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Impact of *Trametes versicolor* and *Phanerochaete chrysosporium* on differential breakdown of pesticide mixtures in soil microcosms at two water potentials and associated respiration and enzyme activity

Silvia Fragoeiro and Naresh Magan

Applied Mycology Group, Cranfield Health, Cranfield University, Bedford MK43 OAL, U.K.

Corresponding author: Prof. N. Magan, Applied Mycology Group, Cranfield Health, Cranfield University, Bedford MK43 OAL, U.K. Tel: 01525 863539; Fax: 01525 863540; E.mail: n.magan@cranfield.ac.uk

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Abstract

This study has examined the **e**ffect of inoculation of soil microcosms with *Trametes versicolor* and *Phanerochaete chrysosporium* on woodchips on differential degradation of pesticides (simazine, trifluralin and dieldrin, 10 mg kg^{-1} soil) at two water potentials (-0.7 and -2.8 MPa) at 15^{o}C. The soil microcosms were destructively sampled after 6/12 weeks and four extracellular enzymes quantified, respiration and pesticides measured with GC and HPLC. The fungal treatments produced extracellular enzymes in soil. Respiratory activity was significantly (P=0.05) enhanced in soil with the inocula, and higher in the pesticide mixtures. Cellulase/dehydrogenase increased in inoculated soil. Laccase increased significantly in the *T. versicolor* treatment. Degradation of the three pesticides by woodchip addition alone was enhanced (20-30%). *T. versicolor* increased degradation of simazine (27-46%), trifluralin (5-17%) and dieldrin (5-11%) and *P. chrysosporium* by 34-48%, 0-30% and 40-46% respectively, when compared with controls after 12 weeks. This study has demonstrated that pesticide mixtures are differentially degraded by fungal inoculants and significant extracellular enzymes are produced in soil, even at -2.8 MPa water potential. This suggests that effective bioremediation of xenobiotic mixtures using woodchips and fungal inoculants is achievable over a relatively wide water potential range when compared with that allowing plant growth (-1.4 MPa).

1. Introduction

Application of fungal technology for the cleanup of contaminants has shown promise since 1985 when the white rot species *Phanerochaete chrysosporium* was found to be able to metabolize a number of important environmental pollutants (Sasek, 2003). White rot fungi possess a number of advantages that can be exploited in bioremediation systems. Since key components of their lignin-degrading system are extracellular, these fungi can degrade insoluble chemicals such as lignin or an extremely diverse range of very persistent or toxic environmental pollutants (Bumpus et al., 1985; Barr and Aust, 1994; Hickey et al., 1994; Arisoy, 1998; Khadrani et al., 1999). The mycelial growth habit is also advantageous as it allows rapid colonisation of substrates, and hyphal extension enables penetration of soil reaching pollutants in ways that other organisms cannot do (Reddy and Mathew, 2001; Magan, 2007). This can maximise physical, mechanical and enzymatic contact with the surrounding environment (Maloney, 2001). White rot fungi can also tolerate a wide range of environmental conditions, such as temperature, pH and moisture levels (Maloney, 2001; Magan, 2007) and do not require pre-conditioning to a particular pollutant, because their degradation system is induced by nutrient deprivation (Barr and Aust, 1994; Pointing, 2001).

A significant amount of research on white rot fungi has been conducted in liquid and/or synthetic media, with less known about bioremediation capabilities in soil, especially under different environmental conditions. Tekere et al. (2001) and Hestbjerg et al. (2003) reported that field conditions did not always enable white rot fungi such as *P. chrysosporium* to achieve optimum activity and therefore it was not a good competitor in the soil environment (Sack and Fritsche, 1997; Hestbjerg et al., 2003). This last point was reinforced by Radtke et al. (1994) who reported bacteria from polluted and agricultural soil

to antagonise the growth of *P. chrysosporium* on solid media. Nevertheless, some studies have described the successful application of *P. chrysosporium* as a bioremediation agent in soil. For example, McFarland et al. (1996) described complete alachlor transformation by this fungus, within 56 days of treatment. Reddy and Mathew (2001) also showed that this species was able to degrade DDT, lindane and atrazine.

Recently, we demonstrated that under different osmotic stress regimes a range of white rot fungi were able to differentially degrade mixtures of pesticides in soil extract broth (Fragoeiro and Magan, 2005). There was also an increase in a range of hydrolytic enzyme production including ligninases, as well as cellulases, even under water stress conditions. Although it is accepted that the extracellular lignolytic enzymes are at least in part responsible for the critical initial reactions of pollutant transformation, the production and activity of these enzymes in contaminated soil under different field conditions have not been examined in detail, although they are critical for successful degradation (Lang *et al.* 1998; Baldrien, 2007).

Most studies of bioremediation of pesticides have concentrated on single contaminants only. For example, Tuomela et al. (1999) showed that *Trametes versicolor* mineralised 29% of added PCP during 42 days of growth in soil. However, soil environmental conditions were not studied in detail, which could have a big impact on degradation rates. However, in contaminated soils pesticides are more commonly found in mixtures (Schoen and Winterlin, 1987; Bending et al., 2006).

The objectives of the present study were to examine the effect of using *T. versicolor* or *P. chrysosporium* as inoculants on a wood chip base in soil microcosms with different water potentials (-0.7, 2.8 MPa) to examine the effect on (a) soil respiration over a period of 12 weeks, (b) dehydrogenase, total liginolytic activity, cellulose and laccase, and (c) differential breakdown of mixtures of simazine, trifluralin and dieldrin.

2. Materials and methods

2.1 Soil

The soil used in this study was a sandy loam soil from Silsoe, Bedfordshire, containing 71.9% sand, 15.8% silt, 12.4% clay, 5.0% organic matter, 81.7 ± 4.1 mg kg^{-1} soil extractable phosphorous, 4.7 ± 0.2 mg kg^{-1} soil nitrate-N, 0.7 ± 0.01 mg kg^{-1} soil ammonium- N, organic matter: furnace 5.01%, titration 1.7% and pH of 6.1 (analysed by School of Applied Sciences, Cranfield University, Bedfordshire, U.K.).

2.2 Soil moisture adsorption curve

Since water availability determines the microbial activity in soil, prior to microcosm experiments being carried out, a moisture adsorption isotherm was developed for soil by adding different volumes of water to 50 g of air dried soil, in the range 0.5-4.0 ml. Soil sub-samples were left to equilibrate overnight at 4^{o}C, before measuring the water potential with an Aqualab-Dewpoint Potentiometer WP4. This enabled accurate modifications of water potential to be made. The relationship between the amount of added water (ml) and resultant water potential is shown in Figure 1. The water potential of each microcosm was adjusted to -0.7 and -2.8 MPa by adding 10 and 5 ml of water to each jar (respectively). Glycerol:water (500-750 mls) solutions were used to maintain the steady-state ERH equivalent to the soil treatment water potential. These were changed regularly during incubation.

