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ORIGINAL PAPER

Potential for bioremediation of agro-industrial effluents with high loads of pesticides by selected fungi

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Abstract Wastewaters from the fruit packaging industry contain a high pesticide load and require treatment before their environmental discharge. We provide first evidence for the potential bioremediation of these wastewaters. Three white rot fungi (WRF) (*Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus*) and an *Aspergillus niger* strain were tested in straw extract medium (StEM) and soil extract medium (SEM) for degrading the pesticides thiabendazole (TBZ), imazalil (IMZ), thiophanate methyl (TM), *ortho*-phenylphenol (OPP), diphenylamine (DPA) and chlorpyrifos (CHL). Peroxidase (LiP, MnP) and laccase (Lac) activity was also determined to investigate their involvement in pesticide degradation. *T. versicolor* and *P. ostreatus* were the most efficient degraders and degraded all pesticides (10 mg l^{-1}) except TBZ, with maximum efficiency in StEM. The phenolic pesticides OPP and DPA were rapidly degraded by these two fungi with a concurrent increase in MnP and Lac activity. In contrast, these enzymes were not associated with the degradation of CHL, IMZ and TM implying the involvement of other enzymes. *T. versicolor* degraded spillage-level pesticide concentrations (50 mg l^{-1}) either fully (DPA, OPP) or partially (TBZ, IMZ). The fungus was also able to rapidly degrade a mixture of TM/DPA (50 mg l^{-1}), whereas it failed to degrade IMZ and TBZ when supplied in a mixture with OPP. Overall, *T. versicolor* and *P. ostreatus* showed great potential for the bioremediation of wastewaters from the fruit packaging industry. However, degradation of TBZ should be also achieved before further scaling up.

Keywords White rot fungi · *Aspergillus niger* · Fruit packaging industrial effluents · Fungicides · Biodegradation

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Introduction

During storage fruits are susceptible to fungal infestations by *Penicilium* sp. and *Geotrichum* sp. which significantly diminish their market value (El Ghaouth et al. 2002). In order to minimize such losses, fruits are subjected to fungicide treatments by spraying during waxing operations or by dipping into concentrated aqueous solutions of fungicides. Both treatments lead to the accumulation of large volumes of wastewaters containing high loads (50–1000 mg l^{-1}) of persistent chemicals like thiabendazole (TBZ), imazalil (IMZ),

ortho-phenylphenol (OPP), thiophanate methyl (TM) and diphenylamine (DPA). TBZ, IMZ, OPP and TM are used for the protection of citrus and pome fruits by *Penicilium* infestations (Holmes and Eckert 1999; Smilanick et al. 2006), whereas DPA is utilized as an antioxidant for the control of the apple scald physiological disorder (Johnson et al. 2006). In addition, insecticides which are applied in the orchards close to harvest could be also present in these wastewaters, with chlorpyrifos (CHL) being a common contaminant (Castillo et al. 2000).

Previous studies have shown that direct discharge of these wastewaters into natural water resources without prior treatment constitutes a serious point-source contamination (Flaim and Toller 1989). Indeed, a monitoring study employed in surface water systems of Costa Rica showed that TBZ and IMZ were amongst the most frequently detected pesticides. This was attributed to the presence of fruit packaging plants adjacent to the river systems monitored (Castillo et al. 2000). Fungicides like TBZ and IMZ were included in Annex I of the 91/414/EC directive under the clause that an efficient treatment of the produced wastewaters should be operative (EU 1997, 2001). Indeed, a depuration system based on pesticide adsorption onto granular activated carbon was patented (Technidex) for the treatment of wastewaters containing high loads of TBZ (Garcia Portillo et al. 2004). Although this system achieved 7000 times reduction in TBZ concentrations the cost for its large scale distribution is prohibitive (EU 2000). Similarly, Flaim and Toller (1989) proposed the use of a filter system composed of a mixture of peat moss, manure, clay and dolomite sand which was efficient in removing >98% of residues of TBZ, benomyl and DPA contained in wastewaters. However this system was not able to treat large wastewater volumes, while the use of materials like peat moss and clay increased the cost for its implementation. Therefore, a sustainable and environmentally acceptable method for the remediation of these wastewaters is urgently needed.

Microbial degradation of pesticides is considered to be the most important process for their irreversible environmental removal (Karpouzas and Singh 2006). It is well established that white rot fungi (WRF) are able to degrade a wide range of organic pollutants (Asgher et al. 2008). They are saprotrophic fungi capable of degrading lignin, using a non-specific and extracellular enzymatic system called lignin mineralizing enzyme (LME) system comprising: two glycosylated heme-containing peroxidases, lignin peroxidase (LiP) and Mn dependent peroxidase (MnP), and a copper-containing phenoloxidase, laccase (Lac) (Hatakka 1994; Wesenberg et al. 2003). The low specificity and the extracellular nature of the LME system of WRF resulted in their application in several industrial processes but also as bioremediation agents for prevention of environmental pollution (Pointing 2001; Gao et al. 2010).

Amongst the WRF tested for their biodegradation capabilities *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor* have showed great promise for the bioremediation of pesticide polluted matrices. Different *P. chrysosporium* strains were able to rapidly degrade the herbicide diuron (Fratila-Apachitei et al. 1999) or the recalcitrant organochlorine insecticide lindane (Mougin et al. 1996). Fragoeiro and Magan (2005) demonstrated that *P. chrysosporium* and *T. versicolor* were able to rapidly degrade a mixture of simazine, dieldrin and trifluralin under osmotic stress. In a similar study, Jauregui et al. (2003) showed that *P. ostreatus* was amongst the most efficient WRF in the degradation of organophosphorus pesticides. In several of the above studies, the direct involvement of the LME system in the degradation of the pesticides was shown (Fratila-Apachitei et al. 1999; Castillo et al. 2001). On the contrary, several studies with WRF have suggested that enzymes other than LMEs were responsible for the degradation of pesticides (Kullman and Matsumura 1996; Mougin et al. 1997). Apart from WRF, *Aspergilus. niger* has also shown high degrading potential against several pesticides including organophosphates (Liu et al. 2001), pyrethroids (Liang et al. 2005) and organochlorines (Bhalerao and Puranik 2007).

Our study aimed (1) to investigate the degrading potential of three WRF (*P. chrysosporium*, *P. ostreatus*, *T. versicolor*) and *A. niger* for the degradation of fungicides and insecticides contained in wastewaters produced by the fruit packaging industry and (2) to identify the potential involvement of LMEs in the degradation of the pesticides by the WRF. The degradation of the selected pesticides was tested in soil extract medium (SEM) and straw extract medium (StEM) which mimic the natural habitat of *A. niger* and WRF, respectively. This study will form the basis for the further utilization of these fungi, grown on appropriate

substrates, in biofiltration systems for the treatment of wastewaters from the fruit packaging industry.

Materials and methods

Fungi

The fungal strains used were purchased by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkultuen GmbH) and were *P. chrysosporium* active culture DSMZ 6909, *P. ostreatus* active culture DSMZ 1020, *T. versicolor* active culture DSMZ 11309 and *A. niger* active culture DSMZ 11167. WRF were routinely grown in malt extract agar (LAB M, UK) at 25°C, while the *A. niger* strain was grown in potato dextrose agar (LAB M, UK) at 25°C.

Media

Pesticide degradation by the selected fungi was studied in: a soil extract medium (SEM) and a straw extract medium (StEM). Both media were selected in accordance to life cycle attributes and ecology of the selected fungi. Thus, the WRF were expected to grow better in the StEM, while the soil-borne fungi *A. niger* was expected to be favored in SEM.

