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Enzymatic activity, osmotic stress and degradation of pesticide mixtures in soil extract liquid broth inoculated with *Phanerochaete chrysosporium* and *Trametes versicolor*

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Summary

In this study we examined the extracellular enzymatic activity of two white rot fungi (*Phanerochaete chrysosporium* and *Trametes versicolor*) in a soil extract broth in relation to differential degradation of a mixture of different concentrations (0–30 p.p.m.) of simazine, dieldrin and trifluralin under different osmotic stress (–0.7 and –2.8 MPa) and quantified enzyme production, relevant to P and N release (phosphomonoesterase, protease), carbon cycling (β-glucosidase, cellulase) and laccase activity, involved in lignin degradation. Our results suggest that *T. versicolor* and *P. chrysosporium* have the ability to degrade different groups of pesticides, supported by the capacity for expression of a range of extracellular enzymes at both –0.7 and –2.8 MPa water potential. *Phanerochaete chrysosporium* was able to degrade this mixture of pesticides independently of laccase activity. In soil extract, *T. versicolor* was able to produce the same range of enzymes as *P. chrysosporium* plus laccase, even in the presence of 30 p.p.m. of the pesticide mixture. Complete degradation of dieldrin and trifluralin was observed, while about 80% of the simazine was degraded regardless of osmotic stress treatment in a nutritionally poor soil extract broth. The capacity of tolerance and degradation of high concentrations of mixtures of pesticides and production of a range of enzymes, even under osmotic stress, suggest potential bioremediation applications.

Introduction

There is growing public concern over the wide range of xenobiotic compounds being introduced inadvertently or deliberately into soil. Such contamination can be long-term and have a significant impact on decomposition processes and nutrient cycling and result in environmental problems in groundwater (Muszkat *et al*., 1993; Valcarcel and Tadeo, 1999; Gadd, 2001). One strategy is the potential of using microbial bioremedial means of treating soil to enhance the breakdown of xenobiotic compounds, thus avoiding expensive excavation and removal/replacement of soil, and conserving soil structure and quality (Balba *et al*., 1998; Hollender *et al*., 2003).

White rot fungi have been proposed as promising bioremediation agents, especially for compounds not readily degraded by bacteria. This ability arises from the production of extracellular enzymes that act on a broad array of organic compounds (Yateem *et al*., 1998). Some of these extracellular enzymes are involved in lignin degradation, such as lignin peroxidase, manganese peroxidase, laccase and oxidases (Levin and Forchiassin, 2001). Only a few groups of microorganisms are capable of degrading complex lignin polymers, and they are best exemplified by the white rot fungi, which cause the greatest degree of mineralization (Field *et al*., 1993). Attributed to the low specificity and strong oxidative abilities of their lignin degradation system, white rot fungi are also capable of degrading a broad spectrum of organic chemicals containing carbon skeletons similar to those found within the lignin polymer (Field *et al*., 1993).

While studies on the capacity of white rot fungi to degrade individual pesticides is extensive (Gadd, 2001), very few have examined the capacity to degrade mixtures of pesticides. Furthermore, while fungal bioremediation agents require aerobic conditions, they also have a foraging mycelial habit, which facilitates effective colonization over a wider range of soil water potentials than many bacteria (Magan, 1997). However, few studies have examined the activities of white rot fungi under interacting conditions of pesticides mixtures and different water availabilities.

The objectives of this study were to examine the interactions and activity of two white rot fungi (*Phanerochaete chrysosporium* and *Trametes versicolor*) in a soil extract broth in relation to (i) degradation of a mixture of different

