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Screening of white rot fungal species for their capacity to degrade lindane and other isomers of hexachlorocyclohexane (HCH)

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Abstract

J.C. Quintero, M.T. Moreira, G. Feijoo, and J.M. Lema. 2008. Screening of white rot fungal species for their capacity to degrade lindane and other isomers of hexachlorocyclohexane (HCH). Cien. Inv. Agr. 35(2):159-167. White-rot fungi have demonstrated a high capacity to degrade organic pollutants, including the insecticide lindane (γ-HCH). The purpose of this study was to evaluate the degradative capacities of several white rot fungi species, *Bjerkandera adusta, Irpex lacteus, Lentinus tigrinus, Phanerochaete chrysosporium, Phanerochaete sordida, Phlebia radiata, Pleurotus eryngii, Poliporus cialatus,* and *Stereum hirsutum.* Fungal tolerance to various concentrations of α-, β-, γ- and δ isomers of hexachlorocyclohexane (HCH) was studied in both liquid and soil media samples. δ- and γ-HCH isomers showed the highest inhibition of fungal growth of all HCH isomers. *P. chrysosporium* and *B. adusta* exhibited a high tolerance to HCH pollution. The δ- and γ-HCH isomers were degraded between 15.1 and 70.8% by six of the nine fungal species, *B. adusta, P. ciliatus, L. tigrinu, S. hirsutum, P. eryngii*, and *I. lacteus*; β-HCH was 56.6, 26.5 and 23.9% degraded by *B. adusta, P. ciliatus* and *P. eryngii*, respectively. In non-sterile soil, all the HCH isomers were degraded between 8.2 and 17.5% by *B. adusta* immobilized on corncobs or woodchips. In nonsterile soil, other soil microflora showed an antagonistic effect on white-rot fungi catalyzed degradation of HCH isomers.

Key words: Hexachlorocyclohexane, degradation, pesticides, *Bjerkandera adusta,* white rot fungi.

Introduction

Hexachlorocyclohexane (HCH) is a mixture of chemical compounds mainly formed by the following isomers: 60-70% α-HCH, 5-12% β-HCH, 10-12% γ-HCH and 6-10% δ-HCH. γ-HCH, known as lindane, has insecticidal activity and has been widely used in commercial formulations either alone or with other isomers (Breivik *et al*., 1999; Li, 1999). HCH isomers are recognized for their toxicity, persistence in the environment, and potential carcinogenic effects. High contamination levels are observed in some soils (Willett *et al*., 1998; Macrae *et al*., 1984). Their environmental persistence is attributed to resistance to microbially catalyzed degradation (Alexander, 1981). The purpose of this study was to identify microbes that are capable of metabolizing HCH isomers.

White-rot fungi degrade environmental pollutants such as polycyclic aromatic hydrocarbons, pesticides, dyes, plastics and explosives (Aust and Benson, 1993; Field *et al*., 1993; Bumpus *et al*., 1985). These fungi possess an extracellular oxidative enzymatic system that degrades wood lignin (Tien and Kirk, 1988). Three ligninolytic enzymes, manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase, also catalyze degradation of organic pollutants (Mester and Tien, 2000; Bucke, 1998). These enzymes are produced under nutrient-limiting conditions (Couto *et al*., 2002; Moreira *et al*., 2000). Their synthesis

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is not induced by the presence of pollutants (Barr and Aust, 1994). Cytochrome P-450 enzymes are also involved in the degradation of organochloride compounds (Masaphy *et al*., 1996; Mougin *et al*., 1996). In addition, reductive dehalogenation reactions serve to degrade organochlorine compounds (Reddy *et al*., 1998).