2.3 Soil microcosms

The pesticide degradation rates, respiratory activity, total microbial populations and the enzyme production in soil inoculated with the selected fungi were evaluated using soil microcosms. Each microcosm comprised 95 g of non-sterile soil and 5 g of inoculated carrier, the same ratio described by Boyle (1995). Since white rot fungi are obligate aerobes (Pointing, 2001) aeration was ensured by using glass vessels for plant tissue culture (V-8630, SIGMA) with vented caps, with a polypropylene membrane 0.22 μm pore size (B-3031, SIGMA).

2.4 Pesticides and incorporation into soil

Analytical grades of each pesticide: simazine (6-chloro-N2,N4-diethyl-1,3,5-triazine-2,4-diamine), trifluralin (a,a,a-trifluro-2,6-dinitro-N,N-dipropyl-*p*-toluidine) and dieldrin (1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanonaphthalene) were obtained from Greyhound, Birkenhead, UK. Simazine is a triazine herbicide, whereas trifluralin is a dinitrotoluidine herbicide. Dieldrin is a chlorinated insecticide. These three compounds are included in the UK red list of toxic substances. Pesticide stock standard solutions were prepared by dissolving analytical standards in methanol and storing in amber bottles at 4ºC. Working standard solutions were obtained by dilution with acetonitrile.

Pesticide solutions were diluted in water and added to each soil microcosm, in order to obtain the desired water potential and a final concentration of 0 and 10 mg kg^{-1} soil, depending on the treatment. The fortified soils were then mixed using a mortar and pestle and left to equilibrate overnight at 4ºC, in the dark.

2.5 Fungal inoculants and inoculation

The isolates used in this study were the white rot fungi: *Phanerochaete chrysosporium* (strain ATCC 35541; ME446) and *Trametes versicolor* (strain FPRL 28A), kindly provided by Dr Mike Challen (HRI-Warwick University, Wellesbourne, U.K.). Isolates were kept as slopes or plates on 5% malt extract agar (MEA) for up to 3 months at 4^{o}C.

The inoculum was prepared for each treatment fungus by growing biomass on moist (50% water content) sterile softwood wood chips for 4 weeks at 25ºC prior to inoculation of the soil. The mixture of pesticides was added to the soil microcosms, and the different treatments were: (a) control + 0 mg kg^{-1} pesticide ; (b) control + 10 mg kg^{-1} pesticide; (c) wood chips + 0 mg kg^{-1} pesticide; (d) wood chips + 10 mg kg^{-1} pesticide; (e) *T. versicolor* + no pesticide; (f) *T. versicolor* + 10 mg kg^{-1} pesticide; (g) *P. chrysosporium* + no pesticide; (h) *P. chrysosporium* + 10 mg kg^{-1} pesticide.

The inoculum was added to the soil, 5 g of inoculum per solid culture vessel (Magenta, Sigma Ltd, U.K.), which can be closed with plastic lids containing a permeable membrane and then incubated at 15ºC, in the dark. Three replicates of each treatment were destructively sampled after 0, 42 and 84 days and carried out twice. The initial soil moisture and fresh:dry weight ratio of each soil sample was determined by drying 8-10 g of fresh soil at 65^{o}C to a constant weight.

2.6 Quantification of pesticide concentrations in soil

10 ml of methanol were added directly to 5 g of wet soil sub-sample in a conical flask, and agitated overnight. After extraction the solvent-soil slurry was poured through a

100 mm top diameter funnel lined with Whatman No.1 filter paper containing 1g of filtering agent Celite 545 (Aldrich, cat. 41993) and collected in a 250 ml beaker. HPLC quantification of all three pesticides was performed with a Gilson HPLC system equipped with a UV detector (117 UV detector, Gilson), Gilson 401C Dilutor, Gilson 231XL Sampling injector, Gilson 306 Pump and Gilson 811C Dynamic Mixer, equipped with a Altima C18 5μm column (4 mm x 250 mm x 4.6 mm). The column was operated at ambient temperature with a flow rate of 1.5 ml min^{-1} and an injection volume of 50 μl.

An isocratic mobile phase system was established using acetonitrile:water at a ratio of 70:30. The HPLC-UV detector was monitored at 215 nm. The HPLC method used enabled the separation and quantification of simazine, dieldrin and trifluralin in a single HPLC run (20 min) with simazine eluting at 3, trifluralin at 11 and dieldrin at 13 min. The limit of detection for the three pesticides was 0.1 mg l^{-1} soil. Standard curves of pesticides were made for each standard in soil extract broth and r-squared values for each curve found to be > 0.99 for all three pesticides. Initial studies were carried out at each treatment water potential to quantify extraction efficiencies (>80% for all) and these were taken account of in final quantification.

2.7 Assessment of fungal growth and metabolic activity in soil

Soil respiration: **Soil respiration was measured by monitoring the concentration of carbon dioxide in the head-space of the microcosm jars, using a Gas Chromatograph (GC) equipped with a packed column (Porapak Q packed glass column) and a thermal conductivity detector (Carlo Erba Instruments, GC 8000 Series MFC800). The conditions of the analysis were the following: column temperature 100ºC; injector temperature: 100^0C; detector temperature 180ºC; filament temperature 230ºC; carrier gas (Helium) and flow rate 40 ml min^{-1}. CO_2 concentration was measured by injecting 3 ml headspace gas and was estimated by reference to a standard calibration gas mixture (10.3 % CO_2 in N_2). The microcosm jars had vented caps, to allow gas exchange.**

In order to obtain a detectable concentration of CO_2 the vented caps were sealed and left at room temperature for 3 hours, prior to CO_2 analysis. Respiration rate was expressed as mg CO_2 h^{-1} g $soil^{-1}$.

Dehydrogenase activity: **2 (p-iodophenyl)-3-(p nitrophenyl)-5-phenyl tetrazolium chloride INT (Acros 146-68-9) was used as substrate for soil dehydrogenase activity (Von Mersi and Schinner, 1991). The INT solution (9.88 mM) was prepared by dissolving 500 mg of INT into 2 ml of N,N-dimethylformamide, followed by the addition of 50 ml of distilled water. The solution was sonicated for 2 minutes and water was added to bring the volume up to 100 ml. The solution was stored in the dark and always used fresh.**

The method is based on the incubation of 0.5 g of moist soil with 375 µl of Tris-HCl buffer (1M, pH 7.0) and 500 µl of the substrate INT at 37^0C for 2h, in the dark followed by colorimetric estimation of the reaction product iodonitrotetrazolium chloride INF (I-7375, Sigma). After the incubation every sample was mixed with 2500 µl of extraction solution ethanol: N,N-dimethylformamide (50:50), and kept in the dark. The samples were shaken vigorously at 20 minutes intervals for 1h to extract the INF, produced in the reaction. After filtration the developed INF was measured at 434 nm against the control. To eliminate the chemical (non-microbial) INT reduction controls were prepared with autoclaved soil (121^oC for 20 min) and treated like the samples. For the calibration curve of INF: standard INF (Sigma I-7375) solutions of known concentrations in a range between 0.324 and 12.96 µg ml^{-1}, were prepared in N,N-dimethylformamide. 875 µl of standard solution was added to 2.5 ml of extracting solution and the absorbance was read at 434 nm.