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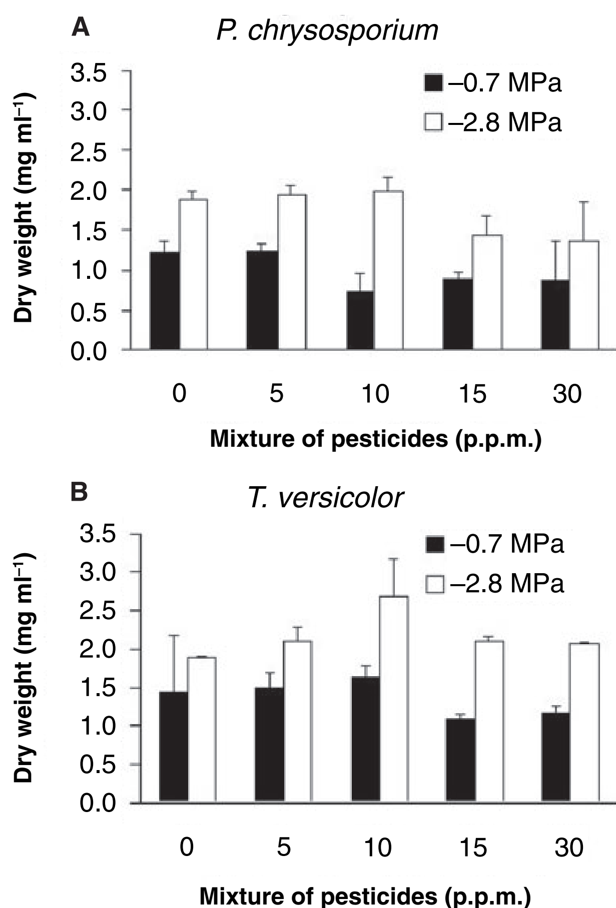

Fig. 1. Dry weight of mycelium of *P. chrysosporium* (A) and *T. versicolor* (B) growing in soil extract supplemented with a mixture of pesticides (0, 5, 10, 15 and 30 p.p.m.), for 25 days, at 27°C, under two different water potential regimes. Error bars represent the standard deviation of the mean ($n = 3$).

concentrations (0–30 p.p.m) of simazine, dieldrin and trifluralin, (ii) interactions with osmotic stress (–0.7 and –2.8 MPa) and (iii) quantify the production of enzymes relevant to P and N release (phosphomonoesterase, protease) and carbon cycling (β-glucosidase, endocellulase) and laccase activity.

Results

Treatment effects on biomass

The effect of mixtures of pesticides, at two different water potential, on biomass of the two fungi after 25-day incubation is shown in Fig. 1. Both *P. chrysosporium* and *T. versicolor* were tolerant of and grew effectively in the presence of up to 30 p.p.m. of the mixture of pesticides in the soil extract broth. The two species showed significantly higher biomass production at –2.8 MPa than at –0.7 MPa ($P < 0.001$). At the two osmotic conditions tested *P. chrysosporium* biomass production was unaffected by pesticide concentration (–0.7 MPa: $P = 0.097$ and –2.8 MPa: $P = 0.067$), which suggests good tolerance to the mixture of pesticides. The *T. versicolor* total biomass was not significantly affected by pesticide treatments at –0.7 MPa ($P = 0.435$). However, at –2.8 MPa, there was a statistically significant increase in biomass when comparing the untreated control in the 10 p.p.m. treatment ($P = 0.022$), suggesting stimulation of growth.

Impact on enzyme activity

Tables 1 and 2 summarizes the impact of the pesticide mixture concentration and water stress treatments on the production of five enzymes by *P. chrysosporium* and *T. versicolor* respectively. *Phanerochaete chrysosporium* produced no laccase in any of the treatments. In contrast, *T. versicolor* produced very high amounts, especially with freely available water. At –0.7 MPa there was a significant increase in laccase production between the control and the treatments ($P = 0.036$). Under osmotic stress laccase production was significantly reduced, although up to 20 units were still detected. At –2.8 MPa osmotic stress there

Table 1. Extracellular enzyme activities of *P. chrysosporium* growing in soil extract supplemented with a mixture of pesticides (0, 5, 10, 15 and 30 p.p.m. total concentrations), for 25 days, at 27°C, under two different water potential regimes.

		Mixture of pesticides (p.p.m.)				
Enzyme (U)	Water potential	0	5	10	15	30
Cellulase	–0.7 MPa	3.4	4.4	0	1.2	1.5
L.S.D. = 2.21	–2.8 MPa	4.2	1.1	0.1	0.9	0.3
Phosphomonoesterase[a]	–0.7 MPa	13.0	26.1	13.9	11.5	16.0
L.S.D. = 12.72	–2.8 MPa	22.1	35.4	26.0	22.9	17.8
β-glucosidase[a]	–0.7 MPa	29.0	9.7	1.4	0.9	3.9
L.S.D. = 13.78	–2.8 MPa	9.3	3.0	1.1	1.0	0.3
Protease	–0.7 MPa	0.1	4.5	23.8	22.4	8.9
L.S.D. = 20.28	–2.8 MPa	6.9	1.7	1.7	0	0.1
Laccase	–0.7 MPa	0	0	0	0	0
	–2.8 MPa	0	0	0	0	0

a. nmol PNP per minute per milligram of protein.
Least significant differences ($P = 0.05$) are for Pesticide–water potential interactions.

