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ORIGINAL PAPER

Bioremediation of a Chilean Andisol contaminated with pentachlorophenol (PCP) by solid substrate cultures of white-rot fungi

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Abstract This study provides a first attempt investigation of a serie of studies on the ability of *Anthracophyllum discolor*, a recently isolated white-rot fungus from forest of southern Chile, for the treatment of soil contaminated with pentachlorophenol (PCP) to future research on potential applications in bioremediation process. Bioremediation of soil contaminated with PCP (250 and 350 mg kg^{-1} soil) was investigated with *A. discolor* and compared with the reference strain *Phanerochaete chrysosporium*. Both strains were incorporated as free and immobilized in wheat grains, a lignocellulosic material previously selected among wheat straw, wheat grains and wood chips through the growth and colonization of *A. discolor*. Wheat grains showed a higher growth and colonization of *A. discolor*, increasing the production of manganese peroxidase (MnP) activity. Moreover, the application of white-rot fungi immobilized in wheat grains to the contaminated soil favored the fungus spread. In turn, with both fungal strains and at the two PCP concentrations a high PCP removal (70–85%) occurred as respect to that measured with the fungus as free mycelium (30–45%). Additionally, the use of wheat grains in soil allowed the proliferation of microorganisms PCP decomposers, showing a synergistic effect with *A. discolor* and *P. chrysosporium* and increasing the PCP removal in the soil.

Keywords Soil contamination · Wheat grains · Immobilized fungi

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Introduction

White-rot fungi are of particular interest due to their ability to degrade a wide range of environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs), numerous synthetic dyes, chlorophenols, among others (Lamar et al. 1990; Walter et al. 2004; D'Annibale et al. 2005; Tortella et al. 2005; Papinutti et al. 2006; Rubilar et al. 2008; Tortella

et al. 2008). The ability of fungi to degrade pollutants has been attributed to the action of an extracellular and non specific ligninolytic enzyme system, composed of laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP) (Heinzkill et al. 1998). Moreover, the filamentous multicellular colonial form of these fungi provides both a high cell to substrate ratio and a mechanical adjunct to substrate breakdown (Pointing 2001).

The most studied fungi have been *P. chrysosporium* and *Trametes versicolor* because they have a fast growth, a high ligninolytic enzyme production and efficient pollutant degradation (Lamar et al. 1990; Moredo et al. 2003; Gao et al. 2005; Borràs et al. 2008). Recent studies with *A. discolor*, a native Chilean fungus previously isolated from temperate forests of southern Chile, have demonstrated that this fungus has a high ligninolytic activity, mainly manganese peroxidase (MnP), and high capability to degrade PCP in both liquid medium and soil slurry (Rubilar et al. 2007; Tortella et al. 2008). Studies developed by Rubilar et al. (2007) demonstrated the formation of intermediate metabolites from PCP degradation in soil slurry culture by *A. discolor.* Pentachloroanisole (PCA), tetrachloro-1,4-dimetoxybenzene, 2,5-dichloro-1,4-dimetoxibenzene, 2-chloro-1,4-dimetoxibenzene, 3,4-dimethoxybenzaldehyde (veratraldehyde) were identified. Similar pathway has been proposed by Reddy and Gold (2000) during the degradation of PCP in liquid media by *P. chrysosporium*. Therefore, it is possible to indicate that in both culture media, liquid or soil slurry, white-rot fungi follow a similar pathway to degrade PCP. However, degradation of PCP in soil by *A. discolor* has not been evaluated, yet.

Soil environment is a complex system, where a variety of agents may affect not only the growth of white-rot fungi but also strongly influence their viability (Lestan and Lamar 1996). The main affecting factors are low nutrient availability and environmental conditions such as temperature, pH, and presence of indigenous microorganisms (Lestan and Lamar 1996). Additionally, pollutant bioavailability to fungi is a factor very important for biodegradation, being pollutants usually less available when aged in soil compared with recent addition (Vernile et al. 2007). However, white-rot fungi have nonspecific extracellular degradation mechanisms that allow them to deplete pollutants with a low availability (Gianfreda and Rao 2004).

Therefore, the use of these fungi in the bioremediation of contaminated soils requires adequate conditions to enhance their growth and the activation of their ligninolytic system at conditions different from those found in their natural environment.

Several investigations have shown that different lignocellulosic materials acting as organic supports may allow the growth of fungi providing a nutritional and structural function and protection of the cells from various environmental stresses. Also, they provide resistance to competition by indigenous microbes (Lestan and Lamar 1996; Walter et al. 2004; Rubilar et al. 2007). The lignocellulosic materials have lignin that prevents the microorganisms proliferation, as this molecule can not be used as the sole carbon and energy source. Therefore, white-rot fungi are forced to degrade lignin to obtain cellulose and hemicellulose, carbon sources present in the wood which is more readily utilizable source of carbon (Lechner and Papinutti 2006). Rubilar et al. (2007) observed in culture slurry highest MnP activity and PCP degradation (95% after 28 days) by *A. discolor* grown on lignocellulosic material distillers dried grains with solubles (DDGS). This substrate positively influenced the kinetics of PCP degradation during the first 14 days, when compared with cultures without lignocellulosic material at the same initial PCP concentration. In this same context, Lamar et al. (1990), demonstrated 96% of PCP degradation by *P. chrysosporium* grown in pulpwood chips after 64 days.

