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Biotransformation of s-Triazine Herbicides and Related Degradation Products in Liquid Cultures by the White Rot Fungus *Phanerochaete chrysosporium*

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Abstract: The ability of the white rot basidiomycete *Phanerochaete chrysosporium* to transform s-triazine herbicides has been investigated in laboratory experiments. The chlorinated metabolites formed during atrazine N- dealkylations were not further transformed by the fungus, whereas hydroxyatrazine was converted to an unknown product. P. *chrysosporium* was also able to carry out the N-dealkylation of the herbicides simazine, propazine and terbuthylazine. Herbicide metabolism was not supported by purified peroxidases. The highest rates of herbicide N-dealkylation were obtained in liquid cultures maintained under moderate temperature allowing a long mycelium growing phase. Atrazine transformation was found to be supported by the mycelium, which contained significant amounts of microsomal cytochrome P450. Herbicide N-dealkylation was decreased in the presence of 1-aminobenzotriazole, in agreement with the involvement of P450 monooxygenases in atrazine metabolism.

Key words: basidiomycete, atrazine, N-dealkylation, P450 monooxygenase

1 Introduction

The white rot fungus *Phanerochaete chrysosporium* Burdsall is able to degrade and/or mineralize a wide range of haloaromatic environmentally persistent pollutants. Its lignin-degrading system (LDS), namely extracellular lignin peroxidases (LiPs) and manganese- dependent peroxidases (MnPs), is thought to catalyse the reactions leading to the metabolization of organic chemicals,

including pesticides,1 although direct proofs are rarely presented. Several authors have also suggested the involvement of intracellular enzymatic systems acting on key steps of xenobiotic transformation.[1] Among these systems were recently mentioned P450 monooxygenases.[2-4]

When using classical liquid culture conditions, we have previously shown that *P. chrysosporium* was able to transform the s-triazine herbicide atrazine [6-chloro- N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine] into polar metabolites, via a main pathway involving oxidative N-dealkylation of the parent molecule.[5] The fungus mineralized the ethyl group of the molecule, but it was unable to carry out the breakdown of the heterocycle. Moreover, in these experimental conditions, biotransformation of atrazine was quite moderate, and results failed to prove the involvement of the LiPs and the MnPs in atrazine transformation, since metabolism of the latter occurred during both the peroxidase production and the fungal growing phases.

At the same time, a study of atrazine biodegradation by *P. chrysosporium* showed that purified peroxidases were not involved in N-dealkylation.[6] In any case, the fungus used in these experiments was unable to transform atrazine in liquid cultures, since neither degradation products nor carbon dioxide were detected.

Because of these opposing results concerning the ability of *P. chrysosporium* to metabolize atrazine in liquid cultures, the present work was firstly aimed at confirming the N-dealkylation of atrazine and related triazines in liquid culture experiments. Secondly, the possible peroxidase involvement in atrazine transformation was clarified. Then, we attempted to localize the enzymatic systems acting on triazines by studying the effects of culture conditions on fungal growth and atrazine transformation, and by incubating the herbicide with separate extracellular fluid and fungal biomass. Finally, additional data were obtained on the involvement of cytochrome P450 in atrazine N-dealkylation, by showing the presence of cytochromes in microsomes isolated from our *P. chrysosporium* cultures, by measuring enzymatic activities in vitro, and by performing assays with known effectors of P450s.

2. Experimental methods

Nomenclature

For convenience, the nomenclature system adopted by Hogrefe et al.[7] is used for the dealkylation products of the herbicides studied, as shown in Table 1, along with their structures.

Chemicals

[ring-U-^{14}C]Atrazine (425 MBq mmol^{-1}, radiochemical purity [96%) was purchased from Sigma (St. Quentin Fallavier, France), and [ring-U-^{14}C]simazine (200 MBq mmol^{-1}, radiochemical purity

[96%) was a generous gift of Dr E. Barriuso (INRA, Grignon, France).

Ring-labelled deethylatrazine (CIAT) and deisopropylatrazine (CEAT) were obtained from fungal transformation of atrazine (3.3 kBq in 10 ml culture medium) by using the following experimental design. The details of fungal culture are provided in the subsequent sections. After a 10-day growth period in presence of the herbicide, the radioactivity of the growth medium was extracted with dichloromethane (3 x 10 ml). The organic extracts were reduced under vacuum, pooled, spotted on thin-layer chromatography silica gel $60F_{254}$ plates (Merck, Chelles, France), and plates were developed with hexane]+ ethyl acetate (5 + 5 by volume). Chromatograms were first examined under UV light and radioactive spots were then located by scanning the plates with a BS 27/N TLC radioanalyser (EG&G, Evry, France). The spots coeluting with standards of CEAT and CIAT were scraped, eluted with acetone and concentrated under nitrogen.