Soil from a field of the National Agricultural Research Foundation of Greece in Larissa was used for the preparation of SEM as described before (Karpouzas et al. 2000). The soil was characterized as sandy loam (sand 64%, clay 11%, loam, 25%) with pH 6.5 and organic matter content 1.2% and total N content of 0.56%. The pH of the SEM prepared was 6.2 ± 0.2. Wheat straw from a local farm was used for the preparation of StEM. Straw had a pH of 5.9, organic carbon content of 45.6% and total N content of 0.19%. Briefly, 100 g of chopped straw were mixed with 1 l of deionized water and sterilized at 120°C for 30 min. After cooling, the sterilized mixture was centrifuged at 10,000 rpm for 10 min and the clear supernatant was collected, sterilized again and maintained at 4°C until used. The pH of the StEM was 5.5 ± 0.2.

Pesticides

Analytical standards of CHL (*O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate*, 99%, Chem-Service, USA), TBZ (*4-(1H-1,3-benzodiazol-2-yl)-1,3-thiazole*) OPP (*ortho-phenylphenol*), IMZ ((*RS*)-*1-(β-allyloxy-2,4-dichlorophenylethyl)imidazole*, 99% Riedel-de-Haën, Germany), DPA (*diphenylamine*, 99.9% Riedel-de-Haën, Germany) and TM (*dimethyl 4,4′-(o-phenylene)bis(3-thioallophanate)*, 99.6%, Fluka, Switzerland) were used in the degradation experiments but also for analytical purposes. Apart from the parent compounds, analytical standards of known metabolic products of CHL and TM like 3,5,6-tricholorpyridinol (TCP, 99%, Fluka, Switzerland) and methyl 2-benzylimidazole carbamate (MBC, 99.2%, Fluka, Switzerland), respectively were used for analytical purposes only. A stock solution of all pesticides in methanol (10,000 mg l^{-1}) was initially prepared and used for obtaining a series of dilutions at the range of 0.1–100 mg l^{-1} which were used for the construction of calibration curves for quantification of pesticide concentrations in HPLC.

Pesticide degradation in StEM and SEM by the selected fungi

An aliquot of the 10,000 mg l^{-1} stock solution of the pesticides in methanol was added in a sterilized Duran bottle and was left to evaporate inside a laminar flow hood under aseptic conditions. After complete evaporation, the residues were re-suspended in appropriate amounts of SEM and StEM to obtain media containing 20 mg l^{-1} of each of the pesticides tested. In order to ensure complete and uniform dissolution pesticide-containing media were placed in an ultrasonic water bath for 30 min. Aliquots (30 ml) of StEM without pesticide were added aseptically into sterilized conical flasks (250 ml) and were immediately inoculated with four agar plugs (1 cm i.d.) taken from the margins of actively growing cultures of each of the studied fungi grown in the appropriate agar media. This inoculation procedure has been used routinely in several previous studies assessing the degradation capacity of WRF (Fragoeiro and Magan 2005; Rigas et al. 2005). Immediately after, inoculation flasks were placed in a shaking incubator at 60 rpm at 25°C and the fungi were allowed to grow in the absence of the pesticides for 3 days. After this period, all flasks were supplemented with 30 ml of StEM containing the pesticide (20 mg l^{-1}), thus obtaining a final pesticide concentration of *ca.* 10 mg l^{-1}. Due to the low water solubility (1.4 mg l^{-1}) of CHL, StEM/SEM containing CHL (10 mg l^{-1}) were prepared as described

above and were directly inoculated with the selected fungi thus allowing the immediate contact of the fungi with the insecticide. Subsequently, all flasks were placed back in the shaking incubator. Immediately after pesticide addition and at (2 h), 2, 5, 10, 15, 20 and 30 days, subsamples of the liquid media were removed and used for pesticide analysis as is described below. In parallel, the activity of MnP, LiP and Lac was also determined at the same sampling dates, in order to identify the possible involvement of LMEs in pesticide degradation.

For each pesticide there were three replicates per fungus grown in the presence of the pesticide. Three enzymatic activity controls for each WRF where the fungi were grown in the absence of the pesticides were also prepared to identify if the presence of the chemical alters the activity of LMEs. In addition, triplicate abiotic controls which contained SEM/StEM + pesticide but no fungi were also prepared to investigate the contribution of abiotic processes in the degradation of the pesticides.

Degradation of high pesticide concentrations by *T. versicolor* in StEM

The ability of *T. versicolor* to degrade spillage-level concentrations (50 mg l^{-1}) of the fungicides TBZ, OPP, IMZ and DPA was tested. Such concentration levels are expected to be found in wastewaters from the fruit packaging industry or in natural water resources contaminated with these wastewaters. Triplicate inoculated and non-inoculated StEM cultures containing 50 mg l^{-1} of each of the tested pesticides were prepared and incubated as described before. Immediately after pesticide addition and 2, 5, 10, 15, 20 and 30 days later samples were removed and used for the determination of pesticide degradation by HPLC analysis.

Degradation of pesticide mixtures by *T. versicolor* in StEM

The degrading ability of *T. versicolor* was tested in StEM where the pesticides (50 mg l^{-1} each) were added as mixtures according to their use in practice. Thus, one mixture comprised TBZ, OPP and IMZ commonly used for the postharvest treatment of citrus fruits. The second mixture comprised of TM and DPA which are used in the postharvest treatment of pome fruits. Triplicate inoculated and non-inoculated StEM cultures containing the pesticides in mixtures were prepared, inoculated and incubated as described above. Immediately after pesticide addition and at 5, 10, 15, 20 and 30 days later subsamples were removed and pesticide residues were determined by HPLC.

Pesticide extraction

Regarding CHL and DPA, 1 and 0.5 ml of the liquid medium, respectively was mixed with 2 and 1 ml of methanol or a mixture of acetonitrile:methanol (80:20 v:v), respectively and vortexed (30 s). The mixtures were subsequently passed through a 0.45 μm syringe filter (Sartorius Stedim Biotech GmbH) and the filtrates were used for HPLC analysis. Regarding TBZ, OPP and IMZ residues an aliquot (0.5 ml) of the liquid medium was centrifuged at 14,500 rpm for 15 min. Subsequently, 0.4 ml of the clear supernatant was mixed with 0.8 ml of methanol, vortexed, centrifuged as above and the clear supernatant was used for HPLC analysis.

The residues of TM and its transformation product MBC were determined in 1 ml of liquid medium which was mixed with 4 ml of ethyl acetate (Merck, Germany). The mixture was vortexed (30 s) and 3 ml from the organic phase were removed and evaporated to complete dryness in a rotary evaporator. The residue was resuspended in 1 ml of a 50:50 (v:v) of acetonitrile:water which were used for HPLC analysis.

Pesticide analysis

Residues of all pesticides were determined in an HPLC-UV system equipped with a RP-C18 (150 mm × 4.6 mm i.d.) Nucleosil® column (Macherey-Nagel GmbH, Germany). In all cases the mobile phase flow rate was set at 1 ml min^{-1}. Recovery tests for all pesticides in the selected media were performed at three fortification levels 20, 2, and 0.2 mg l^{-1}. Extraction recoveries for CHL, TBZ, IMZ, DPA, OPP and TM in StEM were 75–84%, 90–98%, 92–99%, 86–90%, 95–98% and 87–90%, respectively. Similarly, extraction recoveries of the same pesticides in SEM were 77–84%, 89–91%, 87–93%, 94–97%, 91–96% and 92–94%, respectively.

CHL and its hydrolysis product TCP were detected at 300 nm. A gradient pump program was utilized with acetonitrile (pump A) and 20:80 (v:v)

methanol:water + 0.5 ml l^{-1} of acetic acid (pump B). The gradient program followed started with solvent A at 20% for up to 1 min and increased to 80% within the next 5 min where it was maintained for the next 4 min. Subsequently solvent A decreased back to its initial 20% until the end of the run (20 min). Retention times for TCP and CHL were 6.4 and 9.9 min, respectively.