Table 2. Extracellular enzyme activities of *T. versicolor* growing in soil extract supplemented with a mixture of pesticides (0, 5, 10, 15 and 30 p.p.m. total concentration), for 25 days, at 27°C, under two different water potential regimes.

Enzyme (U)	Water potential	Mixture of pesticides (p.p.m.)				
		0	5	10	15	30
Cellulase	–0.7 MPa	2.1	3.6	1.8	4.5	4.2
L.S.D. = 1.86	–2.8 MPa	1.6	1.9	1.9	1.8	2.4
Phosphomonoesterase[a]	–0.7 MPa	9.2	5.9	6.0	2.2	2.8
L.S.D. = 12.67	–2.8 MPa	8.9	8.1	13.7	4.5	5.0
β-glucosidase[a]	–0.7 MPa	14.8	14.2	23.2	16.4	45.1
L.S.D. = 21.18	–2.8 MPa	10.2	51.2	11.0	25.7	3.5
Protease	–0.7 MPa	0	4.1	6.1	2.1	2.3
L.S.D. = 39.23	–2.8 MPa	0.3	58.6	28.3	1.0	0
Laccase	–0.7 MPa	91.9	230.1	206.2	214.4	205.7
L.S.D. = 126.34	–2.8 MPa	9.3	3.2	11.8	6.5	20.2

a. nmol PNP per minute per milligram of protein.
Least significant differences ($P = 0.05$) are for Pesticide–water potential interactions.

was no significant difference between laccase activity in the different pesticide treatments. Cellulase production by *P. chrysosporium* occurred at low levels regardless of treatment ($P = 0.596$), whereas *T. versicolo*r exhibited significantly higher activities of cellulase at –0.7 MPa than at –2.8 MPa ($P = 0.006$) in the pesticide treatments. With regard to other hydrolytic enzymes, *T. versicolor* produced higher concentrations of protease than *P. chrysosporium*. Protease production by *P. chrysosporium* occurred at low levels regardless of treatment. *Trametes versicolor* produced higher protease levels at the lowest water availability ($P < 0.001$) and showed a significant increase in protease levels at 5 p.p.m., under water stress ($P < 0.001$). β-Glucosidase production by *T. versicolor* was significantly higher than *P. chrysosporium* ($P < 0.001$). *Trametes versicolor* produced higher β-glucosidase levels at 5 p.p.m. under water stress ($P < 0.05$). *Phanerochaete chrysosporium* showed the highest production of this enzyme at 0 p.p.m., –0.7 MPa ($P < 0.05$). In contrast to β-glucosidase, phosphomonoesterase production by *P. chrysosporium* was much higher than by *T. versicolor*. The activity of this enzyme was not affected by water potential or pesticide concentration.

Degradation of pesticide mixtures

Figure 2 shows the impact of the two fungal inoculants on degradation of the mixture of pesticides. This shows that practically no dieldrin and trifluralin was detected after a 25-day incubation, regardless of initial concentration of the mixture used or the osmotic stress tested. For simazine, only 20% of the initial concentration was present even in the 30 p.p.m. concentration treatment. For both fungal species there was no significant effect of osmotic stress on simazine disappearance rates (*P. chrysosporium* $P = 0.285$ and *T. versicolor* $P = 0.720$). For *P. chrysosporium* simazine disappearance was significantly higher in the 30 p.p.m. treatment ($P < 0.050$). *Trametes versicolor* also showed the highest disappearance rate in the 30 p.p.m. treatment, regardless of the osmotic potential used ($P < 0.050$).

Discussion

This study has shown that these two white rot species are able to grow effectively in low nutrient status media over a range of concentrations of a mixture of pesticides under osmotic potential conditions optimal for fungal growth (–0.7 MPa) of such white rot fungi, and as well as below the wilting point of plants (–2.8 MPa) (Mswaka and Magan, 1999). Both species produced significantly higher biomass under water stress conditions. This ability may be particularly beneficial in the soil environment where water availability fluctuates significantly. Previous studies in soil extract agar media showed that *T. versicolor* was more tolerant to this mixture of pesticides than *P. chrysosporium* (Fragoeiro and Magan, 2002), however, in the present study in soil extract broth this was not observed.