Total ligninolytic activity: The poly R-478 (polyvinyl sulfonated backbone with anthrapyridone chromophore, violet colour) decolourisation assay was used to study the overall ligninolytic activity in the soil, following the method described by Baheri and Meysami (2002). The assay consisted of mixing 1 g of wet soil with 5 ml of dye poly R-478 (P-1900, Sigma) in aqueous solution (0.02 g l^{-1}). The reaction mixture was kept under light for 24 h for the enzyme reaction to take place. After 24 h the mixture was centrifuged

for 4 min at 5000 rpm (Eppendorf centrifuge: Beckman Microfuge ® Lite) in order to separate the soil particles. Total ligninolytic activity was given as decolourisation degree of the Poly R-478, monitored by the percentage reduction in the absorbance ratio at 520 nm and at 350 nm (Moredo *et al.*, 2003) , calculated as follows:

Colour intensity = absorbance at 530 nm/ absorbance at 350 nm

% Colour of a sample = (absorbance at 530 nm/ absorbance at 350 nm) $_{sample}$ x 100 / (absorbance at 530 nm/ absorbance at 350 nm) $_{poly\ R478}$

% decolourisation = 100 - [(absorbance at 530 nm/ absorbance at 350 nm) $_{sample}$ / (absorbance at 530 nm/ absorbance at 350 nm) $_{poly\ R478}$]

A lower absorbance ratio, means intense decolourisation and higher enzymatic activity.

Laccase and cellulase activities: **Laccase and cellulase activities were quantified on an enzyme extract, obtained from each soil sample. Enzymes in the soil were extracted by mixing 5 g of soil and 20 ml 10 mM phosphate buffer at pH 6.5, agitated in an incubator shaker at a speed of 250 rpm (KS501 Digital IKA Labortechnik) at 4^0C for 1 hour (Criquet *et al.* 1999). This was followed by centrifugation (Beckman Microfuge ®Lite), at 3800 rpm for 6 min, at room temperature. The supernatant obtained contained the fungal enzymes and was stored in 1.5 ml microcentrifuge tubes at –20^oC.**

Laccase activity was determined with ABTS (2,2- azino-bis (3- ethylbenzthiazoline-6-sulfonic acid)) (A-1888, Sigma) at 405 nm, based on the protocol described by Buswell *et al.* (1995). The assay was carried out at ambient temperature, with the ABTS and buffer equilibrated at 37ºC. The reaction mixture, in a total volume of 300 µl (appropriate for 96 well microtitre plates), contained 150 µl sodium acetate buffer, pH 5.0, and 100 µl of enzyme extract. The reaction was initiated by adding 50 µl of 0.55 mM ABTS.

Laccase activity was computed from the increase in A405, recorded in a microtitre plate reader (Dinex Technologies MRX Revelation) set in the kinetic mode (reaction time of 10 minutes, 5 seconds agitation at the beginning). Boiled enzyme was used in the control

sample. One activity unit was defined as the amount of enzyme producing a 0.001 increase in the optical density in 1 min at the conditions of the assay. This assay was first optimised using commercial laccase from *Rhus vernificera*, crude acetone powder, minimum 50 units mg^{-1} solid (L-2157, Sigma), giving a positive result for laccase concentrations as low as 0.03125 mg ml^{-1}, i.e. 0.375 units per well.

Cellulase activity was assessed with carboxymethyl-substituted (CM-) and water soluble polysaccharide derivatives labelled covalently with remazol brilliant blue R (RBB), i.e., CM-cellulose-RBB (Wirth and Wolf, 1992). The assay was performed in microtitre plates. The experimental procedure was as outlined in the Remazol Brilliant Blue R (RBB) protocol, supplied by LOEWE Biochemica. CM-cellulose (50 μl; 4mg ml^{-1}) and buffer (50 μl 0.2M sodium acetate buffer, pH 5) were equilibrated in an incubation chamber at 37ºC. After the addition of 100 μl of enzyme sample the microtitre-plates were sealed with low evaporation lid and incubated for 30 min. The reaction was terminated by the addition of 50 μl of HCl 2N, causing the precipitation of the non-degraded high polymeric substrate. Subsequently the plates were cooled on ice (10 min) and centrifuged at 1450 *g* in a centrifuge equipped with a rotor for microtitre-plates. Supernatants (175 μl) containing soluble dye-labelled degradation products were transferred to a 96-well, half size EIA plate (175 μl, Costar, 1 cm path length) and measured spectrophotometrically at 600 nm. Blanks were prepared similarly (3 replicates per treatment) but without the addition of enzyme sample during incubation.

One unit of enzymatic activity was calculated as absorbance variance (sample absorbance – blank absorbance) x 1000 x min^{-1}. This assay was initially optimised using commercial cellulase from *Aspergillus niger*, minimum 0.3 units mg^{-1} solid, (C-1184, Sigma), giving a positive result for concentrations as low as 0.002 units in the well.

2.8 Data handling and statistics

Data input, data handling/manipulation, linear regression, and graph plotting was carried out using Microsoft Excel 2003 (Microsoft Co.). Other statistical tests (i.e. ANOVA and other statistical tests) were performed using XLSTAT© (Version 5.1) and Statistica (Statsoft, release 8). When required comparison between means was carried out using ANOVA followed by Tukey Multiple Comparisons test (P=0.05).

3. Results

3.1 Effects of treatments on soil respiration

Figure 2 shows the effect of pesticide mixtures, fungal inoculant and water stress treatments on relative respiration rates initially and after 6 and 12 weeks incubation at 15^{o}C. In natural soil the respiratory activity of resident microbial populations was low. Soil amendment with wood chips enhanced respiration. Interestingly, the respiration of treatments containing both fungal inoculants and pesticides resulted in the greatest increased in CO_2 evolution. Maximum respiration activity, as indicated by CO_2 production, increased after 6 weeks incubation. At -2.8 MPa water potential there was a significant increase in respiration rates in the pesticide treatments, especially after 6 weeks incubation. By 12 weeks the relative respiration rates in the inoculated treatments had decreased significantly (P=0.05), although this was still above that in soil alone or that with wood chips only.