For TBZ and OPP, UV detection was achieved at 254 nm, with a mobile phase of 39:60.5:0.5 (v:v:v) acetonitrile:water:30% NH_3 solution. Retention times for TBZ and OPP were approximately 4.3 and 13.1 min, respectively. Regarding IMZ, UV detection was achieved at 204 nm with a mobile phase of 80:20 (v:v) methanol:25% NH_3 solution and its retention time was approximately 4.1 min. DPA UV detection was achieved at 210 nm with a mobile phase of 40:50:10 (v:v:v) acetonitrile:water:methanol and a retention time of 16.3 min. Finally, TM and its transformation product MBC were detected at 270 nm, with a mobile phase of 70:30 (v:v) water:acetonitrile + 0.01% orthophosphoric acid. The retention time of TM and MBC were 10 and 1.9 min, respectively.

Enzymatic activity measurements

Laccase (Lac) activity was determined spectrophotometrically at 425 nm by oxidation of ABTS (2,2-aminobis(3-ethylbenzothiazoline-6-sulphonic acid) (Bourbonnais and Paice 1990). The activity of manganese-peroxidase (MnP) was determined spectrophotometrically at 590 nm by oxidative coupling of MBTH (3-methyl-2-benzothiazoline hydrazone) and DMAB (3-dimethylaminobenzoic acid) (Ngo and Lenhoff 1980). Finally, lignin peroxidase (LiP) activity was determined at 310 nm by monitoring the oxidation of veratryl alcohol (Tien and Kirk 1988). For Lac and Lip activity measurements 0.8 ml of the liquid fungal cultures was utilized, whereas 0.66 ml was used for MnP activity determination.

Statistical analysis

Data from the enzymatic activity were subject to repeated measures ANOVA to identify effects of the main factors (time and pesticide) and their interactions. In cases were significant interactions were observed the LSD test (0.05) was used to identify significant differences between treatments within time.

Results

Pesticide degradation in soil extract medium

Diphenylamine (DPA) (Fig. 1a), *ortho*-phenylphenol (OPP) (Fig. 1b) and thiophanate methyl (TM) (Fig. 1c) were almost completely degraded by *T. versicolor* and *P. ostreatus* within the first 2 days after pesticide addition (dapa). *P. chrysosporium* and *A. niger* showed a slower degradation for DPA (Fig. 1a) and TM (Fig. 1c) with *ca.* 75–85% and 100% loss, respectively at 15 dapa. The same two fungi failed to completely degrade OPP (Fig. 1b). A markedly slower degradation of DPA and TM (*ca.* 50 and 80% at 30 dapa) and a negligible degradation of OPP was noted in the non-inoculated cultures. The initial rapid degradation of TM coincided with the early formation of MBC with maximum concentrations detected in the cultures of *P. chrysosporium* (4.1 mg l^{-1}) at 15 dapa and the lowest in the non-inoculated cultures (1.8 mg l^{-1}) (Fig. 1d).

A rapid loss of chlorpyrifos (CHL) was evident within the first 2 dapa with more than 50% loss observed in all fungal cultures (Fig. 1e). Thereafter, degradation was negligible. A markedly slower degradation was observed in the non-inoculated control cultures. Degradation of CHL led to a slow build up of TCP which did not exceed 1 mg l^{-1} in any of the fungal cultures.

None of the tested fungi was able to degrade thiabendazole (TBZ) and imazalil (IMZ) in SEM during the incubation (data not shown).

Enzymatic activity in soil extract medium

No LiP activity was detected in any of the fungal cultures in both media (SEM and StEM). On the contrary, MnP and Lac activity were detected in the cultures of all WRF tested. Significantly higher Lac (Fig. 2a) and MnP (Fig. 2b) activity ($P < 0.05$) were detected in the *T. versicolor* cultures which had been amended with DPA compared to the non-amended fungal cultures from day 2 onwards. *P. ostreatus* cultures amended with DPA also showed significantly higher Lac activity ($P < 0.05$) compared to the non-amended controls at the beginning (2 and 5 dapa) of the incubation (Fig. 2a), whereas stimulated MnP activity was observed in the non-amended cultures of *P. ostreatus* at the end of the study (20 and 30 dapa)

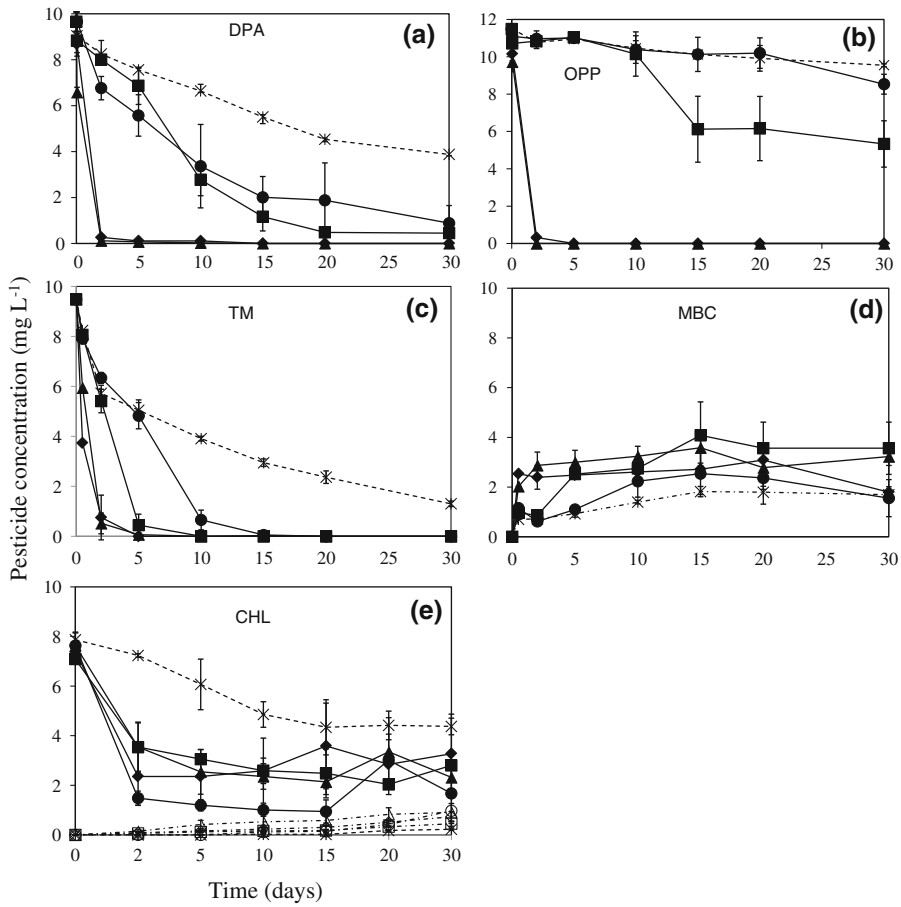

Fig. 1 Degradation of DPA (**a**), OPP (**b**), TM (**c**), formation of its metabolite MBC (**d**), and degradation of CHL (**e**) in SEM inoculated with *Pleurotus ostreatus* (◆), *Phanerochaete chrysosporium* (■), *Trametes verscicolor* (▲), *Aspergillus niger* (●) and in non-inoculated control (×). For CHL the formation of its hydrolysis product TCP is also presented (open symbols). Error bars represent the standard deviation of the means

(Fig. 2b). *P. chrysosporium* showed no Lac activity and little MnP activity throughout the incubation regardless of pesticide presence. In accordance with DPA, significantly higher Lac activity ($P < 0.05$) was detected in the OPP-amended cultures of *P. ostreatus*, which lasted for the first 15 days of the incubation (Fig. 2c), while MnP was not responsive to the presence of OPP (Fig. 2d).