Several prior studies have reported the degradation of lindane by white-rot fungi in liquid and solid medium. However, no prior study has reported degradation of α-, β- and δ-HCH. Prior reports on the degradation of γ-HCH by *Phanerochaete chrysosporium* show percent decompositions between 10.6% (Arisoy, 1998) and 90% (Singh and Kuhad, 1999), and mineralization values between 3.9% (Mougin *et al*., 1996) and more than 90% (Bumpus *et al*., 1985). In soil bioremediation assays, less γ-HCH is eliminated than in liquid medium (Mougin *et al*., 1997). This is due to the difference in mass transfer rates between soil and liquid. As a result of mass transfer effects, HCH isomers show a much slower rate of metabolism in soil than in liquid media. To increase the levels of degradation in soil, fungal species were immobilized on lignocellulosic material supports such as wood chips, corncobs, and wheat straw and then innoculated into polluted soils (Kennedy *et al*., 1990; Andersson and Henrysson, 1996). These cellulosic materials are a plentiful source of nutrients that favor fungal growth and soil colonization. Additionally, cellulosic substrates induce the production of ligninolytic enzymes (Quintero *et al*., 2006a; Castillo *et al*., 2001; Fujian *et al*., 2001).

In this study, fungal tolerance to growth in different HCH concentrations was measured. In addition, the degradative capacity of nine white rot fungi species toward α-, β-, γ- and δ-HCH isomers was measured. The best strain was selected for further bioremediation studies.

Materials and methods

Chemicals

Hexachlorocyclohexane, isomers α-, β- and δ- (Riedel-de Haën-Fluka, Seelze, Germany), the isomer γ-HCH (Sigma-Aldrich, Steinheim, Germany) and Poly R-478 dye (Sigma-Aldrich) with chemical purities between 98 to 99% were used in these studies.

Microorganisms and culture media

The following fungal strains were used for screening: *Bjerkandera adusta, Irpex lacteus, Lentinus tigrinus, Phanerochaete chrysosporium*, *Phanerochaete sordida, Phlebia radiata, Pleurotus eryngii, Poliporus cialatus* and *Stereum hirsutum*. Isolates of these fungi were obtained from the culture collection of the Bioprocess and Environmental Engineering Group (School of Engineering, Universidad de Santiago de Compostela, España). Initially, the fungi were cultured in Petri plates containing per liter: 15 g agar, 3.5 g malt extrac, and 10 g glucose, pH 5.5 (MG). The fungi were grown at 30ºC for 5 d before to excise 6-mm agar plugs from colonized Petri plates and used to inoculate 1L Fernsbach flasks containing 100 mL culture medium containing per liter: glucose (10 g), peptone (5 g), sodium acetate (20 mM, pH 4.5) and BIII nutrients solution (10 mL) (Tien and Kirk, 1988). Cultures were incubated at 30ºC for 7 d. Before inoculation, mycelium was blended for 60 s in a Waring blender.

Effect of HCH isomers on fungal growth

The fungal growth inhibitory effect of the HCH isomers was examined in Petri plates containing MG media with 0, 1, 5 and 10 $mg \cdot L^{-1}$ of the respective HCH isomer. The HCH isomers were prepared as stock solutions dissolved in acetone (concentration of 2 $g \cdot L^{-1}$) and added to sterilized MG media after autoclaving and cooling to 50ºC. Mycelium plugs (6-mm) was punched from MG agar plates and placed in triplicate in the center of Petri plates. Cultures were incubated at 30ºC and the colony diameter measured every one day for nine days.

Biodegradation assays in liquid medium

Biodegradation assays were performed in triplicate in 100 mL Erlenmeyer flasks containing 2 mL of homogenized mycelial suspensions (blended mycelium) (1.4 $g \cdot L^{-1}$

of dry biomass) and 18 mL of media with a composition identical to MG media except that the peptone content was reduced from 5 to 2 $g \cdot L^{-1}$. To each flash 25 µL of a 2.0 $g \cdot L^{-1}$ HCH stock solution (in acetone) were added to a final concentration of 2.5 $mg \cdot L^{-1}$. Abiotic controls, without mycelial suspensions, were included to measure non-specific loss of target compounds. The cultures were incubated under continuous agitation at 150 rpm for 10 d at 30ºC. Samples were withdrawn for HCH analysis at the beginning of the experiment and after 10 d of incubation.