3.2 Enzyme activity in soil microcosms

Dehydrogenase activity: The effect of treatments on the relative amounts of dehydrogenase produced by treatments at both soil water potentials is shown in Table 3. For this parameter the differences between treatments with fungal inoculants were not as marked as for respiratory activity. Overall, the activity of this enzyme was higher in soil inoculated with the fungal species + woodchips. For example, *T. versicolor* dehydrogenase activity in the 10 mg kg^{-1} pesticide soil mixture was 80-100 % higher than in natural soil after 6 weeks in the dry soil treatment (-2.8 MPa). With *P. chrysosporium* the levels of dehydrogense were significantly higher (P=0.05) initially and then decreased with incubation period.

Total ligninolytic activity: The ability to decolourise Poly-R478 in soil under different pesticide and water stress treatments was used as an indicator of total ligninolytic activity (Figure 4). In natural soil the total ligninolytic activity was not affected by water availability or pesticide treatment (P=0.681, P=0.454 respectively). However, incorporation of wood chips did impact on ligninolytic activity, especially in the drier soil. The decolourisation rates were significantly higher in the pesticide treatments compared with the control (P=0.05). In soil inoculated with *T. versicolor* the total activity was significantly higher at -0.7 MPa (P=0.013). There were no significant differences between pesticide treatments, which suggested that the fungal inoculants were probably tolerant of these compounds producing a similar level of decolourisation with or without pesticides being present. A similar trend was observed for *P. chysosporium*.

Laccase activities: Laccase is an important enzyme exclusively produced by the fungal inoculants. This gave a good estimate of the ability of the fungal treatments to produce this key extracellular enzyme and colonise the soil treatments. In natural soil the laccase levels were very low (Table 1). In soil amended with wood chips there was a slight increase in laccase activity, especially after 12 weeks incubation. Soil inoculated with *T.*

versicolor showed the highest laccase activity after 6 weeks incubation under both water regimes. Water availability did not appear to have any significant affect on laccase production in soil. In contrast, *P. chrysosporium* produced very low levels of laccase, regardless of pesticide or water treatments.

Cellulase activities: Production of cellulase varied with water availability and with fungal inoculants used (Table 2). The lowest production was in natural soil. This was slightly increased in wood chip inoculated soil. However, cellulase production was unaffected by *T. versicolor* growth in relation to pesticide or soil water potential. For *P. chrysosporium* there was higher cellulase activity in the drier soil treatment (-2.8 MPa), however there was no effect of pesticide treatment.

3.3 Degradation of the pesticide mixtures in soil microcosms

Table 3 shows the relative percentages (%) degraded of each of the three pesticides in the soil microcosm treatments after 6 and 12 weeks incubation at both -0.7 and -2.8 MPa soil water potential levels. In natural soil the percentage pesticide of the mixtures degraded varied from 25-30% simazine, 50-60% trifluralin and 40-50% dieldrin after 6 and 12 weeks incubation. Woodchip incorporation (5%) into soil microcosms also resulted in a significant increase in degradation rates of simazine and trifluralin but not of dieldrin.

In soil inoculated with *T. versicolor* there were good degradation rates of the three pesticides after 6 weeks at -2.8 MPa. The increase in degradation was about 40% for simazine, 50% for both trifluralin and dieldrin when compared to the controls. *P. chrysopsorium* had a significant effect on degradation rates, especially after 12 weeks. The degradation rates for each pesticide in the mixture were about 64, 94 and 80% respectively after 12 weeks at -2.8 MPa. Indeed the degradation rates were better than that in wetter soil, except for dieldrin which was completely degraded by *P. chrysosporium*.

4. Discussion

There have been very few studies which have examined the degradation of mixtures of pesticides by bioremedial microorganisms in soil in relation to different soil water potentials. In the present study we used a ratio of 5 g inoculant to 95 g soil. Other authors have used very different ratios. For example, Novotny et al. (2003) described dye degradation in soil using a 50:50 soil:straw based inoculant of *Irpex lacteus*; Canet *et al.* (2001) used a 40% incorporation rate with straw based inoculum; Ryan and Bumpus (1989) used a 25% straw-based inoculam; Elyassi (1997) used 10% straw inoculum; Morgan et al. (1993) used 4 g ground maize cobs to 1 g soil (=400%). We believe that some of these are very unrealistic from a practical and economic point of view for bioremediation of xenobiotics in contaminated soils. Furthermore, few if any examined the impact of water potential or effect on mixtures of pesticides. Novotny et al. (1999) used the same species used in the present study and *Pleurotus ostreatus*, and found the latter species to be better than both *P. chrysosporium* and *T. versicolor*. However, they used sterile soil only, devoid of any of the natural microbial communities which would be present.

Pesticides degradation rates showed that treatments with wood chips alone or that with the fungal inoculants gave significantly increased degradation rates. However, there were differential effects on pecticides when they were in soil as mixtures. *T. versicolor* increased degradation of simazine by between 27-46%, trifluralin by 5-17% and dieldrin by 5-11% at the two water potentials and *P. chrysosporium* by 34-48%, 0-30% and 40-46% respectively, when compared to untreated controls after 12 weeks.

An important result was the incorporation of woodchips. This gave a significant enhancement of breakdown by probably providing foci for colonisation by microorganisms

including fungi of the substrate and by thus facilitating production of a wide range of key hydrolytic enzymes including laccases and ligninases which would enable degradation of the pesticide mixture.

However, the relative amounts of each pesticide remaining varied with inoculant fungal species. Thus enhancement of degradation of mixed xenobiotics may be complex and depend on the actual microorganism used, the mixture of pesticides present, the soil type and environmental conditions. White rot fungi grow into wood fibres secreting ligninolytic enzymes which depolymerises the lignin (Meyami and Baheri, 2003). However, they do not normally grow in soil unless provided with a substrate source (e.g. straw, wood chips or saw dust).

This was supported by the respiration measurements of the different treatments. Higher CO_2 production rates indicated higher respiration rates, suggesting potentially higher mineralization rates. This is usually a good indicator that biodegradation of pesticides by native or introduced fungi was occurring. Respiratory activity appeared to peak at about 6 weeks and decreased by 12 weeks. This could suggest exhaustion of readily degradable organic fractions during this period (Balba et al., 1998). In the present study soil modification with wood chips resulted in an increase in respiratory activity of the total microbial populations in the soil microcosms by about 17% (Fragoeiro, 2005). This was probably due to colonisation of woodchips by native soil microorganisms. There was a further significant increase by the presence of fungal inoculants and wood chips. Soil inoculated with *T. versicolor* or *P. chrysosporium* showed improved respiration rates in all treatments with CO_2 concentrations 11 and 14-fold higher in the 10 mg kg soil^{-1} pesticide mixture treatments. Although we only used a 5% woodchip incorporation, this process probably also increased aeration of soil, enhancing metabolic activity of aerobic

microorganisms. Similar increases have been obtained with organic amendments such as alfalfa and bran (Boyle 1995).