These materials can stimulate ligninolytic enzyme production, fungus growth and consequently pollutant degradation (Lestan and Lamar 1996; Walter et al. 2004), being the organic supports most commonly used: ear corn, sawdust, wood chips, sugarcane bagasse and wheat straw (Moredo et al. 2003; Dzul-Puc et al. 2005; Mohammadi and Nasernejad 2009; Lu et al. 2009).

The aim of this investigation was to select a lignocellulosic material suitable as support for the colonization and inoculation of *A. discolor* and *P. chrysosporium* in soil contaminated with pentachlorophenol (PCP) and to evaluate the effect of native soil microflora on the degradation of PCP in non-sterile soil inoculated with white-rot fungi.

Materials and methods

Microorganisms

The fungal strains used were *Anthracophyllum discolor* obtained from the culture collection of the Environmental Microbiology Laboratory of the University of La Frontera (Chile) and *Phanerochaete chrysosporium* CECT-2798 obtained from the Spanish type culture collection of the University of Valencia (Spain). The fungi were transferred from slant culture tubes (maintained at 4°C and transferred every 6 months) to malt extract agar plates (15 g l^{-1} agar, 3.5 g l^{-1} malt extract and 10 g l^{-1} glucose) and kept at 25°C for 5–7 days before being used for inoculum preparation.

Preparation of inoculum

One hundred mL of modified Kirk medium, containing 10 g l^{-1} glucose, 2 g l^{-1} peptone, g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4$, 0.1 g l^{-1} $CaCl_2$, 2 mg l^{-1} thiamine, and mineral salts (10 ml l^{-1}) (Moreira et al. 1997) was placed in 2 l Erlenmeyer flasks and then inoculated with five malt agar plugs (6 mm diameter) of active mycelium of *A. discolor* and *P. chrysosporium.* The fungal cultures were incubated at 25°C and after 7 days they were homogenized in a sterilized blender for 1 min. The blended mycelia were used as the inoculum.

Preparation of lignocellulosic material

Wheat straw (1 cm length), wheat grains and wood chips were used as lignocellulosic materials. Each material (2 g) was transferred to glass test tubes and 2 ml of distilled water was added. The test tubes were autoclaved at 121°C and 1 atm for 40 min twice consecutively.

Preparation of soil

An Andisol soil collected from the Temuco Series, located in southern Chile, was used for the experiments. The soil was collected at a depth of 0–20 cm and the pre-treatment included sieving to select a particle size lower than 2 mm and air-drying. Sterile soil autoclaved at 121°C and 1 atm for 40 min twice consecutively was used as abiotic control. The major physical–chemical properties of the soil are summarized in Table 1.

Table 1 Physical-chemical properties of soil

Parameter	Value[a]
pH (in water)	5.9 ± 0.20
N (%)	0.72 ± 0.02
C (%)	8.06 ± 0.95
P (mg/kg^{-1})	23 ± 2.46
MO (%)	14 ± 1.68
C/N	11 ± 1.25

[a] Values of mean ($n = 3$) ± standard deviation

The soil was spiked with a stock solution of PCP (Aldrich, 98% of purity) diluted in acetone to reach concentrations of 250 or 350 mg PCP kg^{-1} soil, homogenized by vigorous shaking and kept under a fume hood for 24 h for the solvent evaporation.

Selection of the lignocellulosic material for white-rot fungi immobilization

Malt extract agar plates were inoculated with one agar disks of 6 mm diameter, previously immobilized with *A. discolor*. Later, the lignocellulosic material (wheat straw, wheat grains and wood chips) was added around the agar disks at a distance of 4 cm approximately. The plates were incubated at 25°C for 7 days. The malt extract agar without lignocellulosic material was used as control.

The growth of the mycelium in the lignocelullosic material was measured and confirmed by Scanning Electronic Microscopy (SEM). The material that showed the higher growth and colonization of the fungus was selected for further studies.

Production of manganese peroxidase by *A. discolor* and *P. chrysosporium* cultivated with lignocellulosic material

The lignocellulosic material previously selected (2 g), 6 ml of sterile distilled water and 4 ml of inoculum of *A. discolor* or *P. chrysosporium*, previously homogenized in a blender, were added to a 100 ml Erlenmeyer flask. Cultures were incubated at 25°C for 28 days. MnP activity was periodically monitored in the liquid culture. The weight lost of lignocellulosic material was measured by dry weight

at 105°C at the beginning and the end of the incubation period, the difference was used as the degradation by the fungi. Experimental assays were run in triplicate.