Remediation assays of HCH polluted soil

Based on the screening results, *B. adusta* and *P. chrysosporium* were selected for remediation assays. Sandy slime soil obtained from a forest, taken at a depth of 15 cm, was used for these experiments. Bioremediation laboratory assays were performed in triplicate in 100 mL Erlenmeyer flasks with 10 g of sterilized (121°C for 15 min) or non-sterilized soil samples. Soil was contaminated with 12.5 µL of HCH stock solution (2.0 $g \cdot L^{-1}$ of each isomer in acetone) to achieve a concentration of 2.5 $mg \cdot kg^{-1}$ for each isomer and then inoculated with 1.25 g of fungus immobilized in lignocellulosic materials (0.5 mm diameter woodchips or corncobs). The cultures were incubated at 30ºC for 5 weeks. Moisture was added once a week, by adding sterilized water according to the observed weight loss. Abiotic controls were performed mixing soil with non colonized lignocellulosic materials. For HCH, analyses were performed in triplicate. Each of the three flasks was assayed at the beginning of the experiment and after 35 d of incubation.

Extraction and GC-MS analysis

HCH extraction of liquid and soil samples was performed by adding 20 mL of 1:1 hexane-acetone solution to each Erlenmeyer flask. The flasks were tightly sealed with a cap rubber and vigorously shaken for 2 h at 300 $osc \cdot min^{-1}$ in an oscillating agitator. After extraction, samples were subjected to ultrasonic treatment for 15 min in order to separate the organic solid phase from aqueous phases. Then a sample of the organic solid phase was withdrawn for GC-MS analysis. Measured extraction yields for liquid medium and for soil were between 90-100% and 75-80% respectively.

Residual HCH and intermediate HCH-metabolites were analyzed in a Varian-Saturn GC/MS (CP 3900) equipped with a split-splitless injection port and an automatic injector (CP-8400) and connected to an ion trap mass spectrometer (Varian Saturn 2100). A CP-Sil 8 CB Low Bleed/MS fused silica WCOT capillary column (30 m length and 0.25 mm ID) was used. The oven temperature was programmed as follows: hold at 60°C for 2 min; ramp increase rate of $20ºC \cdot min^{-1}$ to final temperature of 180°C; ramp increase rate of $5ºC \cdot min^{-1}$ to final temperature of 200°C and a ramp increase rate at $10ºC \cdot min^{-1}$ to a final temperature of 300ºC. An injection volume of 1 µL was used. The injections were made via a split-less injection at 280ºC. Helium was used as a carrier at a flow rate of 1.0 $mL \cdot min^{-1}$. The retention times of HCH isomers were: α-HCH (8.90 min), β-HCH (9.50 min), γ-HCH (9.80 min) and δ-HCH (10.60 min). HCH was quantified using a linear standard calibration curve with a range from 0.05 to 5 $mg \cdot L^{-1}$. The percent biodegradation was calculated on the basis of the proportionate change between HCH remaining in abiotic controls and HCH in liquid and soil assays.

Results

Effect of HCH isomers on fungal growth

Independent of the fungi species, the δ-HCH isomer had the highest detrimental effect on the mycelial growth, followed by lesser effects by γ-HCH, β-HCH and α-HCH isomers, respectively. There was a dose response effect with the highest mycelial growth inhibition obtained with 5 and 10 $mg \cdot L^{-1}$ of the respective HCH isomers (Figure 1). A concentration of 10 $mg \cdot L^{-1}$ δ-HCH completely inhibited mycelial growth of *S. hirsutum,* and more than 80% inhibited mycelia growth of *L. tigrinus*, *P. radiata*, *P. ciliatus* and *P. sordida*. At the same concentration, γ-HCH 60% inhibited mycelial growth of *P. sordida*, *P. radiata* and 40% inhibited mycelial growth of *B. adusta* and *P. ciliatus*. Among fungal species, *P.*

chrysosporium and *B. adusta* were the most tolerant of HCH isomers (Figure 1).

Degradation of HCH in liquid culture

The highest rate of degradation of γ-HCH was obtained with *B. adusta* (42.4%) and *P. eryngii* (48.5%). The highest rate of degradation of δ-HCH was obtained with *P. eryngii* (70.8%), followed by *P. ciliatus* (60.4%), *B. adusta* (54.2%) and *I. lacteus* (53.8%) (Table 1). The β-HCH isomer was the most resistant to degradation with the highest rates observed with *B. adusta* (56.6%), followed by *P. ciliatus* (26.5%) and *P. eryngii* (22.7%).