The present study on the degradation of the mixture of pesticides showed good capacity by both fungi at both osmotic potential treatments, regardless of the initial concentrations of pesticides between 0 and 30 p.p.m. Interestingly, greater degradation rates were obtained in the treatments with higher initial concentration of pesticides. We have concentrated on the direct impact of kosmotropic solutes such as NaCl, PEG 600 (Brown, 1990). It is not known whether increasing concentrations of mixtures of pesticides can act as anthropogenic chaotropic solutes (e.g. phenols, urea, ethanol, benzyl alcohols), which may further interact with osmotic stress and influence the effects observed. However, few studies have considered these interactions (Hallsworth *et al*., 2003).

Previously Tekere and colleagues (2002) reported degradation rates of about 82% for the pesticide lindane (an

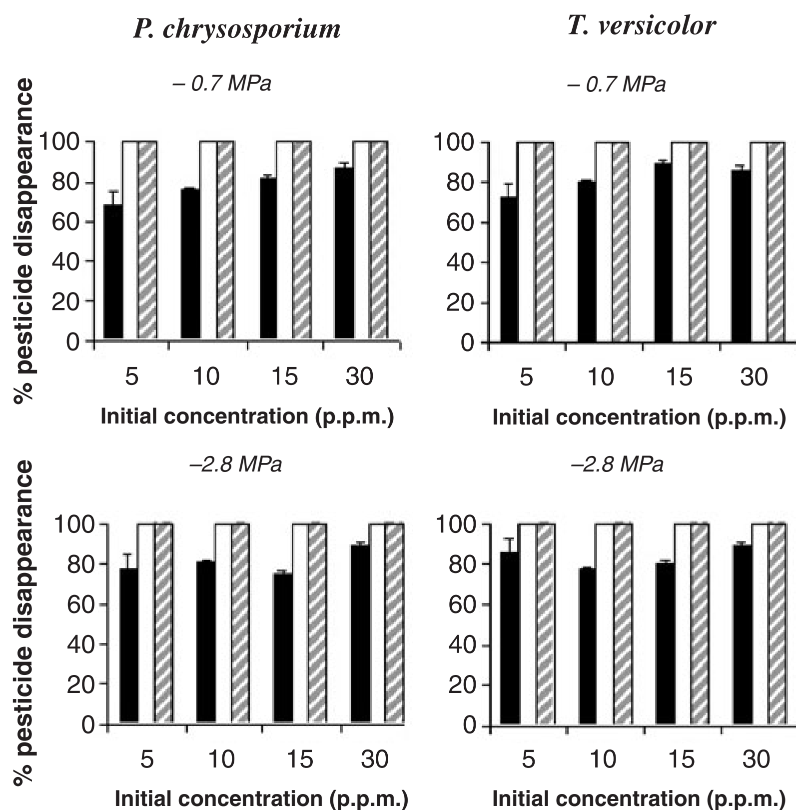

Fig. 2. Percentage pesticide disappearance in soil extract, inoculated with *P. chrysosporium* and *T. versicolor* for 25 days, at 27°C, under two different water potential regimes (key: simazine ■, dieldrin □, trifluralin ▨). Error bars represent the standard deviation of the mean ($n = 3$).

organochlorine, like dieldrin) by *P. chrysosporium* but the highest degradation rates were achieved when the initial concentrations were as low as 5 and 10 p.p.m. Lower degradation was obtained at initial concentrations of 20 and 40 p.p.m. Surprisingly, few studies on the degradation of mixtures of pesticides have been carried out (Yavad and Reddy, 1993; Bending *et al.*, 2002). Yavad and Reddy (1993) described comineralization of a mixture of the pesticides 2,4-D and 2,4,5-T by *P. chrysosporium* wild-type and a putative peroxidase mutant in nutrient-rich broth, with a small amount remaining in the mycelial fractions (5%). Bending and colleagues (2002) showed degradation rates of metalaxyl, atrazine, terbuthylazine and diuron by white rot fungi in nutrient solution of >86% for atrazine and terbuthylazine. However, nutritionally rich media were used and water stress interactions were not considered.