No significant differences ($P > 0.05$) in Lac (Fig. 2e) and MnP (Fig. 2f) activity was observed in the TM-amended cultures of *T. versicolor* and *P. ostreatus* compared to the corresponding non-amended cultures. On the other hand, in the presence of CHL a significantly higher Lac (Fig. 2g) and MnP (Fig. 2h) activity ($P < 0.05$) was measured in the *T. versicolor* cultures compared to the non-amended cultures from day 10 onwards. The presence of TM and CHL in the *P. chrysosporium* cultures caused a significant reduction in MnP activity compared to the corresponding non-amended cultures from 5 up to 15 dapa (Fig. 2f) and throughout the incubation (Fig. 2h), respectively.

No significant differences in the MnP and Lac activity were evident in the fungal cultures in the presence of IMZ and TBZ (data not shown).

Pesticide degradation in straw extract medium

Diphenylamine (DPA) (Fig. 3a), *ortho*-phenylphenol (OPP) (Fig. 3b) and thiophanate methyl (TM) (Fig. 3c) were rapidly degraded by *T. versicolor* and *P. ostreatus* within the first 2 h after inoculation. DPA was also rapidly degraded by *P. chrysosporium* and *A. niger* with a complete loss observed within 5 and 2 dapa, respectively (Fig. 3a). On the contrary, *P. chrysosporium* showed a lower degrading ability for OPP (Fig. 3b) and TM (Fig. 3c) with 70 and 95% degradation, respectively observed at 15 dapa. The slower degradation among all fungal cultures for OPP (Fig. 3b) and TM (Fig. 3c) was evident in the *A. niger* cultures, which was comparable

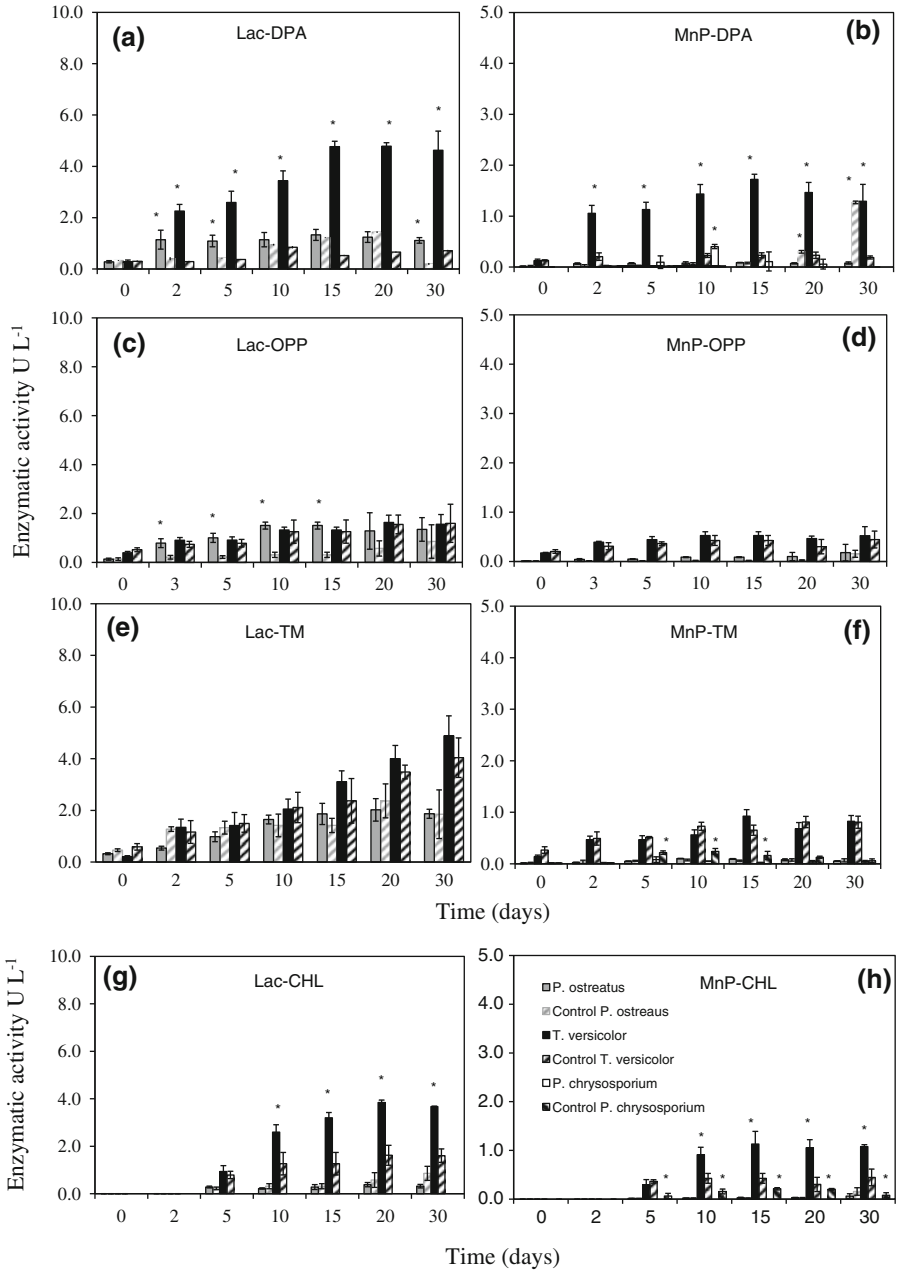

Fig. 2 The enzymatic activity of Lac (**a**, **c**, **e**, **g**) and MnP (**b**, **d**, **f**, **h**) in SEM cultures of the white rot fungi (WRF) which were amended or not amended with DPA (**a**, **b**); OPP (**c**, **d**); TM (**e**, **f**) and CHL (**g**, **h**). The error bars represent the standard deviation of the means. Bars designated with an asterisk indicate a statistically significant difference in the enzymatic activity between pesticide-amended and non-amended cultures (control) for the same fungus within each sampling time

to the reduced degradation observed in the non-inoculated cultures. The major metabolite of TM, MBC was detected in all fungal cultures (Fig. 3d) and its early formation coincided with the rapid degradation of TM. Maximum concentrations of MBC were observed in the cultures of *P. ostreatus* (3.2 mg l^{-1}) at 2 dapa, whereas a slower formation of MBC was evident in the non-inoculated cultures.

Degradation of chlorpyrifos (CHL) followed the same pattern as in SEM in all fungal cultures with a