Ring-labelled and ethyl-labelled OIET were obtained separately by acidic hydrolysis of [ring-U-^{14}C]atrazine and [*ethyl*-1-^{14}C]atrazine (880 MBq mmol^{-1}, Amersham, Les Ulis, France). Ethanolic solutions of atrazine (1 ml; 250 KBq) were diluted with hydrochloric acid (4 M; 3 ml) and kept overnight at 60¡C. In these conditions, hydrolytic dechlorination of the triazine was complete. A stock solution of each labelled OIET was prepared by adding to the previous acidic solutions the unlabelled molecule and ultra-pure water for dilution. Final concentration of the solution (125 KBq mmol^{-1}) was 100 µM.

Radiochemical purity of the labelled metabolites was up to 99%. Their chemical structure was verified by GC-MS (atrazine and CEAT), and TSP-LC-MS (OIET).[8] Stock solutions of transformation products were stored at 4°C without any further transformation for several months.

Except for CBAT, high-purity standards of s-triazines and metabolites were obtained from Promochem (Molsheim, France) and Cluzeau Info Labo (Ste Foy la Grande, France). Aminobenzotriazole, 2,4-dichlorophenoxyacetic acid, phenobarbital and ergosterol were purchased from Janssen (Noisy le Grand, France), Sigma and Fluka (St. Quentin Fallavier, France), respectively.

Fungus

P. chrysosporium BKM-F-1767 (ATCC 24725) was used. The strain was maintained on malt agar slant cultures at 37°C. Spores were collected by washing slants with sterile distilled water and were stored at 4°C.

Incubation conditions for liquid cultures

Conidiospores (2 x 10^6) were inoculated in 150-ml Erlenmeyer flasks containing the growth medium (10 ml).[5,9] The medium was supplemented with 2 µM substrate: atrazine or one of its

transformation products (CIAT, CEAT, OIET), or other s-triazine herbicides (simazine, propazine, terbuthylazine). That treatment consisted of a mixture of unlabelled and labelled (1.5-5 kBq) 5 kBq) pesticides dissolved in acetone (10 µl) for chlorinated compounds, or sterile acidic solution (200 µl) for OIET. The Erlenmeyer flasks were enclosed in 1-litre sealed flasks with two vials containing sodium hydroxide solution (1 M; 10 ml, to trap carbon dioxide) and water (10 ml, to keep constant moisture). They were incubated without shaking in the dark5 at 25¡C, although some experiments were performed at 15°C or combination of both temperatures, as stated in the text. Every two days, all cultures were flushed with ambient air and sodium hydroxide solutions were replaced, and samples were analysed for degradation over 16-20-day periods. Biotransformation of simazine, propazine and terbuthylazine was only studied in eight-day experiments.

Fungal growth was assessed by measurements of the mycelium dry weight after filtration on glass-fibre filters (GF/D, Whatman), washing with Milli-Q water (10 ml) and drying one day at 90°C.

Studies were also undertaken to determine if P450s (heme-thiolate monooxygenases) are involved in CIET biotransformation. For that purpose, the ability of P450 effectors to modify the herbicide transformation rate in *P. chrysosporium* cultures was investigated at 25°C. Effectors included 1-aminobenzotriazole [ABT, a classical mechanism-based inactivator of P450s from mammals and higher plants],[10,11] phenobarbital and 2,4-dichlorophenoxyacetic acid [inducers of P450s in higher plant tissues].[12,13] The chemicals were added to the cultures in acetone (maximal volume of 100 µl), without any effect of the solvent on fungal growth. Samples were analysed for degradation 4, 8 and 12 days after treatment.

Enzymatic assays with peroxidases

Lignin peroxidase activity in the extracellular fluid (400-µl aliquots) was determined by the rate of oxidation of veratryl alcohol to veratraldehyde at 30°C as described by Tien and Kirk.[14] Manganese peroxidase activity was also determined spectrophotometrically by the method of Paszczynski et al.[15] with vanillylacetone as a substrate, at 30°C. Reaction mixtures contained extracellular fluid (100 or 200 µl). The reaction was started by adding hydrogen peroxide, and the rate of disappearance of vanillylacetone was measured. Enzymatic activities were expressed in nanokatals: 1 nkat ml^{-1} is equivalent to 60 U $litre^{-1}$.

