters (Belamie, 1992; Deo et al., 1994; de Cruz et al., 1996). Soil bioremediation is an effective means to reduce the concentration of pesticides in the environment. For many years, various research groups have been interested in the development of white rot fungus technology for th biodegradation of organic environmental pollutants. Among these fungi,

Phanerochaete chrysosporium has been extensively studied because of its ability to degrade a wide variety of organic pollutants (Barr and Aust, 1994). *P. chrysosporium* was able to mineralize the chlorinated insecticide lindane (1α,2α,3β,4α,5α,6β-hexachlorocyclohexane, CAS no. 58-89-9) in liquid cultures (Bumpus et al., 1985; Kennedy et al., 1990). However, little is known about the mechanisms involved in the lindane breakdown by the fungus. Mougin et al. (1996) shed some light on these mechanisms, by identifying the main metabolites during lindane breakdown in *P. chrysosporium* liquid cultures. They suggested the involvement of P450s in some reactions leading to the insecticide degradation. In soils, Kennedy et al. (1990) observed a significant mineralization of lindane over 60 d following inoculation with the fungus established on corn-cobs. We have evaluated the effect of bioaugmentation on lindane transformation in sterile and non-sterile soils using *P. chrysosporium*.

Materials and methods

Soil preparation and incubation

Soil samples were collected in early March 1994, in the 10-20 cm layer in Versailles (France). Soil was a silt loam, comprising 25.5% sand, 55.0% silt and 19.5% clay. The content of organic matte in the soil was 1.65%. The soil pH was 8.1, and the cation exchange capacity was 10.2 meq 100 g^{-1} The soil was gently air-dried for 4-5 d in the laboratory, sieved (2 mm) and stored at l 5°C for less than 2 months. Before use for experimental work, a portion of the soil sample was sterilized by autoclaving at 121°C for 20 min.

Soil incubations were done using 25 g dry soil in 150-ml Erlenmeyer flasks, inoculated with spores of *P. chrysosporium* BKM-F-1767 (ATCC 24725). An aliquot of 7-8 ml of culture medium (Mougin et al., 1994) was added to achieve 35% (w/w) soil moisture content. The moisture content was kept constant during the experiments by adding sterile water. The medium was supplemented with a mixture of unlabelled and labelled (3.3 kBq) lindane (l191 MBq mmo1^{-1}, radiochemical purity > 99.5%, Rhone-Poulenc Agrochimie, Lyon, France) to give a final concentration of 0.8 µg g^{-1}. The Erlenmeyer flasks were sealed with cotton plugs and incubated in 1-1 sealed flasks with three vials containing 10 ml of I N NaOH (to trap CO_2), 10 ml water (to keep moisture constant), and 10 ml n-hexane (to trap volatile organics), respectively. The headspace of the flasks

was flushed with air at the begining of the experiments, and then every 7 d. $^{14}CO_2$ production and lindane degradation were assessed for 9 wk. Samples were incubated in darkness at 25°C.

Fungal biomass determination

Fungal biomass in soils was estimated from extractable ergosterol, according to the modified method of Seitz et al. (1977). Each flask was supplemented with 150 ml methanol, 50 ml ethanol and 20 g KOH, and refluxed for 60 min at 90°C. The cooled saponified mixture was filtered on a Buchner funnel, prior to clean-up. Twenty millilitres of the filtrate were applied on to a glass column packed with a small wad of glass wool and 20 g Extrelut (Merck, Chelles, France). The column was left for 30 min before elution in darkness with 90 ml n-hexane. The organic fraction was evaporated to complete dryness. The residue was dissolved in 20 ml ethanol and sonicated for 1 min. Ergosterol in the ethanolic solution was quantified by HPLC. Usually, l00 µl of the solution were injected through a 7125 Rheodyne valve onto the analytical column ODS-80TM (25 cm x 4.6 mm i.d.) (Varian). A Varian 9010 pump delivered the mobile phase consisting of the mixture acetonitrile-ethanol-H_3PO_4 (55/45/0.05; v/v/v) at a rate of 1 ml min^{-1}. Detection was done in a Varian 9050 detector at 282 nm. A calibration curve was made with authentic ergosterol (Fluka, St. Quentin Fallavier, France), and a correlation curve between dry fungal biomass and culture ergosterol content was established. This gave a linear equation Y (biomass d.w., µg) = 1.9331 + 0.1892X (ergosterol, µg) with a regression coefficient R = 0.992 (data not shown). The ergosterol-to-biomass ratio was around 5.0 mg g^{-1}, in agreement with the reported value of mg g^{-1} for *P. chrysosporium* cultures (Davis and Lamar, 1992).

Analytical procedure

The radioactivity in soils samples was extracted by adding 50 ml of methanol- water (80/20; v/v) and shaking for 60 min. The liquid and solid phases were separated by centrifugation, and the solid fraction was extracted a second time. The extracts were pooled, and 80 ml aliquots were diluted with 240 ml of distilled water in a separatory funnel, and then the radioactivity was extracted three times with 100 ml of dichloromethane. After concentration of the organic phase and dissolution in 500 µl methanol, 100 µl aliquots were analysed by HPLC.

Labeled compounds were analysed with the HPLC equipment described above, with a solvent system composed of acetonitrile and water, each acidified with 0.1% H_3PO_4; it began with 1% acetonitrile for 3 min, followed by a linear increase to l00% acetonitrile over 15 min, and a stationary phase of 10 min. The radioactivity of the column eluate was monitored by an HPLC LB 507 A radioactivity system (EG and G, Evry, France). Non-extractable radioactivity in the soil samples was determined by combustion in a model 307 oxidizer (Packard Instruments, Rungis, France).