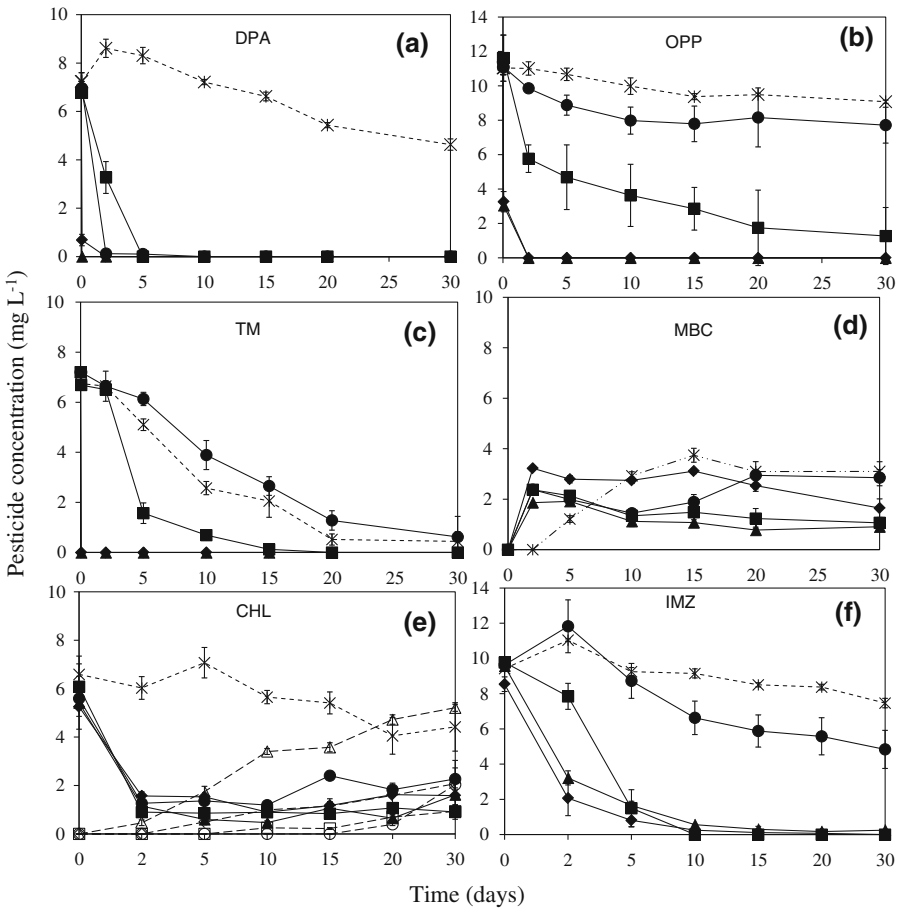

Fig. 3 Degradation of DPA (**a**), OPP (**b**), TM (**c**), formation of its metabolite MBC (**d**), and degradation of CHL (**e**) and IMZ (**f**) in StEM inoculated with *Pleurotus ostreatus* (◆), *Phanerochaete chrysosporium* (■), *Trametes verscicolor* (▲), *Aspergillus niger* (●) and in non-inoculated control (×). For CHL (**c**) the formation of its hydrolysis product TCP is also presented (open symbols). Error bars represent the standard deviation of the means

rapid loss (*ca*. 80%) by 2 dapa and a slow degradation thereafter (Fig. 3e). However, the slowest degradation was observed in the non-inoculated cultures. The metabolite of CHL, TCP was gradually formed and reached the highest concentration in the cultures of *T. versicolor* (5.2 mg l^{-1}) at 30 dapa, while no TCP was detected in the non-inoculated cultures (Fig. 3e).

Similarly to SEM, thiabendazole (TBZ) was not degraded in StEM by any of the fungi tested (data not shown). However, imazalil (IMZ) was fully degraded by the WRF within 10 dapa, while *A. niger* degraded less than 50% of the initial amount by the end of the incubation (Fig. 3f). Minor degradation was observed in the non-inoculated cultures.

Enzymatic activity in straw extract medium

Significantly higher Lac activity ($P < 0.05$) was detected in the DPA—amended cultures of *P. ostreatus* compared to the corresponding non-amended cultures at 2, 10 and 15 dapa, (Fig. 4a). Significantly higher MnP activity ($P < 0.05$) was evident in the DPA-amended cultures of *T. versicolor* and *P. chrysosporium* compared to the corresponding control cultures at 0, 2, 30 dapa and at 2 and 5 dapa, respectively (Fig. 4b). Similarly to DPA, the presence of OPP in the cultures of *T. versicolor* and *P. ostreatus* stimulated Lac (Fig. 4c) and MnP (Fig. 4d) activity ($P < 0.05$) compared to the corresponding non-OPP amended cultures at the beginning of the incubation (0 and 2 dapa). This stimulation of Lac activity persisted for up to 15 dapa in the *T. versicolor* cultures. On the contrary, OPP significantly reduced ($P < 0.05$) MnP activity in the *P. chrysosporium* cultures from day 2 onwards (Fig. 4d).

TM did not appear to significantly affect ($P > 0.05$) Lac and MnP activity in the fungal cultures (Fig. 4e, f). The only exception was observed in the *T. versicolor*

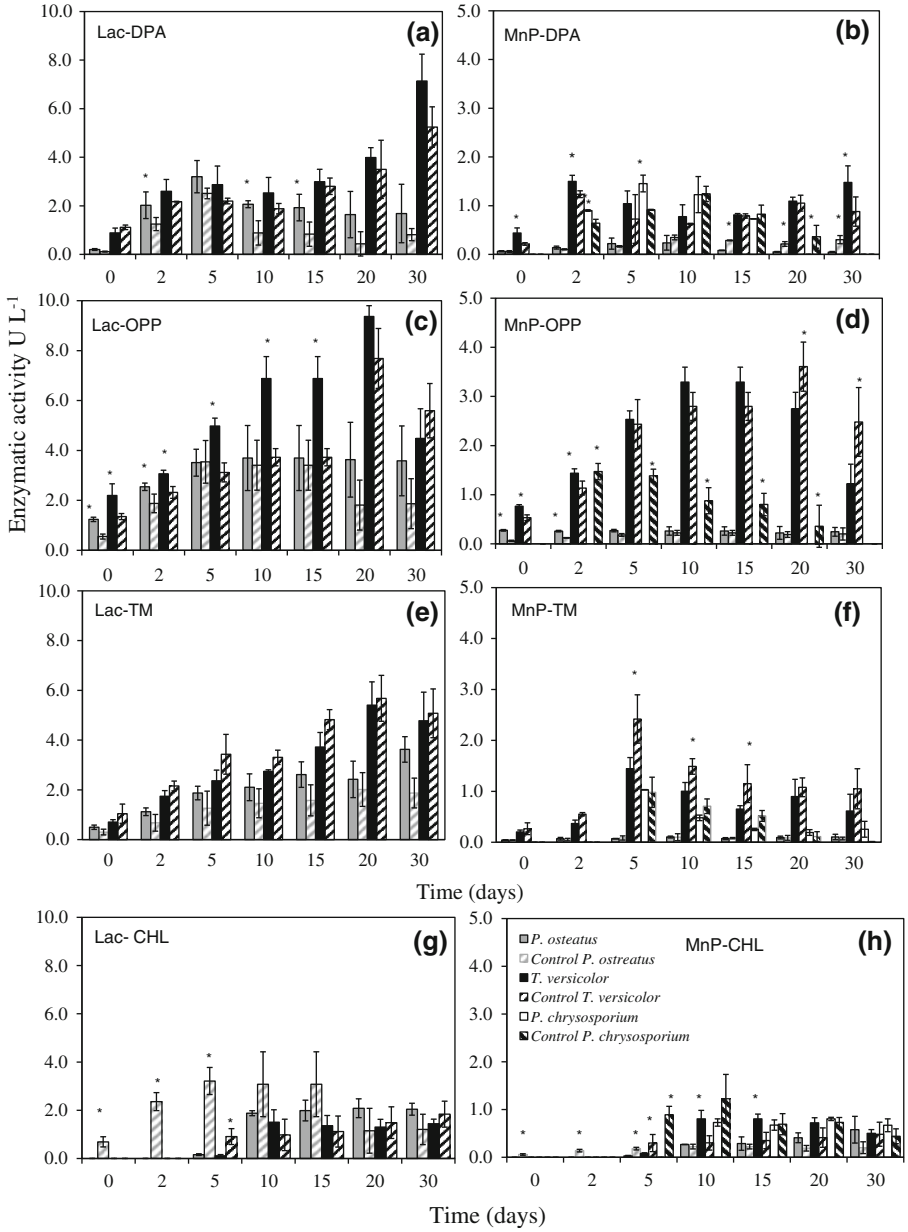

Fig. 4 The enzymatic activity of Lac (**a**, **c**, **e**, **g**) and MnP (**b**, **d**, **f**, **h**) in StEM cultures of the white rot fungi (WRF) which were amended or not amended with DPA (**a**, **b**); OPP (**c**, **d**); TM (**e**, **f**) and CHL (**g**, **h**). Error bars represent the standard deviation of the means. Bars designated with an asterisk indicate a statistically significant difference in the enzymatic activity between pesticide-amended and non-amended cultures (control) for each fungus within each sampling time

cultures, where the presence of TM significantly reduced MnP activity ($P < 0.05$) from day 5 up to day 15 compared to the MnP activity in the non-amended fungal cultures (Fig. 3f).