Water potential did have an effect on respiration rates, but this varied with the inoculant fungal species used. While plant growth is limited to -1.4 MPa, the present study have clearly shown that the activity of microbial populations, especially fungi are effective over a much wider soil water potential range. There are few other studies which have examined this important factor and the impact it has on microbial activity. Conant et al. (2004) showed that respiration rates were higher at -0.03 to -0.05 MPa than in drier (-1.0 to -1.5 MPa) soils. Other studies have suggested that fungal activity and enzyme production were optimum at 30-50% (w/w of dry soil) although the actual soil water potentials were not determined (Meysami and Baheri, 2003).

Enzyme production is a critical process to soil function, such as organic matter decomposition and synthesis, nutrient cycling and decomposition of xenobiotics (Acosta-Martinez et al., 2003). In the present study detailed analyses has been carried out for the first time to examine a wide range of enzymes activities in relation to both degradatioin of mixtures of pesticides and water potential status of the soil. We have demonstrated that there are significant changes in dehydrogenase, total ligninolytic acivity, laccase and cellulase activities in soil depending on soil treatment, inoculant growth and pesticide mixture used.

The addition of pesticides to soil resulted in an increase in dehydrogenase activity in most treatments. In the control soil the addition of the pesticides alone enhanced activity with higher levels at -0.7 than at -2.8 MPa. Soils modified with wood chips + pesticides produced the highest levels of this enzyme after 6 weeks indicating a significant stimulation in microbial activity. In the fungal inoculated treatments there was also an increase in dehydrogenase activity. For example, soil inoculated with *T. versicolor* had enhanced

activity after both 6 and 12 weeks at both water regimes. Higher activity was observed at -0.7 MPa. For *P. chrysosporium* there was a decrease in activity over the 12 week incubation period. Although previous studies have suggested that there is an increase in dehydrogenase activity the wetter the soil (Quilchano and Maranon, 2002) we found no particular pattern in this enzyme in our steady state water potential treatments. Previous studies have correlated single pesticide degradation with dehydrogenase activity. Min et al. (2001) reported that increasing concentrations of butachlor (22 mg kg^{-1} soil) in soil enhanced activity of this enzyme by the 16^{th} day of incubation. Baran et al. (2004) reported higher activity of this enzyme in presence of PAHs. Felsot and Dzantor (1995) suggested that alachlor + organic amendments using corn meal resulted in an increase in enzyme activity and degradation rates. They also suggested that organic amendments may enhance co-metabolism of high concentrations of pesticides in soil. In contrast, McGrath and Singleton (2000) reported that while PCP transformation in soil was rapid (250 to 2 mg Kg^{-1} soil) after 6 weeks remediation dehydrogenase activity remained low throughout. They suggested that initial very high concentrations of addition of the PCPs may have been toxic to the microbes while *P. chrysosporium* did not improve remediation.

The activity of dehydrogenase is considered a good indicator of oxidative metabolism in soils and thus of microbiological activity because of it being exclusively intracellular and being linked to viable cells (Quilcano and Maranon, 2002). It has also been suggested that it is short term substrate–induced activity which may reflect the impact of chemicals on the physiologically active biomass of the soil microflora.

Total ligninolytic activity was used in this study as it gives information on the activity of the whole set of enzymes involved in lignin degradation. The results in this study showed that in all treatments the highest decolourisation occurred after 6 weeks incubation in soil amended with *T. versicolor*. There was no effect of pesticide treatments suggesting

that the inoculants were tolerant of the pesticide mixture used. This is supported by our previous studies with these same fungi in vitro where ligninolytic activity increased even when -2.8 water potential stress was imposed (Fragoeiro and Magan, 2005). There does not appear to be direct relationship between the ligninolytic activity and pesticide degradation rates in the present study. This has been previously observed with mixtures of diuron, metalaxyl atrazine and terbuthylazine (Bending et al., 2002) although Alcalde et al. (2002) observed correlation with oxidation of PAHS mediated by laccases.

In natural soil the level of laccases produced was very low or insignificant, whereas that amended with wood chips showed some laccase production in some treatments, especially after 12 weeks incubation. The highest level of activity for soil-amended with wood chips was observed in the 10 mg kg^{-1} soil treatment under water stress (-2.8 MPa; 22 U g $soil^{-1}$). The incorporation of *T. versioclor* resulted in the highest laccase activity, after 6 weeks in both water potential treatments. It is interesting to note that even under water stress this species was very active at producing the extracellular enzyme. *P. chrysosporium* produced very low amounts of laccase which confirms our previous in vitro studies (Fragoeiro and Magan, 2005) and by Novotny et al. (1999) in soil-based studies. Previous studies have also shown different results with remediation of other pesticides. For example, Sannino et al. (1999) showed that *Cerrena unicolor* did not produce laccase in the presence of 0.5-7 mg l^{-1} simazine. However, few studies have examined the implications of water stress on the enzymatic activity of soil inoculants. Boyle (1995) found that *T. versicolor* did not produce laccase in soil at -3.4 MPa but higher activities were recorded at -0.9 to -0.4 MPa water potential.

Cellulase activity was increased by the presence of wood chips alone or by the inoculant *P. chrysopsorium*, especially in the -2.8 MPa treatments. Wood chip degradation required production of cellulases and hemicellulases by the native microbial populations

and this was evident from the cellulase production in this treatment. *T. versicolor*, which produced higher amounts of the other hydrolytic enzymes discussed earlier produced much less cellulases than *P. chrysosporium* in these studies over the 12 week incubation period.

Overall, there were some differences between enzyme production in soil microcosms and that in soil extract broth (Fragoeiro and Magan, 2005). The main differences were that while in soil extract-based liquid culture laccase and cellulase production was much higher at -0.7 MPa while in soil microcosms the optimum was at -2.8 MPa. This study has confirmed the differential rates of pesticide degradation observed previously in vitro (Fragoeiro and Magan, 2005). However, in soil microcosms the relative rates of breakdown of individual pesticides within the mixture were different although significant enhancement in overall breakdown of the mixture was achieved by the addition of the fungal inoculants when compared to untreated soil.

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Table 1. Laccase activity (U g^{-1} soil) in soil microcosm treatments at both -0.7 and -2.8 MPa water potentials at 15°C over periods of 12 weeks. *, significantly different from the control (P=0.05).