In liquid medium, the concentration of α-HCH decreased substantially and the concentration of γ-HCH showed a limited decrease. The difference between abiotic controls and fungi assays represents biological degradation. Degradation of α-HCH was detected only with *P. ciliatus* (10.8%) and *P. eryngii* (23.9%).

The analysis of the HCH taken from samples in liquid medium showed no detectable metabolites. However, in the culture extracts of *B. adusta*, other organochloride compounds like 3-chloro-4-metoxiphenil ethanone and 2,4-dichloro-3-metoxibenzene-carbonyl chloride were detected (Figure 2).

Soil bioremediation assays

For assays with *P. chrysosporium*, no degradation of pollutants was observed (Figure 3). However, fungal colonization of the soil was observed. For incubations performed with *B. adusta*, all the isomers present in the soil were between 8 and 17% degraded with woodchips and between 13 and 17% degraded with corncobs after 4 weeks incubation. An extensive colonization of the soil by fungus was observed.

To evaluate the effect of the soil microflora on the degradation of HCH and its possible

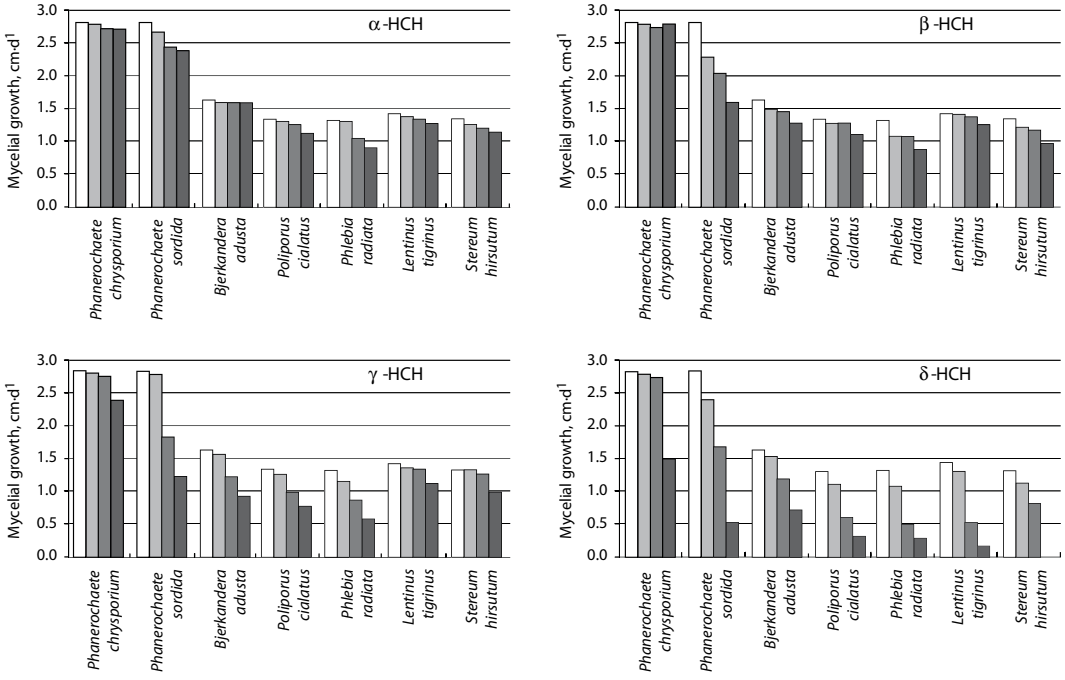

Figure 1. Effect of HCH isomers on mycelial growth of seven white rot fungi at 30ºC determined in agar plates. White bar = control without HCH, light grey bar = 1 mg·L^{-1}, and medium grey bar = 5 mg·L^{-1}, dark grey bar = 10 mg·L^{-1}. Standard deviation < 15% of each value.