CHL in the cultures of *T. versicolor* and *P. ostreatus* significantly ($P < 0.05$) inhibited Lac (Fig. 4g) and MnP (Fig. 4h) activity for up to 5 dapa. On the contrary, a significantly higher MnP activity ($P < 0.05$) was observed in the CHL-amended cultures of *T. versicolor* at 10 and 15 dapa (Fig. 4f).

IMZ and TBZ did not induce changes in the MnP and Lac activity (data not shown).

Degradation of high pesticide concentrations by *T. versicolor*

StEM appeared to favour pesticide degradation by the WRF and *T. versicolor* showed the highest and broader degrading efficiency thus its ability to degrade higher pesticides concentration (50 mg l^{-1}) was assessed in StEM. Indeed, *T. versicolor* completely degraded OPP and DPA within 10 and 15 days, respectively (Fig. 5) and degraded more than 50% of the initial amount of TBZ and IMZ within 30 days. Degradation of all pesticides was negligible in the non-inoculated control cultures.

Degradation of fungicide mixtures by *T. versicolor*

T. versicolor was also tested against mixtures of fungicides which were prepared based on their application practice. Thus TBZ, OPP and IMZ were included in the first pesticide mixture, while DPA and TM in the second. The fungus nearly fully degraded OPP within 2 dapa, while negligible degradation of TBZ and only partial of IMZ was evident during the 30 day incubation (Fig. 6a). On the other hand, *T. versicolor* completely degraded DPA and TM by day 5 (Fig. 6b). Negligible degradation of all fungicides was observed in the non-inoculated controls.

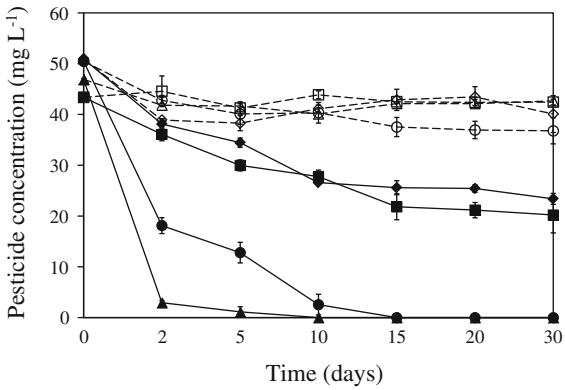

Fig. 5 Degradation of high concentration levels (50 mg l^{-1}) of OPP (▲), DPA (●), IMZ (■) and TBZ (◆) in StEM inoculated (solid line, closed symbols) and non-inoculated (dashed line, open symbols) with *T. versicolor*. Error bars represent the standard deviation of the means

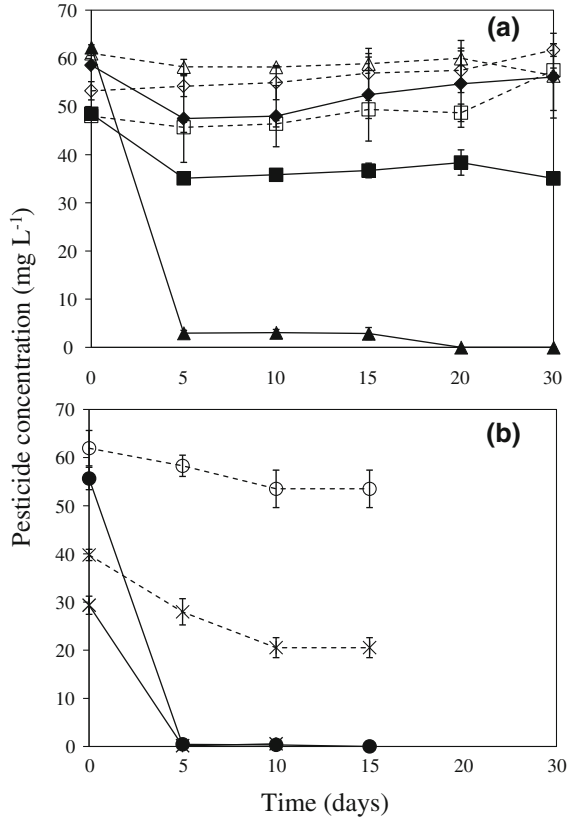

Fig. 6 Degradation of mixtures of (**a**) OPP (▲), TBZ (◆) and IMZ (■); and (**b**) DPA (●) and TM (×) in StEM inoculated (solid line, closed symbols) and non-inoculated (dashed line, open symbols) with *T. versicolor*. Error bars represent the standard deviation of the means

Discussion

White-rot fungi were able to rapidly degrade most of the pesticides tested including diphenylamine (DPA), *ortho*-phenylphenol (OPP), chlorpyrifos (CHL), thiophanate methyl (TM) and imazalil (IMZ) when grown in StEM. On the contrary their degradation efficiency was diminished when grown at SEM. In the case of IMZ, WRF failed to degrade it in SEM, while they managed to completely degrade it in StEM (Fig. 3f). The highest degradation efficiency of WRF in the StEM compared to SEM is in accordance with their saprophytic nature which favors their prolific growth onto N-poor ligninocellulosic materials like straw (Fog 1988; Reddy 1993), compared to soil (Lang et al. 1998). In addition, Tuomela et al. (2002) showed that straw may serve as co-substrate, whereas soil may repress lignin mineralization. *P. chrysosporium* was

the WRF that presented the most pronounced reduction in degradation ability in SEM (Figs. 1, 3). Previous studies have indicated a drastic reduction in the degradation efficiency of *P. chrysosporium* when inoculated in soil (Hickey et al. 1994), compared to *P. ostreatus* (Martens and Zatrazil 1998; Baldrian et al. 2000) and *T. versicolor* (Mswaka and Magan 1999) which were able to grow and degrade organic pollutants in soil conditions.

Trametes versicolor and *P. ostreatus* demonstrated the highest degradation efficiency for all the pesticides tested, while *P. chrysosporium* degraded pesticides either at a slower rate (DPA, TM) or only partially (OPP). Indeed, *T. versicolor* and *P. ostreatus* completely degraded the phenolic pesticides DPA, OPP and the benzimidazolic fungicide TM within few hours in StEM. So far degradation studies have focused on *P. chrysosporium* (Kullman and Matsumura 1996; Mougin et al. 1996, 1997), while fewer studies have investigated the biodegradation potential of *T. versicolor* (Hiratsuka et al. 2001) and *P. ostreatus* (Rigas et al. 2005).

Degradation of CHL by the tested fungi was rapid during the first days of incubation and it was followed by a gradual formation of TCP. This metabolic pattern of CHL implies that its initial degradation proceeded via an unknown intermediate metabolite which was subsequently slowly hydrolyzed to TCP. The latter compound was the only metabolite detected under our analytical conditions. Early studies by Bumpus et al. (1993) reported that *P. chrysosporium* mineralized 27.5% of CHL in liquid culture. The initial rapid degradation of CHL and its negligible degradation thereafter might indicate a potential toxic effect by the gradual accumulation of TCP. Previous studies have showed that TCP possess strong antimicrobial activities and its accumulation might lead to complete inhibition of degradation of the parent compound (Racke et al. 1990; Singh et al. 2003). The rapid disappearance of CHL observed at the early stages of the incubation in all fungal cultures might have been also a result of its rapid adsorption on the fungal mycelium. Previous studies with PAHs have suggested that sorption onto the fungal mycelium is a significant process for the dissipation of such lipophilic molecules (Barclay et al. 1995). However, the fungal mycelium was not adequately developed at the early stages of the incubation (2–5 days) since agar plugs were added in a medium already containing CHL, in contrast to the other pesticides where a 3-day incubation period was allowed before the fungal mycelium comes into contact with the pesticide. Therefore, CHL sorption onto fungal biomass is unlikely to have resulted in such a rapid dissipation of CHL in the fungal liquid cultures. Further studies with autoclaved fungal mycelium might provide conclusive evidence for this process.