Water potential (-MPa)	0.7					
Pesticide Mixture (mg kg^{-1} soil)	0			10		
Time (weeks)	0	6	12	0	6	12
Control	0	0	0	0	0	0
Wood chips	0	0	3.1	0	0	1.7
T. versicolor	0	26.7	368.5*	0	74.4	21.3
P. chrysosporium	0	0	0	0	5.9*	1.8
Water potential (- MPa)	2.8					
Control	0	0	0	0	0	0
Wood chips	0	0	5.4	0	19.2	0
T. versicolor	0	93.3	61.8	0	562.2*	22.5
P. chrysosporium	0	0	0	0	13.3*	1.1

Table 2. Cellulase activity (Units g^{-1} soil) in soil microcosms amended with a mixture of pesticides (dieldrin, simazine and trifluranil, 10 mg g^{-1} soil) inoculated with fungi (*T. versicolor*, *P. chrysosporium*) at -0.7 and -2.8 MPa water potentials and 15^{o}C. *. Significantly different from the untreated control treatment (P=0.05).

Water potential (-MPa)	0.7					
Pesticide Mixture (mg kg^{-1} soil)	0			10		
Time (weeks)	0	6	12	0	6	12
Control	64.7	3.9*	1.3*	73.9	4.0*	1.3*
Wood chips	88.0	50.7	50.4	102.1	12.0*	5.3*
T. versicolor	87.0	33.1	93.8	102.1	13.9	28.9
P. chrysosporium	88.1	36.1	52.9	102.2	75.9	54.5
Water potential (- MPa)	2.8					

Control	55.4	10.9	18.7	92.7	34.1	105.2*
Wood chips	30.0	45.2	33.6	62.3	10.0	107.6*
T. versicolor	94.8	28.0	42.7	62.3	98.7	31.7
P. chrysosporium	94.8	97.5	61.2	62.3	128.7	122.7

Table 3. Comparison of effect of woodchips, and fungal inoculants on percentage pesticide (%) (simazine, trifluralin, dieldrin, 10 mg kg^{-1}) degraded after 6 and 12 weeks at -7.0 and -2.8 MPa water potentials in soil microcosms at 15^{o}C. Figures in parentheses are for comparison with degradation in natural soil. *, significantly different from the controls based on actual concentration using HPLC (P=0.05).

			Percentage pesticide degraded		
Incubation (weeks)	W.potential (-MPa)	Treatment	Simazine	Trifluralin	Dieldrin
6	0.7	Woodchips	41.4* *(2.5)*	56.0 *(58.4)*	71.2 *(23.7)*
		T. versicolor	89.9*	77.7	48.2*
		P. chrysosporium	63.8*	74.7	87.3
6	2.8	Woodchips	13.8 *(21.2)*	75.2 *(57.1)*	61.8 *(40.0)*
		T. versicolor	57.1*	81.7*	70.7*
		P. chrysosporium	64.4*	85.5*	69.9*
12	0.7	Woodchips	46.6* *(27.5)*	67.5 *(62.4)*	79.4 *(53.8)*
		T. versicolor	73.5*	76.5	52.7
		P. chrysopsporium	75.6*	57.3	100*
12	2.8	Woodchips	75.7* *(29.9)*	92.1* *(64.2)*	61.6 *(40.2)*

T. versicolor	57.3	80.9*	51.0
P. chrysopsporium	64.3*	93.7*	79.7*

Figure legends

Figure 1. Water potential (MPa) after addition of various volumes of water to 50 g of soil. Vertical bars represent the mean standard deviation (n=3).

Figure 2. Changes in CO_2 concentration in soil containated with a mixture of dieldrin, simazine and trifluralin (10 mg kg^{-1}) treated with either wood chips alone or the two fungal inoculants (*T. versicolor* or *P. chrysosporium*) on wood chips (5% w/w) over 12 weeks in soil microcosms at -0.7 and -2.8 MPa water potentials and 15^{o}C. Bars indicate standard error of the mean. Key to treatments : C0, control ; C10, control + 10 mg kg^{-1} pesticide mixture ; WC, woodchips ; TV, *T. versicolor* ; PC, *P. chrysopsporium*.

Figure 3. Dehydrogenase activity (expressed as μg INF produced in 2h g^{-1} soil) of soil microcosms containing a mixture of three pesticides (dieldrin, simazine and trifluranil) and inoculated with fungi (*T. versicolor* or *P. chrysosporium*) on wood chips (5% w/w) over 12 weeks at -0.7 and -2.8 MPa water potentials and 15^{o}C. Bars indicate standard error of the mean. Key to treatments : C0, control ; C10, control + 10 mg kg^{-1} pesticide mixture ; WC, woodchips ; TV, *T. versicolor* ; PC, *P. chrysopsporium*.

Figure 4. Total lignolytic activity (expressed as percentage decolouration of Poly R478) in soil microcosms containing a mixture of three pesticides (dieldrin, simazine and trifluranil) and inoculated with fungi (*T. versicolor* or *P. chrysosporium*) on wood chips (5% w/w) over 12 weeks at -0.7 and -2.8 MPa water potentials and 15^{o}C. Bars indicate standard error of the mean. Key to treatments: C0, control ; C10, control + 10 mg kg^{-1} pesticide mixture ; WC, woodchips ; TV, *T. versicolor* ; PC, *P. chrysopsporium*.