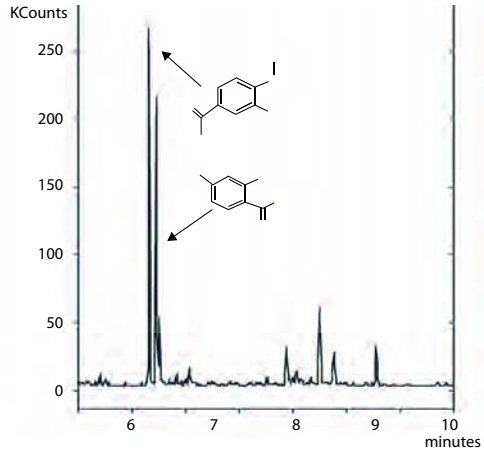

Figure 2. Chromatogram of organochloride compounds detected in the biodegradation studies of hexachlorocyclohexane(HCH) isomers with *Bjerkandera adusta* in liquid medium after 10 d of treatment. The retention times were as follows: α-HCH (8.90 min), β-HCH (9.50 min), γ-HCH (9.8 min), and δ-HCH (10.6 min), ethanone, [1-(3-chloro-4-methoxyphenyl)] (6.3 min) and 1-benzenecarbonyl chloride, (2,4-dichloro-3-methoxy) (6.5 min).

synergistic effect with the ligninolytic fungi, experimental results with sterile and unsterile soil were compared. Maximum degradations between 12 and 16% for α-HCH and around 60% for γ-HCH were achieved after 28 days. However, for β- and δ-HCH, no degradation was possible under any evaluated conditions (Table 2).

Discussion

The isomers γ- and δ-HCH were substantially inhibitory to fungal growth, while α- and β-HCH had no detrimental effect on fungal growth at the concentrations used in this study. A similar effect was obtained in toxicity assays with methanogenic bacteria (Quintero *et al*., 2006b). However, growth inhibition was not observed with white-rot fungi *Cyathus bulleri* and *Phanerochaete sordida*. At 80 $\mu g \cdot L^{-1}$ γ-HCH, which was the lowest concentration studies, little or no growth inhibitory effect was observed (Singh and Kuhad, 2000). Pentachlorophenol was also highly inhibitory to fungal growth (Alleman *et al*., 1992). The insecticidal properties of γ-HCH explain its more negative effect on fungal growth (Willett *et al*., 1998). The present study is the first report of the effect of all HCH isomers on white rot fungi.

Several studies have reported the degradation of lindane by white-rot fungi. However, these studies lack data on degradation of α-, β- and δ HCH isomers. Our results showed the potential use of white-rot fungi for the degradation of β- and δ-HCH isomers, which are considered to be highly resistant to degradation both in aerobic and anaerobic conditions (Bachmann *et al*., 1988). The capability of white-rot fungi to degrade these isomers is demonstrated in this study.

Previous reports on the degradation of γ-HCH by

Table 1. Biodegradation of hexachlorocyclohexane (HCH) isomers by white rot fungi after 10 d in liquid medium.

White rot fungi	HCH isomers degraded[1], %			
	α-HCH	β-HCH	γ-HCH	δ-HCH
Phanerochaete chrysosporium	nd2	0.0	nd	0.0
Phanerochaete sordida	nd	0.0	nd	0.0
Bjerkandera adusta	nd	56.6 ± 7.1	42.4 ± 9.4	54.2 ± 12.4
Poliporus ciliatus	10.8 ± 2.5	26.5 ± 3.8	32.5 ± 1.8	60.4 ± 9.1
Phlebia radiata	nd	0.0	nd	24.9 ± 5.9
Lentinus tigrinus	nd	0.0	15.1 ± 5.1	39.5 ± 4.3
Stereum hirsutum	nd	0.0	24.6 ± 1.9	29.9 ± 2.5
Pleurotus eryngii	23.9 ± 0.5	22.7 ± 2.6	48.5 ± 0.0	70.8 ± 1.4
Irpex lacteus	nd	0.0	33.7 ± 1.3	53.8 ± 2.1

[1] Each value is the mean of three replicates. ± standard deviation.
[2] nd: not determined.