PCP removal in soil by fungal mycelium free and immobilized in lignocellulosic material

The assay was performed in 100 ml Erlenmeyer flasks containing 10 g soil (dry weight) contaminated with 250 or 350 mg PCP kg^{-1} soil. The soil was inoculated with *A. discolor* and *P. chrysosporium* as free mycelium and immobilized in lignocellulosic material.

For assays with free mycelium: the flasks with PCP contaminated soil were inoculated with 4 ml of inoculum homogenized in a blender and 2 ml of sterile distilled water, in order to provide 35% humidity.

For assays with mycelium immobilized in the lignocellulosic material: 4 ml of inoculum of fungi homogenized in a blender, 2 g of lignocellulosic material selected for immobilization of the fungi and 4 ml of sterile distilled water were added to 100 ml Erlenmeyer flasks and incubated at 25°C for 7 days. Then, the flasks with fungi previously grown in the lignocellulosic material were transferred to flasks with soil contaminated with PCP at 35% humidity.

Moreover, biotic (natural soil without fungus) and abiotic control (sterile soil without fungus) were incubated with 6 ml of sterile distilled water. Both controls were prepared with (2 g) and without lignocellulosic material.

Each experiment was carried out in triplicate under destructive sampling mode (8 treatments with total of 24 flasks per time point for each PCP concentration).

All flasks were incubated at 25°C in total darkness for 28 days. The MnP activity and residual PCP concentration were periodically measured at 0, 7, 14, 21 and 28 days.

Linear regression equations describing the relationship between PCP soil concentration and time were used to calculate removal half-lives of the treatments according to Eq. 1:

$$\mathrm{Ln}\ C = \mathrm{Ln}\ C_0 - kt \quad (1)$$

where Ln C is the natural logarithm of soil PCP concentration at t time, Ln C_0 is the natural logarithm of initial soil PCP concentration and k is the removal rate constant (d^{-1}). The time required to reach a PCP removal of 50% ($t_{1/2}$) was also calculated.

Manganese peroxidase activity assays

Five mililiter of 250 mM sodium tartrate (pH 4.5) were added to three flasks containing soil (10 g). The flasks were agitated for 20 min at 200 rpm in an orbital shaker. Then an aliquot of 1.5 ml of supernatant was centrifuged at 6000 rpm for 10 min and the MnP activity measured from the supernatant through monitoring the oxidation of 2,6-dimethoxyphenol (DMP) spectrophotometrically at 30°C (Spectronic Genesys 2PC). The reaction mixture (1 ml) contained 200 µl (250 mM, pH 4.5) of sodium malonate, 50 µl (20 mM) of 2,6-DMP, 50 µl (20 mM) of $MnSO_4 \cdot H_2O$, and 600 µl of supernatant. The reaction was initiated by adding 0.4 mM H_2O_2. One MnP activity unit was defined as the amount of enzyme transforming 1 µmol DMP per minute (Moreira et al. 1997).

Extraction and analysis of PCP

PCP extraction from soil was performed from flasks after MnP activity assays. The analysis was developed as follows: (i) 20 ml hexane:acetone mixture (1:1, v/v) were added to each flask containing soil (10 g), shaken for 2 h and centrifuged (2500 rpm for 10 min) for the separation of the organic and solid phases; (ii) the contents were sonicated for 15 min for separation of the organic solvent and aqueous phases; (iii) an aliquot of the organic phase was used for analysis.

Residual PCP was determined by high performance liquid chromatography (HPLC) with an instrument equipped with a Merck-Hitachi L-7100 pump, a Rheodyne 7725 injector with a 20 µl loop, a Merck-Hitachi L-7455 diode array detector operating at 215 nm, and a Hitachi D-7000 data processor. A Lichrosphere 60 RP select B 250 mm × 4 mm column of 5 µm particle size with a LichroCART 4–4 guard column (Merck) was used. The mobile phase consisted of acetonitrile and phosphoric acid (1% aqueous solution) 1:1 (v/v) with a flow rate of 1 ml min^{-1} (PCP retention time was 12 min). Instrument calibrations and quantifications were performed against the pure reference standard (0.05–5 mg l^{-1}). The procedure described was checked for PCP

recovery (which ranged from 86 to 100%). Detection limit was 0.03 mg l^{-1}, considering the noise-to-signal ratio greater than 2.

Electronic microscopy

Photomicrographs were taken using a scanning electron microscope (SEM) (JEOL JSM-6380LV model). Three pieces of lignocellulosic material of 0.5 cm × 0.5 cm with a thickness of 0.3 or 0.5 cm were transferred to a Petri plate and dried for 12 h at 50°C. The pieces of lignocellulosic material were submerged in glutaraldehyde (2.5%) for 15 min and washed with 0.1 M phosphate buffer pH 7.0. Then, the samples were placed in osmium tetraoxide for 2 h and dehydrated by immersion in ethanol (different concentrations, 70, 80, 90 and 100%). The samples were deposited over the sample holder and coated with gold to make observations using the scanning electron microscope.