Our results suggest that the hydrolytic and ligninolytic enzymes are not only secreted in nutrient-rich substrates but are produced by mycelia growing in weak nutritional matrices. Of particular interest is the capacity for production of these enzymes in the presence of up to a 30 p.p.m. mixture of the pesticides. In this study, *T. versicolor* exhibited very high laccase activity, for example, in the 30 p.p.m. treatment at –0.7 MPa laccase activity of 680 units ml^{-1} was measured, although growing in a weak soil extract medium. Previous studies suggest that a fungus showing laccase activity of 120–1000 units ml^{-1} in compost is a potential commercial source for laccase (Trejo-Hernandez *et al.*, 2001). Thus, the levels of laccase produced by *T. versicolor* in our study may have some applications.

Under the treatment conditions used in this study *P. chrysosporium* did not produce laccase, although previous studies with other isolates of this species (Shim and Kawamoto, 2002) reported high activities of laccase by this fungus. The conditions of their assay were different as the fungus was grown in a bioreactor and the culture medium was enriched with veratryl alcohol, an inducer of laccase activity. Laccase is a copper-containing phenoloxidase involved in the degradation of lignin (Pointing, 2001). The catabolic role of fungal laccase in lignin biodegradation is not well understood (Eggen, 1999; Trejo-Hernandez *et al.*, 2001) but this enzyme has already attracted considerable interest for biodegradation of xenobiotic compounds with lignin-like structures (Trejo-Hernandez *et al.*, 2001).

Previously, it was assumed that degradation of xenobiotics by white rot fungi is mediated by enzymes involved in lignin degradation but Jackson and colleagues (1999) reported degradation of TNT by non-ligninolytic strains of *P. chrysosporium*. Other studies with *P. chrysosporium* in liquid culture have reported biotransformation of the insecticide lindane independently of the production of ligninolytic enzymes (Mougin *et al.*, 1996), and Bending and colleagues (2002) showed >86% degradation of atrazine

and terbuthylazine by white rot fungi in liquid culture. However, no relationship between the degradation rates and ligninolytic activity was found.

β-Glucosidase and phosphatase are enzymes that carry our specific hydrolyses and were selected in these experiments because they catalyse reactions involved in the biogeochemical transformations of C and P and are likely to be an essential component of any assessment of substrate mineralization (Taylor *et al*., 2002).

Our results show a strong production of phosphomonoesterase by the two test species, independently of pesticide concentration. None of the pesticides used in this study contain phosphorous, thus this enzyme may not act directly on the pesticide mixture but might be involved in degradation metabolism. Phosphomonoesterases are associated with the phosphorous cycle: they form an important group of enzymes catalysing the hydrolysis of organic P esters to orthophosphates (Vuorinen and Saharinen, 1996).

Production of β-glucosidase by *P. chrysosporium* was strongly inhibited in the presence of the mixture of pesticides. In contrast, *T. versicolor* showed stimulation in the activity of this enzyme. As β-glucosidase is associated with the carbon cycle this result may suggest that *T. versicolor* may have a better capacity for utilizing this mixture of pesticides as a source of carbon.

Proteases are rate-limiting enzymes in nitrogen mineralization processes (Horra *et al*., 2003). These were produced at both osmotic stress levels and the range of pesticide concentrations used. These have been monitored previously in relation to soil quality status but not in relation to bioremediation aspects. They could play an important role in enhancing degradation of mixtures of xenobiotic compounds in soil systems.

In summary, the present results suggest that *T. versicolor* and *P. chrysosporium* have the capacity to degrade different groups of pesticides, supported by the ability for expression of a range of extracellular enzymes regardless of imposed osmotic stress at –2.8 MPa. *Phanerochaete chrysosporium* was also able to degrade this mixture of pesticides independently of laccase activity. Although *P. chrysosporium* has received significant interest as a bioremediation agent it has difficulties in colonizing soil (Sack and Fritsche, 1997) and competing with native soil microflora (Tucker *et al*., 1995). Thus, *T. versicolor,* which is tolerant of low water potential, may survive fluctuating soil moisture regimes (Mswaka and Magan, 1999) and be an interesting candidate, especially for degradation of a mixture of xenobiotic compounds.

Experimental procedures

Fungal species

Phanerochetae chrysosporium ATCC 35541 and *Trametes versicolor* FPRL 28 A were used in this study. Stock cultures were maintained on 2% malt extract agar slants at 4°C. These strains were kindly supplied and held in the culture collection of HRI-Warwick, Wellesbourne, Warwick, UK. (Dr M. Challen).