Degradation of TM proceeded rapidly with rapid formation of its common metabolite MBC in all fungal cultures, while a slower formation of MBC was evident in the non-inoculated cultures suggesting the involvement fungi in this initial transformation. This is in line with previous findings which reported that TM is rapidly transformed in soil to MBC and that this conversion is microbially driven (Fleeker et al. 1974).

The enzymatic activity of LMEs was measured in the cultures of the WRF in order to investigate their possible involvement in the degradation of the pesticides tested. Activity of MnP and Lac were detected in all cultures of *T. versicolor* and *P. ostreatus* which is in accordance with previous studies with these two fungi (Leatham and Kirk 1983; Waldner et al. 1988). No LiP activity was detected in any of the fungal cultures tested, although *P. chrysosporium* is well-known for its ability to produce this extracellular enzyme (Tien and Kirk 1988). Previous studies by Castillo et al. (1997) have reported a problem with the measurement of LiP activity in substrates rich in lignocellulosic materials and it was attributed to the production of phenolic substances which could act as substrates for the oxidation of veratryl alcohol hampering LiP detection.

The rapid degradation of the pesticides DPA and OPP by *T. versicolor* and *P. ostreatus* coincided, in most cases, with a significant increase in the activity of Lac and MnP in both media. This finding strongly suggests that the LMEs of *T. versicolor* and *P. ostreatus* are involved in the degradation of DPA and OPP which are characterized by two phenolic rings in their molecules. This is in line with the substrate affinities of MnP and Lac which are known to oxidize phenol rings of lignin (Hatakka 1994). The active involvement of LMEs in the degradation of different pesticides has been shown in previous studies. For example, Pizzul et al. (2009) showed in vitro that MnP induced the degradation of most of the 22 pesticides tested.

On the other hand, degradation of the fungicides TM and IMZ proceeded without a significant increase

in the activity of Lac and MnP in the fungal cultures compared to the corresponding controls. These findings indicate that the LMEs are not involved in the degradation of these pesticides and probably other enzymes are responsible. This is in line with several previous studies which have not found any correlation between pesticide degradation and LMEs. For example, Hiratsuka et al. (2001) reported that *T. versicolor* was able to degrade a range of diphenyl-ether herbicides without the involvement of LMEs. Instead, cytochorome P450 monoxygenases were found to mediate the initially hydroxylation of the herbicides.

Aspergillus niger was not as efficient as WRF in the degradation of the pesticides tested. Thus *A. niger* failed to degrade the fungicides OPP, IMZ, TBZ and only slowly degraded DPA and TM. However, *A. niger* degraded more than 80% of CHL within 5 days. Previous studies by Gopal (1996) showed that *A. niger* was able to achieve more than 70% degradation of CHL in liquid medium without any formation of TCP. The reduced ability of *A. niger* to degrade most of the pesticides tested even in SEM, which represents a medium resembling its natural soil habitat was not expected and could be attributed to a direct toxicity effect by the fungicides. This is further supported by the finding that *A. niger* showed a high degradation efficiency for the insecticide CHL and the antioxidant DPA but a reduced degradation ability for all fungicides tested. The close phylogenetic association of *Aspergillus* with *Penicillium*, which are the prime fungal pathogens targeted by the fungicides tested, further reinforces the possibility of a direct toxicity effect by the fungicides. Indeed, *Aspergillus* and *Penicillium* belong to the ascomycetous family Trichocomaceae and share common morphological and ecophysiological characteristics (Ogawa and Sugiyama 2000).

Further studies with the most efficient degrading fungus *T. versicolor* verified its high degrading ability against spillage level concentrations (50 mg l^{-1}) of the phenolic pesticides OPP and DPA, while it only partially degraded the fungicides TBZ and IMZ when supplied at similarly high concentration. The partial degradation of the high concentration of IMZ (50 mg l^{-1}) by *T. versicolor* in StEM is in contrast with its complete degradation, at a lower concentration (10 mg l^{-1}), by the same fungi in the same medium. This discrepancy could be attributed to a potential toxicity effect of the high fungicide concentration or any intermediates which were produced during its degradation. TBZ was also partially degraded by *T. versicolor* in StEM when supplied at high concentration levels (50 mg l^{-1}). In contrast, no TBZ degradation was evident in the StEM cultures of *T. versicolor* when a low concentration of the fungicide was tested (10 mg l^{-1}) or when 50 mg l^{-1} of TBZ was supplied together with IMZ and OPP. The reason for this discrepancy is currently unknown.

Further studies investigated the ability of *T. versicolor* to degrade mixtures of the fungicides relevant to their combined uses in the fruit packaging industry. *T. versicolor* degraded rapidly DPA and TM which are both used in the postharvest treatment of apples. Only few studies so far have investigated the degrading potential of WRF against mixtures of pesticides. For example Bending et al. (2002) reported that WRF degraded >80% of terbuthylazine, atrazine and diuron in liquid culture supplemented with a mixture of these pesticides. More recently, Fragoeiro and Magan (2005) showed that *T. versicolor* and *P. chrysosporium* were able to degrade a mixture of simazine, dieldrin and trifluralin in SEM. Our findings suggest that *T. versicolor* has a high potential for the biopurification of wastewaters produced from the apple packaging industry. On the contrary, *T. versicolor* failed to degrade TBZ and only partially degraded IMZ but rapidly degraded OPP. These fungicides are all used in the citrus fruits packaging industry. The inability of *T. versicolor* to efficiently degrade the high concentrations of TBZ and IMZ is in accordance with the partial degradation of these fungicides by the same fungus when they were supplied individually and not in mixture. Ongoing efforts have focused on the isolation of bacterial populations able to rapidly degrade TBZ and IMZ but also in the up-scaling of the fungal biodegradation system towards the development of an operative system for the depuration of wastewaters from the fruit packaging industry.

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References

Asgher M, Bhatti HN, Ashraf M, Legge RL (2008) Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system. Biodegradation 19:771–783

Baldrian P, in der Wiesche C, Gabriel J, Nerud F, Zadrazil F (2000) Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in soil. Appl Environ Microbiol 66:2471–2478

Barclay CD, Farquhar GF, Legge RL (1995) Biodegradation and sorption of polyaromatic hydrocarbons by *Phanerochaete chrysosporium*. Appl Microbiol Biotechnol 42:958–963

Bending G, Friloux M, Walker A (2002) Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential. FEMS Microbiol Lett 212:59–63

Bhalerao TS, Puranik PR (2007) Biodegradation of organochlorine pesticide, endosulfan by a fungal soil isolate, *Aspergillus niger*. Int Biodet Biodeg 59:315–321

Bourbonnais R, Paice MG (1990) Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation. FEBS Lett 267:99–102

Bumpus JA, Kakar SN, Coleman RD (1993) Fungal degradation of organophosphorus insecticides. Appl Biochem Biotechnol 39–40:715–726

Castillo MdP, Ander P, Stenstrom J (1997) Lignin and manganese peroxidase activity in extracts from straw solid substrate fermentations. Biotech Technol 11:701–706

Castillo LE, Ruepert C, Solis F (2000) Pesticide residues in the aquatic environment of banana plantation areas in the North Atlantic Zone of Costa Rica. Environ Toxicol Chem 19:1942–1950

Castillo MP, Wiren-Lehr S, Scheunert I, Torstensson L (2001) Degradation of isoproturon by the white rot fungus *Phanerochaete chrysosporium*. Biol Fertil Soils 33:521–528