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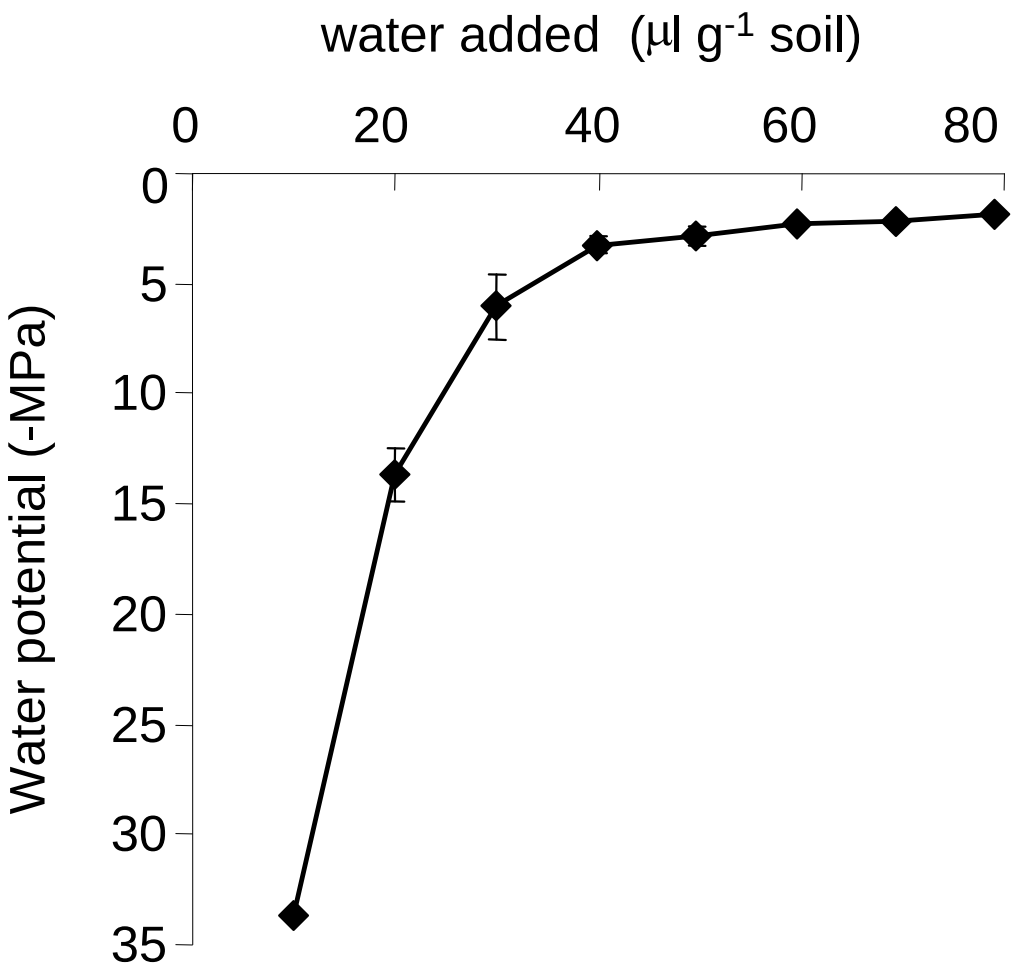