Pesticides

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 hydrocarbon insecticide (Fig. 3).

Liquid culture system

A soil extract liquid medium was used in this study. The soil was a sandy loam from Silsoe, Bedfordshire with 71.78% sand, 15.79% silt, 12.43% clay, 5.01% organic matter, pH of 6.07, extractable phosphorous (mg kg^{-1} soil) 81.7 ± 4.06, nitrate-N (mg kg^{-1} soil) 4.7 ± 0.17, ammonium- N (mg kg^{-1} soil) 0.7 ± 0.035, organic matter: furnace 5.01%, titration 1.67% (analysed by Soil Research Institute, Silsoe, Bedfordshire). Soil extract broth was prepared by using 200 g of untreated field-moist soil in 400 ml of tap water. The soil/water mixture was autoclaved for 30 min, centrifuged at 2400 *g* for 20 min and filtered through filter paper (Whatman

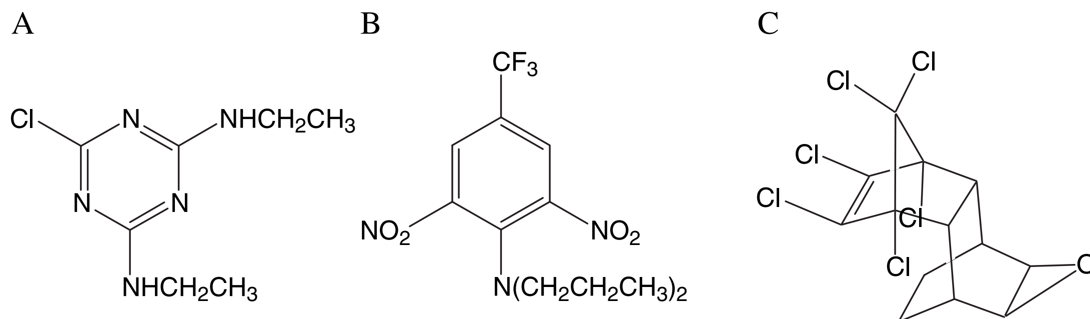

Fig. 3. Chemical structures of the pesticides: simazine (A), trifluralin (B) and dieldrin (C).

no. 1), using a vacuum pump. The water potential of the basic medium was –0.7 MPa. This was modified to –2.8 MPa using the ionic solute KCl (3.73 g/100 ml).

Erlenmeyer flasks (250 ml) containing 100 ml of soil extract were supplemented with a mixture of simazine, dieldrin and trifluralin to give final concentrations of 0, 5, 10, 15 and 30 p.p.m. (total concentration). Four plugs (5-mm diameter) of actively growing mycelium were taken from agar plates and then inoculated in each flask, at 27 ± 1°C, for 25 days with constant agitation at 150 r.p.m. The experiment was carried out with three replicates per treatment and repeated. This data set is from one experiment.

Sampling and dry weight determination

After incubation the mycelium was filtered through Whatman no. 1 paper filters and biomass determined by drying the mycelium for 48 h at 80°C. The fresh filtrate was frozen at –20°C and used later for pesticide quantification, protein determination and various enzymatic determinations.

Pesticide analysis

Samples were filtered through 0.2-μm filter and diluted with acetonitrile (75% sample : 25% acetonitrile) prior to injection in the HPLC system. 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 5-μm column (4 mm × 250 mm × 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 were 0.1 p.p.m. Standard curves of pesticides were made for each standard in soil extract broth and r-squared values for the linear regression were found to be >0.99 for all three pesticides.

Quantification of enzyme production

Laccase

Laccase activity was determined with ABTS (2,2-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid) (A-1888, Sigma) spectrophotometrically at 405 nm, based on the protocol described by Buswell and colleagues (1995). The assay was carried out at room temperature (20°C), with the ABTS and buffer equilibrated at 37°C. The reaction mixture in a total volume of 300 μl, appropriate for 96-well microplates contained 150 μl of 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 microplate reader (Dinex Technologies MRX Revelation) set in the kinetic mode (reaction time of 10 min, 5-s 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 optimized 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.

Protease

The protease activity in the filtrate was quantified using sulfanilamide azocasein substrate (Germano *et al.*, 2002), purchased from Sigma (A-2765), and the assay was optimized to 96-well microplates instead of cuvettes. Azocasein is a chemically modified protein, prepared by adding sulfanamide groups to casein, which are orange and are covalently linked to the peptide bonds. When azocasein is subject to proteolytic action short peptides and amino acids are liberated from the chain and remain in solution, giving an orange colour to the solution. The greater the proteolytic activity the more intense the orange colour of the solution.