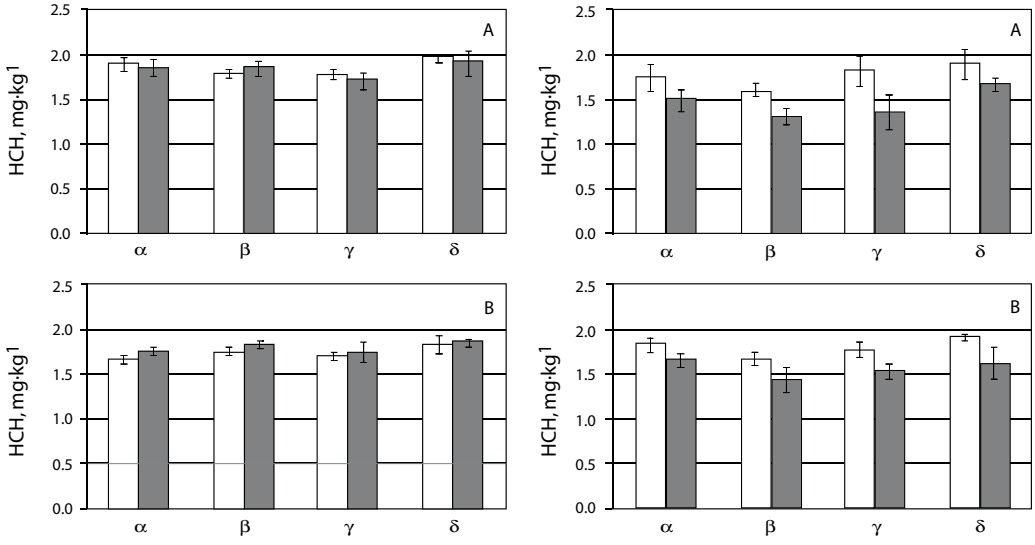

Figure 3. Biodegradation of isomers α-, β-, γ- and δ of hexachlorocyclohexane (HCH) in sterilized and infested soil employing the white rot fungi *Phanerochaete chrysosporium* (left) and *Bjerkandera adusta* (right) after 35 d of treatment. Soil was infested with inoculated corncobs (A) and inoculated woodchips (B). White bars = abiotic controls and grey bars = fungal treatment.

P. chrysosporium indicate variable degradation rates ranging from 10.6% (Arisoy, 1997) to 90% (Singh and Kuhad, 1999) with mineralization values ranging from 3.9% (Mougin *et al*., 1996) to more than 90% (Bumpus *et al*., 1985). Our results show 42% degradation of γ-HCH with *B. adusta* in liquid medium and 8 to 17% degradation of the same isomer with the same fungi in a sandy soil system. Mass transfer effects limit the accessibility of HCH in soil compared to liquid media (Mougin *et al*., 1997).

In the degradation of lindane, intermediate metabolites such as tetrachlorocyclohexene (TCCH) and tetrachlorocyclohexenol (TCCOL) have been detected previously (Singh and Kuhad, 2000; Mougin *et al*., 1996). However, these compounds were not detected in this work. Instead, organochloride compounds such as ethanone 1-(3-chloro-4-methoxyphenyl)- and 1-benzenecarbonyl chloride, 2,4-dichloro-3-methoxy were detected due to fungal biosynthetic capacity. The endogenous production of these two compounds as secondary metabolites was previously reported (Mester *et al*., 1997; Field *et al*., 1995). The biosynthetic routes of the organochlorine metabolites are still unknown. It is hypothesized that the humus or other organic material provide chlorine atoms required for metabolite formation (Silk *et al*., 2001). In this study, the degradation of HCH provides a possible supply source for these chlorine atoms. However additional experimentation will be needed to prove this hypothesis.

To increase the levels of degradation in soil, some researchers have inoculated polluted soils with various fungal species immobilized on different lignocellulosic supports (e.g., woodchips, corncobs, and wheat straw) (Andersson and Henrysson, 1996; Kennedy *et al*., 1990). These reports show the beneficial effect of immobilization on degradation of organic cyclic compounds. The white rot fungus *P. chrysosporium* immobilized in corncobs achieved 35% degradation of TNT after 18 d of treatment (Tudor *et al*., 1990), 62% degradation of 2,4,5-trichlorophenoxyacetic acid after 30 d of treatment (Ryan and Bumpus, 1989), 10% degradation of DDT after 60 d treatment (Bumpus *et al*., 1988), and 23% degradation of chlordane after 60 d of treatment (Kennedy *et al*., 1990).

Table 2. Biodegradation of hexachlorocyclohexane (HCH) isomers in sandy soil infested with *Bjerkandera adusta* after four weeks of incubation.