Results and discussion

Selection of the lignocellulosic material for the colonization by white-rot fungi

The effect of wheat straw, wheat grains and wood chips on the growth of *A. discolor* in malt extract agar plates was evaluated. Figure 1 shows the growth of *A. discolor* in different lignocellulosic supports after 7 days of incubation. Fungal growth occurred in all supports and colonization of wheat grains was higher and faster (100%) that observed with wheat straw (50%) and wood chips (25%) (Table 2).

The immobilization of *A. discolor* in wheat grains, wheat straw and wood chips were also investigated with SEM to corroborate the previous results of growth and colonization of fungus. Results shown in Fig. 2 seem to confirm that *A. discolor* growth was higher in wheat grains, than in wheat straw and wood chips. The SEM micrographs were developed also to demonstrate the coating and colonization of wheat grain with fungus, the growth of the fungus on the surface of wheat grains, and the hyphal growth onto the wheat grains (Fig. 3).

Table 2 Growth of *A. discolor* in malt extract agar with lignocellulosic materials after 7 days

Lignocellulosic material	Growth
Wheat straw	+++
Wheat grains	++++
Wood chip	+

+: 0–25%, +++: 25–75%, ++++: 75–100% of growth

Figure 3 shows that the fungus faster and tightly adhered on the surface of wheat grains. Moreover, the fungus was adsorbed on wheat grains and grew not only on the surface but also in the interspaces of it.

Our results confirm that the fungal growth depends on the type of lignocellulosic material used during incubation. Dzul-Puc et al. (2005) demonstrated that the use of lignocellulosic residues as substrate depends of their chemical and physical characteristics, such as compounds content (lignin, hemicellulose, cellulose, carbohydrates, etc.), type of components and their structure. In fact, the higher growth of the fungus observed on wheat grains is probably due to the high content of carbohydrates and proteins of that material. Wheat grains provide a major source of energy for the fungus and thus a higher growth is achieved. Moreover, the use of this material could be a convenient condition for the industrial production and application of white rot fungi. This high growth in this support promotes their spread in media that are not usually natural environments.

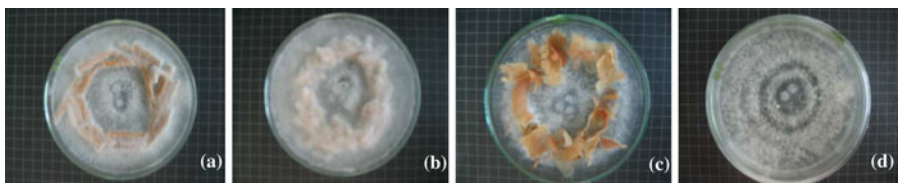

Fig. 1 Colonization of *A. discolor* on different lignocellulosic materials. Wheat straw (**a**), wheat grains (**b**), wood chip (**c**) and control (only malt extract agar) (**d**)

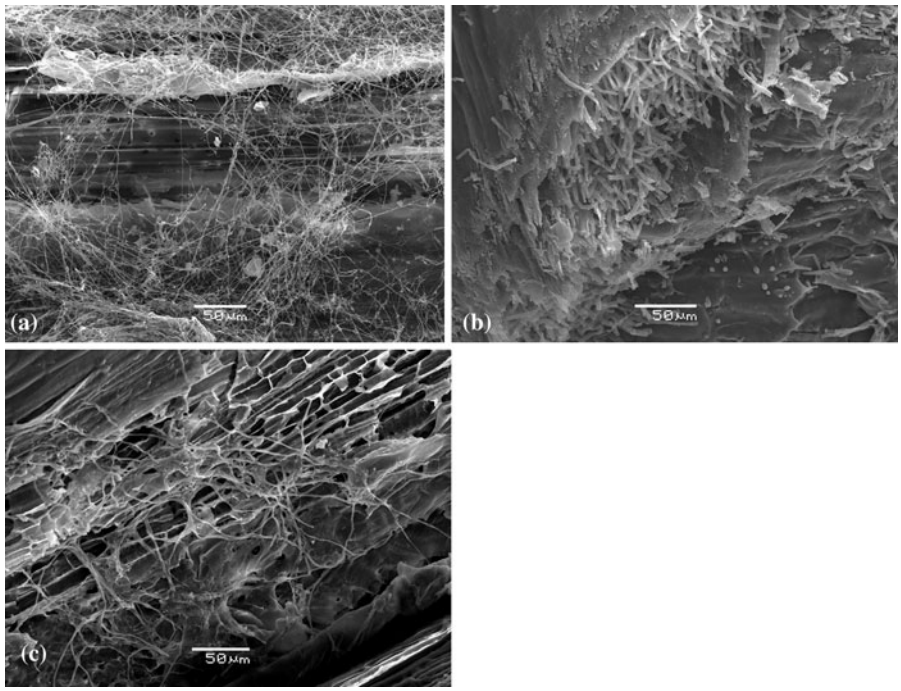

Fig. 2 SEM micrographs of *A. discolor* immobilized in wheat straw (**a**), wheat grains (**b**) and wood chip (**c**)