Following incubation the assay was carried out at 45°C using 30 μl of azocasein (1% in 0.2 M Tris-HCl buffer, pH 7.5) and the reaction started by adding 20 μl of enzyme solution. After incubation for 1 h the enzyme was inactivated by the addition of 150 μl of trichloroacetic solution (10% W/V) and this solution was neutralized using 50 μl of 1 M NaOH.

Trichloroacetic acid (TCA) was added to stop the reaction and to precipitate macromolecules, including the enzymes and the undigested azocasein. These were then removed by centrifuging the microplate at 958 *g*, for 10 min, in a centrifuge equipped with a rotor for microplates. Subsequently, supernatants (150 μl) were transferred to a 96-well, half-size EIA plate (175-μl cavities, Costar, 1-cm path length). The absorbance was measured spectrophotometrically at 440 nm against a blank prepared similarly but with the enzyme solution inactivated (100°C, 10 min). One unit of enzymatic activity was calculated as absorbance variance (sample absorbance – boiled sample absorbance) × 1000 min^{-1}. This assay was first optimized using commercial protease from *Aspergillus oryzae*, 500 units g^{-1} (P-6110, Sigma), giving a positive result for concentrations as low as 0.0005 units of protease in the well.

Cellulase

Carboxymethyl-substituted (CM-) and water soluble polysaccharide derivatives labelled covalently with Remazol Brilliant Blue R (RBB), i.e. CM-cellulose-RBB was used as a substrate for cellulase (Wirth and Wolf, 1992). The assay was performed in microtitre plates.

The experimental procedure was as outlined in the RBB protocol supplied by LOEWE Biochemica. One unit of enzymatic activity was calculated as absorbance variance (sample absorbance – boiled sample absorbance) × 1000 min^{-1}.

This assay was first optimized 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.

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β-D-glucosidase and phosphomonoesterase

β-D-glucosidase and phosphomonoesterase activities were assayed using p-nitrophenyl substrates (Keshri and Magan, 2000): 4-nitrophenyl-β-D-glucopyranoside and 4-nitrophenyl phosphate disodium salt, respectively, in 96-well microplates. The reaction was started by adding 80 μl of enzyme extract, with 40 μl of acetate buffer (0.05 M pH 4.85) and 80 μl of substrate: 25 mM for 4-nitrophenyl-b-D-glucopyranoside (Acros Organics, 2492-87-7) and 15 mM for 4-nitrophenyl phosphate disodium salt (Acros Organics, 4264-83-9), followed by incubation at 37°C. The control included boiled enzyme extract and was treated in the same way as the samples.

After 1 h 10 μl of 1 M sodium carbonate solution (Sigma Chemical, UK) was added to stop the reaction and the plates were left for 3 min before reading absorbance at 405 nm. The increase in absorbance corresponded to the liberation of p-nitrophenol by enzymatic hydrolysis of the substrate. Total enzymatic activity was calculated from the calibration curve of 4-nitrophenol.

Calibration curve of 4-nitrophenol: Standard 4-nitrophenol solutions (Spectrophotometric grade, N/3200/48, Fisher Chemicals) of known concentrations in a range between 3.28 and 210 μg ml^{-1} were prepared using 0.05 M acetate buffer pH 4.85. The standard solutions were treated in the same way as the samples: by mixing 80 μl of p-nitrophenol solution with 120 μl of buffer, followed by incubation for 1 h at 37°C. Sodium carbonate solution (10 μl, 1 M) was added and the microplate was left for 3 min before absorbance was read at 405 nm.

Protein determination

Protein concentration was quantified with Bicinchoninic Acid Kit for Protein determination (BCA-1, Sigma), using bovine albumin (A-3059, Sigma) as standard. The quantification assay was carried out at 37°C, in 96-well microplates, by mixing 200 μl of reagent solution, provided with this kit, with 25 μl of sample. A linear concentration range between 200 and 1000 μg ml^{-1} or 5–25 μg of albumin is obtained, at 530 nm.

Statistical treatment

Two-way analysis of variance was used to compare different treatments. To isolate which groups differed a pairwise multiple comparison procedure (Tukey Test) was run.

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