El Ghaouth A, Wilson CL, Wisniewski M, Droby S, Smilanick JL, Korsten L (2002) Biological control of postharvest diseases of citrus fruits. In: Gnanamanickam SS (ed) Biological Control of Crop Diseases. University of Madras-Guindy Chennai, Tamil Nadu, India, pp 291–312

European Union (1997) Review report for the active substance imazalil. 1688/VI/97-final

European Union (2000) Opinion of the scientific committee on plants regarding the evaluation of thiabendazole in the context of council directive 91/414/EEC concerning the placing of plant protection products on the market—The Scientific Committee on Plants, SCP/THIABEN/002-Final, p 7

European Union (2001) Review report for the active substance thiabendazole, 7603/VI/97-final, p 38

Flaim GM, Toller G (1989) Treatment of postharvest pesticide residues. Agric Ecos Environ 27:505–511

Fleeker JR, Lacy HM, Schultz IR, Houkom EC (1974) Persistence and metabolism of thiophanate methyl in soil. J Agric Food Chem 22:592–595

Fog K (1988) The effect of added nitrogen on the rate of decomposition of organic matter. Biol Rev 63:433–462

Fragoeiro S, Magan N (2005) Enzymatic activity, osmotic stress and degradation of pesticide mixtures in soil extract liquid broth inoculated with *Phanerochaete chrysosporium* and *Trametes versicolor*. Environ Microbiol 7:348–355

Fratila-Apachitei LE, Hirst JA, Siebel MA, Gijzen HJ (1999) Diuron degradation by *Phanerochaete chrysosporium* BKM-F-1767 in synthetic and natural media. Biotechnol Lett 21:147–154

Gao D, Du L, Yang J, Wu W-M, Liang H (2010) A critical review of the application of white rot fungus to environmental pollution control. Crit Rev Biotechnol 30:70–77

Garcia Portillo M, Avino ES, Vicente JO, De Andres RL, Jimenez MdlAS, Blanco JPL (2004) Purification system for wastewater coming from fruit and vegetable processing plants and phytosanitary treatments in the field. United States Patent, US 6,709,585 B1, p 9

Gopal M (1996) Degradation of chlorpyrifos by two soil fungi *Aspergillus niger* and *Trichoderma viride*. Toxicol Environ Chem 57:145–151

Hatakka A (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol Rev 13:125–135

Hickey WJ, Fuster DJ, Lamar RT (1994) Transformation of atrazine in soil by *Phanerochaete chrysosporium*. Soil Biol Biochem 26:1665–1671

Hiratsuka N, Wariishi H, Tanaka H (2001) Degradation of diphenyl ether herbicides by the lignin-degrading basidiomycete *Coriolus versicolor*. Appl Microbiol Biotechnol 57:563–571

Holmes GJ, Eckert JW (1999) Sensitivity of *Penicillium digitatum* and *P. italicum* to postharvest citrus fungicides in California. Phytopathology 89:716–721

Jauregui J, Valderrama B, Albores A, Vazquez-Duhalt A (2003) Microsomal transformation of organophosphorus pesticides by white rot fungi. Biodegradation 14:397–406

Johnson DS, Allen JG, Warman TM (2006) Post-harvest application of diphenylamine and ethoxyquin for the control of superficial scald on Bramley's seedling apples. J Sci Food Agric 31:1189–1194

Karpouzas DG, Singh BK (2006) Microbial degradation of organophosphorus xenobiotics: metabolic pathways and molecular basis. Adv Microb Physiol 51:119–186

Karpouzas DG, Morgan JAW, Walker A (2000) Isolation and characterization of ethoprophos degrading bacteria. FEMS Microbiol Ecol 33:209–218

Kullman SW, Matsumura F (1996) Metabolic pathways utilized by *Phanerochaete chrysosporium* for degradation of the cyclodiene pesticide endosulfan. Appl Environ Microbiol 62:593–600

Lang E, Nerud F, Zadrazil F (1998) Production of ligninolytic enzymes by *Pleurotus* sp. and Dichomitus squalens in soil and lignocellulose substrate as influenced by soil microorganisms. FEMS Microbiol Lett 167:239–244

Leatham GF, Kirk TK (1983) Regulation of ligninolytic activity by nutrient nitrogen in white-rot basidiomycetes. FEMS Microbiol Lett 16:65–67

Liang WQ, Wang ZY, Li H et al (2005) Purification and characterization of a novel pyrethroid hydrolase from *Aspergillus niger* ZD11. J Agric Food Chem 53:7415–7420

Liu Y, Chung Y, Xiong Y (2001) Purification and characterization of a dimethoate-degrading enzyme of *Aspergillus*

niger ZHY256, isolated from sewage. Appl Environ Microbiol 67:3746–3749

Martens R, Zadrazil F (1998) Screening of white-rot fungi for their ability to mineralize polycyclic aromatic hydrocarbons in soil. Folia Microbiol 43:97–103

Mougin C, Pericaude C, Malosse C, Laugero C, Asther M (1996) Biotransformation of the insecticide lindane by the white rot basidiomycete *Phanerochaete chrysosporium*. Pest Sci 47:51–59

Mougin C, Laugero C, Asther M, Chaplain V (1997) Biotransformation of s-triazine herbicides and related degradation products in liquid cultures by the white rot fungus *Phanerochaete chrysosporium*. Pest Sci 49:169–177

Mswaka A, Magan N (1999) Water and temperature relations of *Trametes* and related species from the indigenous forests of Zimbabwe. Mycol Res 103:1309–1317

Ngo TT, Lenhoff HM (1980) A sensitive and versatile assay for peroxidase and peroxidase-coupled reactions. Anal Biochem 105:389–397

Ogawa H, Sugiyama J (2000) Evolutionary relationships of the cleistothecial genera with *Penicillium*, *Geosmithia*, *Meribla* and *Sarophorum* anamorphs as inferred from 18S rDNA sequence divergence. In: Sampson RA, Pitt JA (eds) Integration of modern taxonomic methods for *Penicillium and Aspergillus classification*. Harwood Academic Publishers, Amsterdam, The Netherlands, pp 149–161

Pizzul L, Castillo MdP, Stenstrom J (2009) Degradation of glyphosate and other enzymes by lignolytic enzymes. Biodegradation 20:751–759

Pointing SB (2001) Feasibility of bioremediation by white-rot fungi. Appl Microbiol Biotechnol 57:20–33

Racke KD, Laskowsky DA, Shultz MR (1990) Resistance of chlorpyrifos to enhanced biodegradation in soil. J Agric Food Chem 38:1430–1436

Reddy CA (1993) An overview of the recent advances on the physiology and molecular biology of lignin peroxidases of *Phanerochaete chrysosporium*. J Biotechnol 30:91–107

Rigas F, Dritsa V, Marchant R, Papadopoulou K, Avramides EJ, Hatzianestis I (2005) Biodegradation of lindane by *Pleurotus ostreatus* via central composite design. Environ Int 31:191–196

Singh BK, Walker A, Morgan JAW, Wright DJ (2003) Effects of soil pH on the biodegradation of chlorpyrifos and isolation of a chlorpyrifos-degrading bacterium. Appl Environ Microbiol 69:5198–5206

Smilanick JL, Mansour MF, Sorenson D (2006) Pre- and postharvest treatments to control green mold of citrus fruit during ethylene degreening. Plant Dis 90:89–96

Tien M, Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. Meth Enzymol 161:238–249

Tuomela M, Oivanen P, Hatakka A (2002) Degradation of synthetic ^{14}C-lignin by various white-rot fungi in soil. Soil Biol Biochem 34:1613–1620

Waldner R, Leisola MSA, Fiechter A (1988) Comparison of ligninolytic activities of selected white-rot fungi. Appl Environ Microbiol 29:400–407

Wesenberg D, Kyriakides I, Agathos SN (2003) White rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol Adv 22:161–187