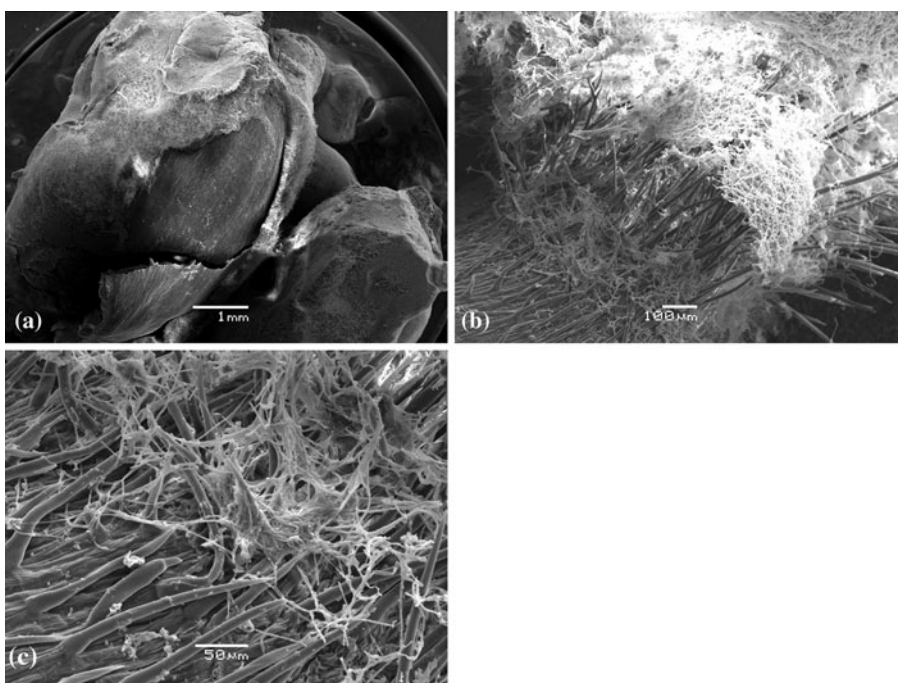

Fig. 3 SEM micrographs of *A. discolor* immobilized in wheat grains (**a**) coating of wheat grain with fungus, (**b**) growth of fungus on the surface of wheat grains and (**c**) hyphal growth onto the wheat grains

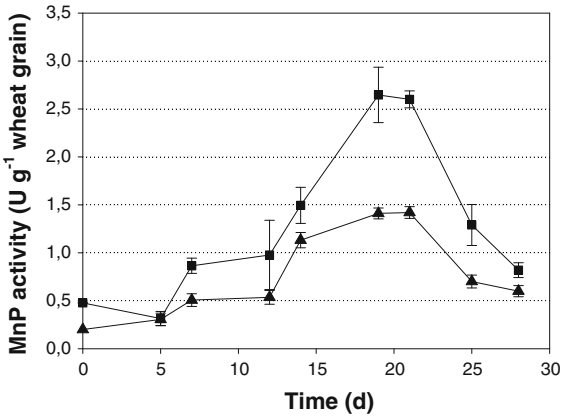

Fig. 4 MnP production by *A. discolor* (■) and *P. chrysosporium* (▲) cultivated with wheat grains. *Bars* represent SDs

Evaluation of lignocellulosic material as substrate for MnP production by *A. discolor* and *P. chrysosporium*

Wheat grains as substrate for *A. discolor* was evaluated also for MnP production and degradation of wheat grains after 28 days of incubation. *P. chrysosporium* was used as strain control for comparative effects.

Figure 4 shows that the maximum value of MnP activity was obtained at 19 days of cultivation, with 2.6 and 1.4 U g^{-1} wheat grains for *A. discolor* and *P. chrysosporium*, respectively. Subsequently, the activity decreased by 69 and 58% for *A. discolor* and *P. chrysosporium,* respectively, at 28 days of incubation.

By assimilating the production of MnP activity to the growth curve of microorganisms in batch culture, it was possible to calculate that the two fungi produced MnP activity at a quite similar constant rate (0.123 and 0.141 Units of MnP d^{-1} for P *A. discolor* and *P. chrysosporium*, respectively) in the interval 0–19 days (corresponding to the exponential phase of a typical microbial growth), at least under used experimental conditions.

Lechner and Papinutti (2006) demonstrated that MnP activity production by *Lentinula edodes* in cultures with wheat straw showed high enzyme production during colonization (until 20 days), then it declined drastically. This effect is probably due to the accessibility of nutrient′s content in the medium that depends on the particular lignocellulosic material (Lechner and Papinutti 2006). Nutrients from lignocellulosic material became available after lignin degradation by ligninolytic enzymes capable to reach other carbon sources present in the wood such as cellulose and hemicelluloses, which are a more readily utilizable carbon source.