Experimental error

Each experiment was done in duplicate and repeated twice. Results are expressed as means. The standard deviation, excepted in the case of unextractable radioactivity in sterile soils, was less than 10% of the mean. Data presented in Table 1 were submitted to ANOVA to test for differences among soil treatments. In the case of significant differences, means were then compared using the Newmann-Keuls test (Snedecor and Cochran, 1967).

Results and discussion

Figure 1(A) shows the growth curves for *P. chrysosporium* in soils. No fungal growth was observed in sterile and non-sterile soils supplemented with culture medium alone. After spore inoculation of a sterilized soil, the fungal biomass increased rapidly during the first week, and then proceeded at a lower but steady rate during the following 8 wk to a final biomass value of 112.4 mg d.w. The biomass in soil was found to be consistent with the proliferation of white mycelium observed throughout the soil. Conversely, only a limited fungal growth (12.l mg d.w.) was detected in non-sterile soils after inoculation with *P. chrysosporium*. The amount of fungal biomass decreased slowly to 6.8 mg by the end of the experiment. The low fungal biomass in non-sterile soil is due to the competition of the fungus with the indigenous microflora. In general, the fungus is grown on organic substrates, such as corn-cobs, before inoculation to soil. Our results indicate that a liquid inoculum, consisting of culture medium supplemented with fungal spores, allows fungal growth in soils, with a uniform distribution of the mycelium. That might ensure a greater pesticide transformation.

After a short lag phase, the released CO_2 resulting from indigenous microflora activity

amounted to 21.6% of the initial radioactivity after 9 wk [Fig. l(B)]. This increased to 49. l% following fun- gal inoculation. In contrast, no or negligible mineralization (less than 1.0%) occurred in autoclaved soils, with or without *P. chrysosporium*.

Effects of sterilization and fungal inoculation on lindane dissipation are shown Fig. l(C). The amount of extracted lindane was found to be considerably reduced in the non-sterilized soils (8.0 and 7.3% of the initial radioactivity), whether with or without the fungus addition. By contrast, much higher amounts of extractable lindane remained in autoclaved soils (67.6 and 71.1%), and appeared almost unaffected by the presence of *P. chrysosporium*. No metabolites formed from lindane have been extracted from any of the sterile and non-sterile samples.

Mass-balance analysis of 9-wk *P. chrysosporium* incubations are shown in Table l. In sterilized soils, total 14 C recovered was 81.0-83.0% of the initial radioactivity. The loss of 14C (around 20.0%) could be partly due to adsorption to the glass flasks. Although a high soil moisture content may favour lindane volatilization (Waliszewski, 1993), labeled volatiles were not trapped under these incubation conditions. Conversely, in non-sterilized and non-inoculated soil, total 14C recovered represented only 52.4% of the initial radioactivity. An explanation for this loss of radioactive material could be the volatilization of degradation intermediates, as 2.6% of the initial radioactivity were counted during the 9-wk incubations in hexane-containing traps. In agreement with this result, microbial degradation of lindane has been reported to produce volatile transformation products, such as low chlorinated benzenes and phenols, and chlorine-free compounds (Deo et al., 1994). Headspace solid-phase microextraction (SPME) and g.c. analysis (either with f.i.d. or e.c.d.; de Cruz et al., 1996) were performed. Non-chlorinated and chlorinated compounds, more polar than lindane, were detected under these conditions. However, their concentrations were too low for identification by mass spectrometry. Fungal in-oculation of non-sterilized soil led to a better 14C recovery, whereas no volatiles were trapped under these conditions. The fungus seems to be involved in the modification of the insecticide degradation pathway, by increasing the conversion of volatile chemical intermediates produced by the indigenous microflora to CO_2.

The above results confirm that the bioremediation of soils may be achieved by using a bioaugmentation approach with *P. chrysosporium*. The fungus appears to be a potential tool to reduce the environmenal contamination by xenobiotics. At least, in the case of

lindane, it may establish synergistic relationships with the indigenous microbial community.

Acknowledgements

We gratefully acknowledge Rhone-Poulenc Agrochimie (Lyon, France) for the generous gift of labeled lindane.

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Table 1. Mass-balance analysis of ^{14}C in soil cultures after 9-wk incubations in the presence of labeled lindane.

Treatment	Fraction (% of initial radioactivity)[a]				
	Mineralized[b]	Extractable[c]	Non-extractable	Volatiles	Total
Sterile—not inoculated	0.0	77.7 a	5.2	n.d.	82.9 a
Sterile—inoculated	0.7	74.3 a	6.4	n.d.	81.4 a
Not sterile—not inoculated	21.6 a	9.7 b	18.4	2.6	52.4 c
Not sterile—inoculated	49.1 b	9.1 b	14.4	n.d.	72.6 b

n.d., not detected.
[a]Within a column, means followed by the same letter do not significantly differ at $P = 0.05$.
[b]Data from sterile soils are not taken into account for ANOVA.
[c]Values are corrected with minor amounts of ^{14}C recovered after a 24-h soil extraction following the two extractions of 60 min.

Fig. 1. Fungal growth (A), lindane mineralization (B) and extraction (C) in soil cultures. Symbols refer to soil treatments: O, sterile-not inoculated; ●, sterile-inoculated; △, not sterile-not inoculated; and ▲, not sterile-inoculated.