Figure 1. Fragoiero and Magan

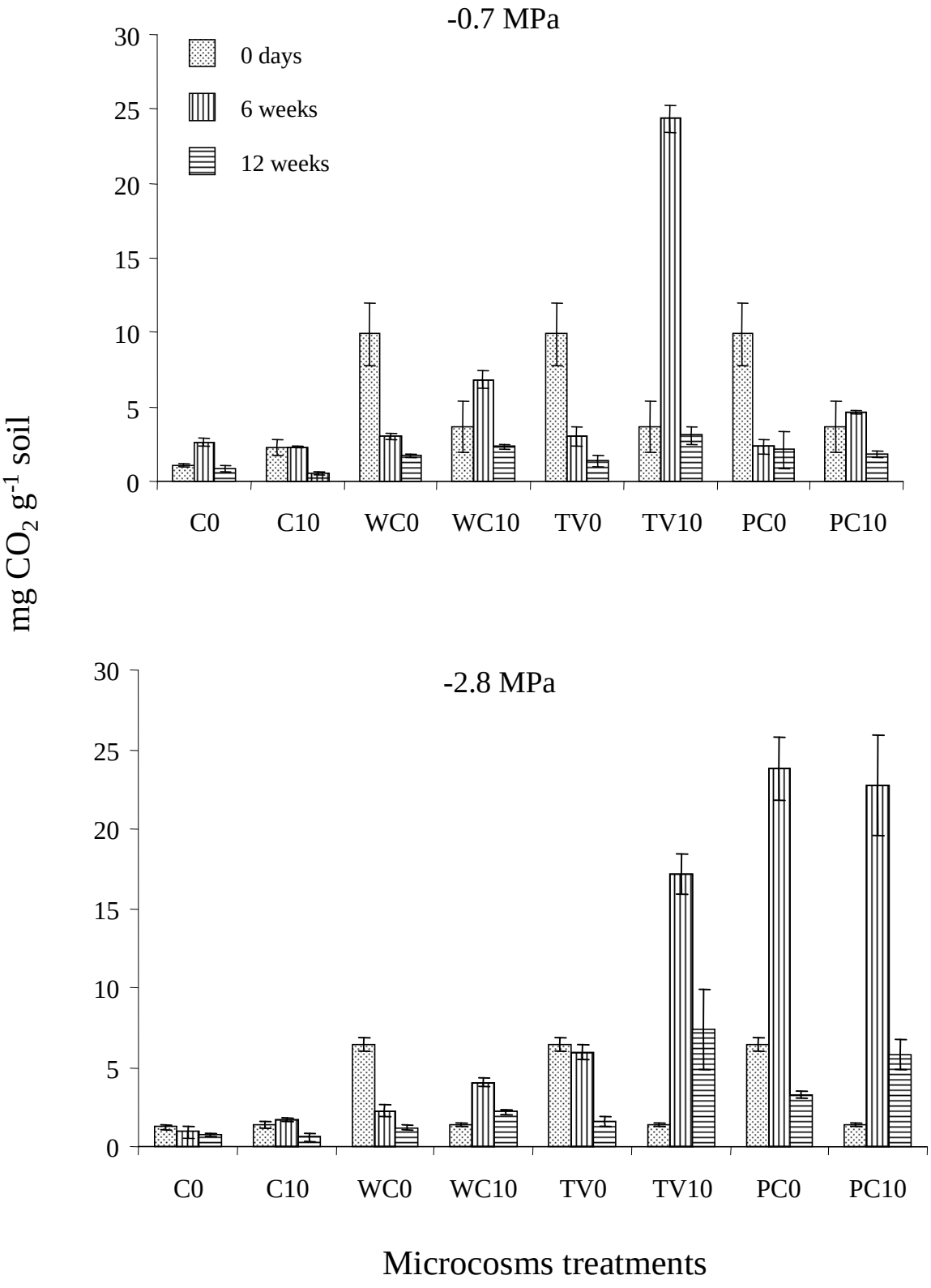

Figure 2. Fragoiero and Magan

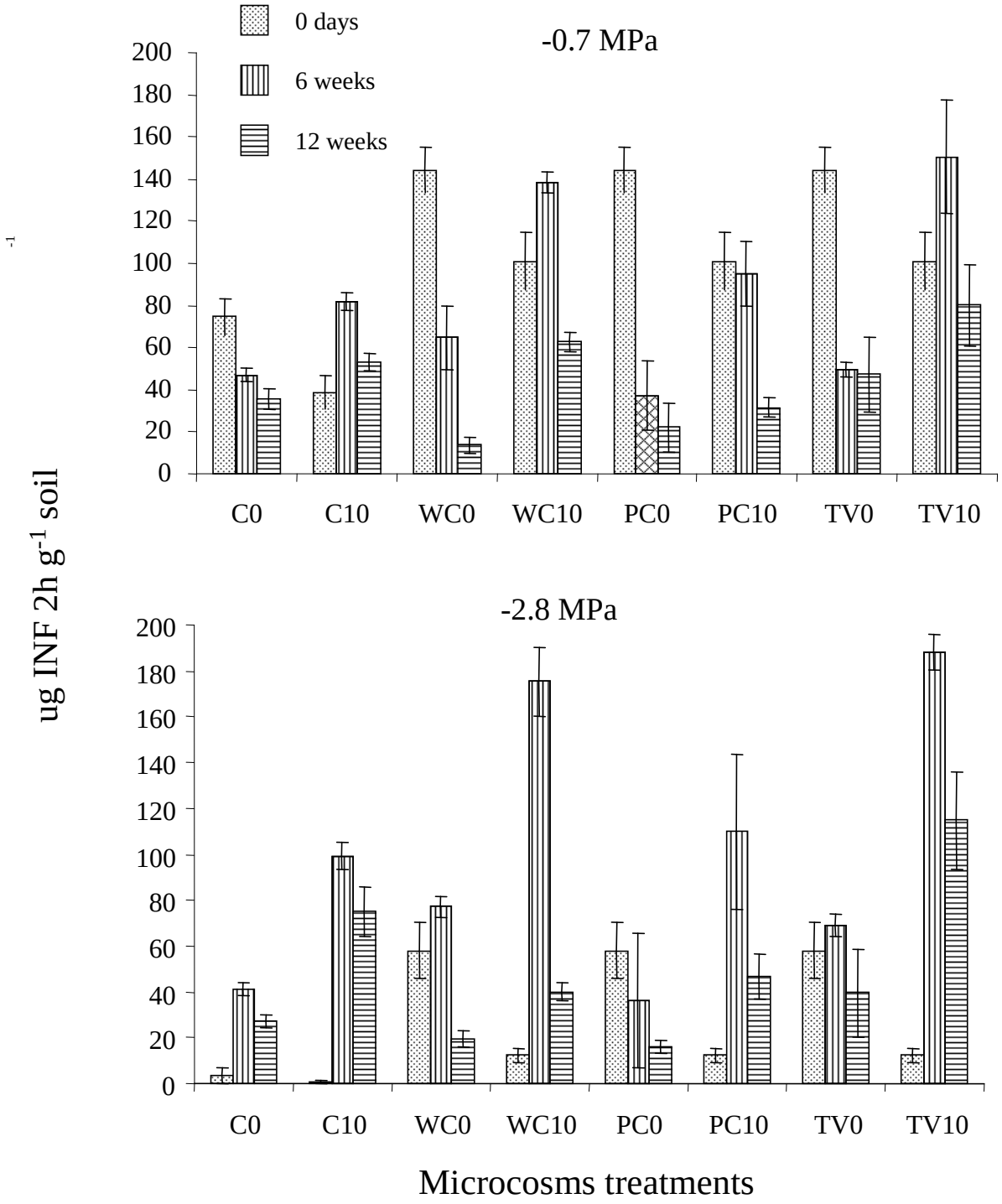

Figure 3. Fragoeiro and Magan

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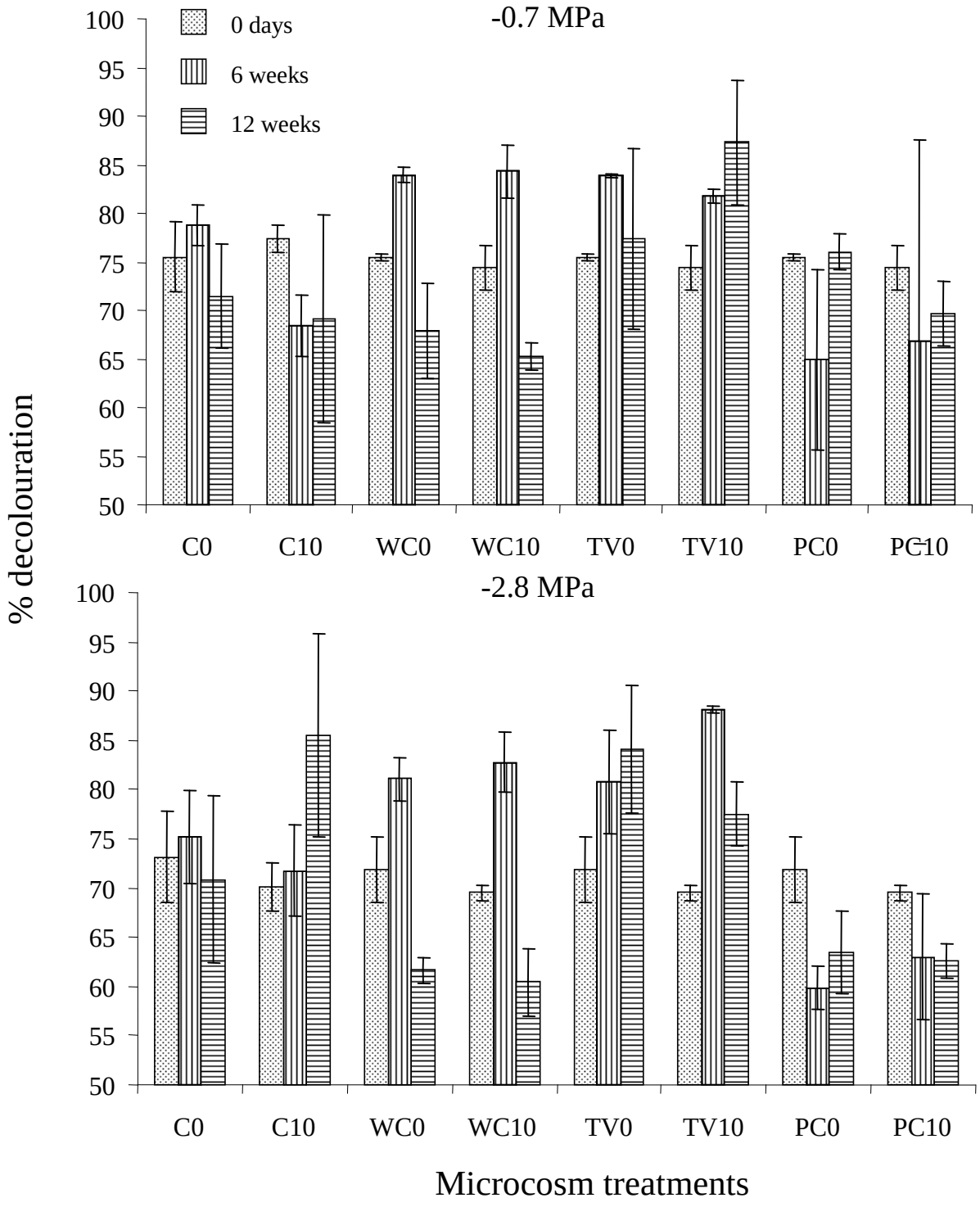

Figure 4. Fragoeiro and Magan