The degradation of wheat grains after 28 days of incubation (measured as weight loss of total solids) was 19.9 and 21.4% for *P. chrysosporium* and *A. discolor*, respectively. Although degradation of wheat grains by both strains was very similar, the maximum value of MnP activity produced by *A. discolor* was 1.9 fold greater than that detected in the culture with *P. chrysosporium.* This effect has been observed in Kraft paste bleaching with ligninolytic fungi, where an increment of ligninolytic activity does not provide a great potential for bleaching (Feijoo et al. 1997).

Although diverse lignocellulosic materials have been used to support the growth of fungi in soil, there are few studies related to the production of ligninolytic enzymes with wheat grains. In this context, the most commonly substrate used to support the growth of white-rot fungi in contaminated soil has been wheat straw (Dorado et al. 1999; Hofrichter et al. 1999; Lechner and Papinutti 2006). Cultures of *P. chrysosporium* precultivated with wheat straw showed maximal MnP activity of 2.2 U g^{-1} of dry support (Xu et al. 2001), whereas other fungi as *Nematoloma frowardii* cultivated in the same lignocellulosic material produced up to 16 U g^{-1} support (Hofrichter et al. 1999).

On the other hand, Dorado et al. (1999) demonstrated the use of wheat straw as a substrate for *Pleurotus eryngii, P. chrysosporium, Phlebia radiata* and *Ceriporiopsis subvermispora*, with weight loss of lignocellulosic material between 10 and 25% after 30 days of incubation.

Soil PCP degradation by mycelium free and immobilized in wheat grains

PCP degradation in a soil contaminated with two PCP concentrations (250 and 350 mg kg^{-1} soil) was evaluated with *A. discolor* and *P. chrysosporium* as free and immobilized in wheat grains. Moreover, the effect of the autochthonous soil microflora (soil non-inoculated with white-rot fungi) on PCP degradation and the adsorption of PCP on the soil

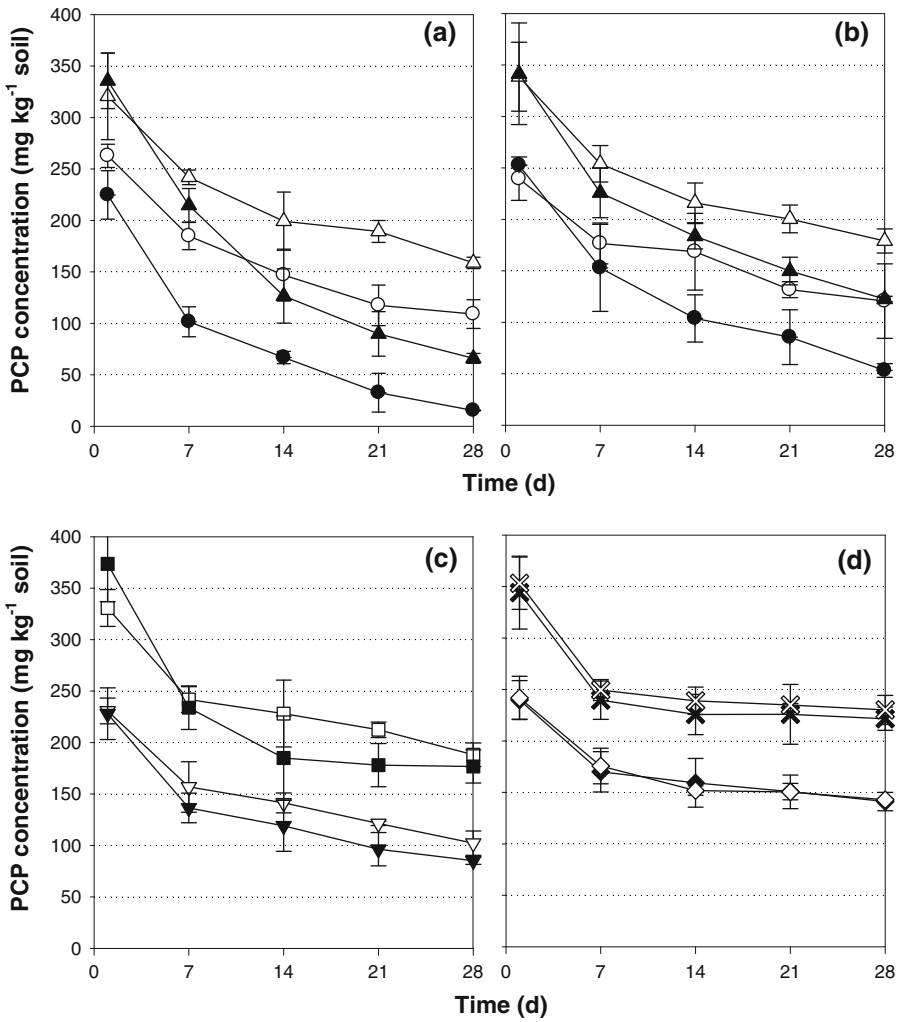

Fig. 5 Residual PCP concentration in soil inoculated with (**a**) *A. discolor*, (**b**) *P. chrysosporium,* (**c**) natural soil without fungus (biotic control) and (**d**) sterile soil without fungus (abiotic control). Open symbols: soil without wheat grains; closed symbols: soil with wheat grains. Bars represent SDs

(non-inoculated sterile soil) were also evaluated (Fig. 5; Table 3).