Systems	HCH isomers degraded[1], %			
	α-HCH	β-HCH	γ-HCH	δ-HCH
Infested soil:				
Corncobs + sterile soil	15.9±4.7	13.8±4.1	17.4±4.4	15.7±5.1
Woodchips + sterile soil	10.1±3.7	14.5±8.1	8.2±5.3	17.5±9.0
Woodchips + Non sterile soil	12.1±5.4	0.0	62.0±4.6	0.0
Non infested soil:				
Non sterilized soil	16.1±2.7	0.0	62.8±8.5	0.0

[1] Each value is the mean of three replicates ± standard deviation.

Plant residues are a plentiful source of nutrients that favor fungal growth and soil colonization. Additionally, plant residues elicit production of adaptative ligninolytic enzymes (Castillo *et al*., 2001; Fujian *et al*., 2001). However, the lower remediation values obtained in soil than liquid medium can be explained by sorption phenomena. Pollutant is retained in the soil pores thereby impeding transfer to the liquid phase (Quintero *et al*., 2005; Rijnaarts *et al*., 1990).

Synergistic effects between white rot fungi and other soil microorganisms were not observed in this study. In contrast, the fungi showed no elimination of α- and γ-HCH in the presence of soil microorganisms. Several white-rot fungi species show potential for possible use in bioremediation of HCH isomers. A similar effect was found in degradation of benzo[a]pyrene by *B. adusta* in the soil in presence of indigenous microflora (Kotterman and Field, 1998; Wiesche, *et a*l., 1996).

In conclusion, several white-rot fungi species have demonstrated a high capacity to degrade organic pollutants such the insecticide lindane (γ-HCH). The δ- and γ-HCH isomers were between 15.1 to 70.8% degraded by *B. adusta, P. ciliatus, L. tigrinus, S. hirsutum, P. eryngii* and *I. lacteus*, while β-HCH was 56.6, 26.5 and 23.9% degraded by *B. adusta, P. ciliatus* and *P. eryngii*, respectively. In non-sterile soil, *B. adusta,* immobilized on corncobs or woodchips, all the HCH isomers were from 8.2 to 17.5% degraded. In a non-sterile soil, an antagonist effect with the soil microflora was observed. This effect could be avoided by increasing the fungal biomass in the inoculation step.

Resumen

Los hongos de la pudrición blanca de la madera han demostrado una alta capacidad para degradar contaminantes orgánicos y entre ellos el insecticida lindane (γ-HCH). Sin embargo, no se conoce su capacidad para degradar los demás isómeros presentes en la formulación de este insecticida, altamente tóxico y recalcitrante en el ambiente. El objetivo de este estudio fue evaluar la sensibilidad de diferentes especies de hongos de la pudrición blanca de la madera a los isómeros de α-, β-, γ- y δ de hexachlorociclohexane (HCH), así como su capacidad para degradarlos en medios líquidos y sólidos. Los hongos evaluados fueron: *Bjerkandera adusta*, *Irpex lacteus*, *Lentinus tigrinus*, *Phanerochaete chrysosporium*, *Phanerochaete sordida*, *Phlebia radiata*, *Pleurotus eryngii*, *Poliporus cialatus* y *Stereum hirsutum*. Los resultados mostraron que δ- y γ-HCH fueron los isómeros que presentaron mayor grado de inhibición en el crecimiento a concentraciones entre 5 y 10 mg·L^{-1}. Además, *P. chrysosporium* and *B. adusta* fueron los que mostraron mayor tolerancia a altas concentraciones de los contaminantes. Los isómeros δ- and γ-HCH fueron considerablemente degradados en medio líquido entre 15,1 and 70,8% por *B. adusta*, *P. ciliatus*, *L. tigrinu*, *S. hirsutum*, *P. eryngii* and *I. lacteus*. Mientras que β-HCH solamente fue degradado por *B. adusta*, *P. ciliatus* and *P. eryngii*, en proporciones de 56,6; 26,5 and

23,9%, respectivamente. En suelo esterilizado *B. adusta* degradó parcialmente todos los isómeros entre 8,2 and 17,5%, mientras que en suelo no esterilizado se observó un efecto antagonista que impidió la acción del hongo.

Palabras clave: *Bjerkandera adusta*, lindano, hongos de la madera, pesticidas.

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