A high removal of PCP occurred at both initial PCP concentrations (250 and 350 mg kg^{-1} soil) mainly in the treatment with *A. discolor* immobilized in wheat grains (Fig. 5a). Moreover, PCP removal happened in two stages, a first, rapid removal (until 7 days of incubation), followed by a slower stage. This behavior was observed in all treatments. For example, PCP degradation rate at 250 mg kg^{-1} with immobilized *A. discolor* was 17.60 mg $(kg\ d)^{-1}$ until 7 days of incubation, therefore the degradation slowed down to 11.28 and 7.48 mg $(kg\ d)^{-1}$ at 14 and 28 days, respectively. Similar results were obtained for immobilized *P. chrysosporium* with a degradation rate of 14.24, 10.63 and 7.12 mg $(kg\ d)^{-1}$ at 7, 14 and 28 days of incubation, respectively.

It has been reported that PCP degradation in soil by white-rot fungi is a biphasic process: a first rapid degradation of the chemical, followed by a slower elimination phase (McGrath and Singleton 2000). This behavior is probably associated with the availability of the chemical to fungus attack, and it is probably due to PCP complexation with soil organic matter during the first days (Czaplicka 2004). Residual PCP concentration of the abiotic controls (sterile soils non-inoculated with fungi) (Fig. 5d) corroborates these results with a faster adsorption rate after 7 days of incubation in both treatments (with and without wheat grains) and at both initial PCP concentrations.

Table 3 PCP removal, half life values ($t_{1/2}$ in days) in soil by *A. discolor* and *P. chrysosporium* as mycelium free and immobilized in wheat grains

Treatments				PCP removal[a]		k (d^{-1})	R^2	$t_{1/2}$
Soil	Wheat grains	Fungi	Initial PCP concentration (mg kg^{-1})	(mg kg^{-1})	(%)			
Natural	−	*A. discolor*	250	153.7 ± 25.4	58.5 ± 7.5	0.026	0.96	26.82
Natural	+	*A. discolor*		209.4 ± 31.3	93.2 ± 10.1	0.091	0.98	7.58
Natural	−	*P. chrysosporium*		118.9 ± 18.4	49.63 ± 7.4	0.019	0.93	34.88
Natural	+	*P. chrysosporium*		199.4 ± 11.2	79.0 ± 4.5	0.048	0.98	14.43
Natural	−	−		128.4 ± 15.8	55.7 ± 6.3	0.020	0.99	33.78
Natural	+	−		142.8 ± 9.4	62.6 ± 3.8	0.023	0.99	29.87
Sterile	−	−		98.7 ± 6.2	41.2 ± 2.4	0.008	0.99	78.90
Sterile	+	−		99.08 ± 3.5	40.9 ± 1.4	0.008	0.84	77.07
Natural	−	*A. discolor*	350	161.9 ± 13.2	50.5 ± 3.8	0.019	0.96	36.90
Natural	+	*A. discolor*		269.6 ± 26.5	80.4 ± 7.6	0.055	0.98	12.49
Natural	−	*P. chrysosporium*		159.6 ± 19.1	47.1 ± 5.5	0.016	0.98	43.11
Natural	+	*P. chrysosporium*		218.9 ± 28.0	64.1 ± 8.5	0.029	0.99	23.83
Natural	−	−		142.6 ± 10.2	43.1 ± 3.0	0.012	0.97	58.84
Natural	+	−		186.9 ± 18.2	52.7 ± 5.2	0.021	0.89	32.06
Sterile	−	−		122.5 ± 11.7	35.6 ± 3.8	0.003	0.79	207.7
Sterile	+	−		122.9 ± 7.2	34.8 ± 2.1	0.003	0.95	188.0

+: with wheat grains, −: without wheat grains and/or without fungi, [a] values of mean ($n = 3$) ± standard deviation

Adsorption has been considered the principal mechanism in the majority of studies of PCP degradation in soil (Tse and Lo 2002). Cea et al. (2005) demonstrated that Chilean Andisols are particularly efficient sorbents for chlorophenols, mainly for allophane–ferrihydrite associations found in accumulate organic matter through the formation of stable complexes with FeOH and AlOH, and these allow hydrogen bond formation between penolic hydroxyl group and pentachlorophenol.

PCP concentration in all treatment substantially decreased after 28 days of incubation (Fig. 5; Table 3). Data of Table 3 indicate that inoculation of soil with both fungi immobilized in wheat grains produced a high PCP removal, being approximately 1.5 fold more effective that in soil inoculated with free mycelium at both PCP concentrations. Additionally, as shown in Table 3 the decreasing trend of PCP concentration over time in the different soil treatments could be well described using a first-order kinetics, which had high correlation coefficients ranging from 0.79 to 0.99. Moreover, $t_{1/2}$ of PCP removal varied greatly among the soil treatments ranging from 7.58 to 207.7 days, and the PCP degradation was faster in the soil inoculated with the white-rot fungus *A. discolor*.

PCP degradation in soil inoculated with free mycelium of *A. discolor* and *P. chrysosporium* was similar to that measured in the biotic control soil (natural, non sterile soil without fungus) without wheat grains, at both PCP concentrations. These results could suggest that PCP degradation under these environmental conditions can be ascribed to the autochthonous soil microflora (Table 3). McGrath and Singleton (2000) demonstrated that remediation of soil contaminated with PCP by *P. chrysosporium* as free mycelium did not improve the PCP remediation over non-inoculated PCP contaminated soil. A possible effect of white-rot fungi inactivity is the absence of available carbon and nitrogen sources (Moreira et al. 2000), as no growth of strains was observed under these cultivation conditions.

In contrast, a difference of PCP removal was observed by comparing the treatments with the fungi immobilized in wheat grains and the biotic control soil (natural, non sterile soil without fungus) but with wheat grains (Table 3). For instance, at an initial PCP concentration of 250 mg kg^{-1} soil, immobilized *A.*

discolor and *P. chrysosporium* removed 209.4 (93.2%) and 199.4 (79.0%) mg kg^{-1} soil, respectively, whereas in biotic control soil with wheat grains only 142.8 mg kg^{-1} soil (62.6%) was removed. This difference of PCP removal could be explained by synergistic effects occurring between fungi and autochthonous microflora of soil, where 66.6 and 56.5 mg kg^{-1} PCP removal were attributed to *A. discolor* and *P. chrysosporium*, respectively. Similarly, at initial concentration of 350 mg kg^{-1}, the PCP removal attributed to the action of immobilized *A. discolor* and *P. chrysosporium* was 82.7 and 32.0 mg kg^{-1}, respectively.

These results seem to suggest that any possible effect of microbial competition decreases when soil is inoculated with fungi previously immobilized in lignocellulosic materials. In a bibliographical review on bacterial associations with decaying wood, Clausen (1996) postulates that an initial colonization of wood by bacteria increases the permeability of wood structure and predisposes wood to fungal attack. Thus, the addition of lignocellulosic material in non-inoculated soil allowed proliferation of indigenous microorganisms with ability to degrade PCP. As a consequence, the pollutant removal increased in comparison to the treatment with non-inoculated soil without lignocellulosic material (Table 3).

Different interactions between white-rot fungi and soil microorganisms have been found in other studies with diverse pollutants. Kotterman et al. (1998) showed positive interaction between *Bjerkandera adusta* and microorganisms of soil in benzo(a)pyrene degradation. Also, pyrene degradation in soil increased between 5 and 20% when the soil was inoculated with *Dichomitus squales* and *Pleurotus* sp, in comparison to non-inoculated soil (Wiesche et al. 1996). However, in other cases the autochthonous microflora can affect the colonization of the soil by white-rot fungi through an effect of competition. Studies by Radtke et al. (1994) showed that soil bacteria, mainly *Pseudomonas,* are able to inhibit the growth of *P. chrysosporium* for synthesis of fenazina derivatives. Morevoer, Lang et al. (2000) indicated that a competition can occur when soil microorganisms are capable to use lignocellulosic substrate, which can inhibit the growth of the fungus.

Additionally, MnP activities produced by *A. discolor* and *P. chrysosporium* were, respectively, 2.28 and 0.49 U g^{-1} wheat grains at initial PCP concentration of 250 mg kg^{-1} while they decreased to 1.83 and 0.12 U g^{-1} at 350 mg kg^{-1} for *A. discolor* and *P. chrysosporium*, respectively. The enzyme activity was affected by PCP concentration in soil and only detected when the fungus was immobilized on the support.

Conclusions

Some general, promising conclusions may be derived for the results here obtained:

1. Wheat grains used as lignocellulosic support allowed a greater growth of *A. discolor* and *P. chrysosporium* and colonization of substrates compared to other lignocelullosic materials evaluated.
2. As compared to free mycelia, immobilization of *A. discolor* and *P. chrysosporium* in wheat grains favored the spread of fungi in the soil and consequently also the removal of a pollutant as PCP.
3. Both white-rot fungi *A. discolor* and *P. chrysosporium,* when immobilized in wheat grains, demonstrated similar capacities to degrade PCP.
4. *A. discolor* had a great potential to colonize soil and to compete with the autochthonous microflora of soil.
5. The application of wheat grains in soil allowed proliferation of indigenous microorganisms which showed synergistic effect with inoculated white-rot fungi, causing an increase in the PCP removal in soil.

Finally, the two investigated fungi can be considered as potential inoculum for bioremediation of soil contaminated with PCP.

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