Atrazine transformation was assayed by incubation with purified LiPs and MnPs. The purified enzymes were obtained according to Bonnarme et al.[16] The reaction mixture for in-vitro assays contained (i) 100 mM sodium tartrate (pH 3), 2 mM veratryl alcohol and purified LiPs (10 µl), (ii) 100 mM sodium tartrate (pH 5), 0.1 mM $MnSO_4$ and purified MnPs (10µkl), (iii) 100 mM sodium tartrate (pH 3), 2 mM veratryl alcohol, 0.1 mM $MnSO_4$ and purified LiPs and MnPs (10 µl of each) and finally (iv) 100 mM sodium tartrate (pH 5), 2 mM veratryl alcohol, 0.1 mM $MnSO_4$ and

purified LiPs and MnPs (10 µl of each). All assays contained also 12 µM atrazine and the reaction was started by adding 0.05 mM hydrogen peroxide. Peroxide was added again after 20 and 40 min incubation time and the reaction mixture was analysed after 60 min. Incubation temperature was 30°C.

Preparation of microsomes and P450 measurements

Microsomal fractions were isolated after a seven-day growth period from 1-litre cultures maintained at 25°C and divided between five Roux flasks. All experiments were carried out at 4°C. The cultures were kept for 1 h at 4°C, and then filtered. The fungal biomass was washed with potassium phosphate buffer (0.1 M; 50 ml, pH 7.4). It was disrupted in a Virtis 45 blender at 22 500 rev min^{-1} in phosphate buffer (30 ml) supplemented with glycerol (200 g $litre^{-1}$) and bovine serum albumin (1.5 g $litre^{-1}$), over 1-min period with 3 x 10- and 2 x 15-s bursts separated by 20-s cooling periods. The crude homogenate was filtered and centrifuged at 1000 g for 15 min. The supernatant was centrifuged at 10 000 g for 15 min, followed by centrifugation at 100 000 g for 90 min. The pellets were then resuspended in buffer (10 ml) and sedimented again. The washed pellets, resuspended in phosphate buffer (0.1 M; 3 ml) containing glycerol (300 g $litre^{-1}$), EDTA (0.1 mM) and reduced glutathione (0.1 mM), were referred to as the microsomal fraction. They were stored for up to two weeks at [80°C until used. Microsomal protein content was estimated by using the Biorad kit for protein assay.

Quantitative determination of hemoproteins was carried out using the method of Omura and Sato.[17] NADPH-cytochrome c reductase activity was measured after addition of potassium cyanide (2 mM) to the reaction mixture to minimize cytochrome c reoxidation by mitochondria, according to the method of Benveniste et al.[18] An extinction coefficient of 18 500 M^{-1} cm^{-1} at 550 nm was used for activity determination.

Analytical procedures for pesticide compounds

Liquid cultures were filtered on 0.45-µm membranes (Millipore, St Quentin-Yvelines, France) under vacuum. Then, medium fractions (8 ml) were concentrated on a C guard column MCH-10 (3 cm x 4 mm ID; Varian) at a flow rate of 1 ml min^{-1} with an isocratic pump (Varian 9001). The on-line elution of triazine compounds was achieved onto the analytical column ODS- 80TM (25 cm x 4.6 mm ID; Varian) with a pump (Varian 9010) delivering a solvent system composed of acetonitrile and water, both acidified with phosphoric acid (1 g $litre^{-1}$). It began with 1% acetonitrile for 3 min, followed by a linear increase to 100% acetonitrile over 15 min and a stationary phase for 10 min. The radioactivity of the column eluate was monitored by a Berthold HPLC LB 507 A radioactivity system (Wallac EG&G, Evry, France).

For OIET analysis, a specific solvent gradient was achieved. It began with 1% acetonitrile for 3 min, followed by a linear increase to 35% acetonitrile over 12 min and a stationary phase for 2 min. The acetonitrile content was then increased linearly to 100% in 5 min and maintained for 5 min.
The radioactivity remaining in the cell biomass was determined after dry weight measurements by solubilization. Mycelia were placed in scintillation vials, and hydrated with water (500 µl) for 1 h prior to digestion with Optisolve (2 ml, Wallac EG&G, Evry, France) solubilizer for 5 h. Finally, mixtures were supplemented with Optiphase 3 (10 ml, Wallac EG&G, Evry, France) and liquid scintillation counting was achieved 24 h later.
For analysing atrazine transformation following in-vitro assays, aliquots (100 µl) of the enzymatic medium were injected into HPLC through a 7125 Rheodyne valve. The elution of labelled compounds was then achieved under the conditions described above.
The disappearance of unlabelled s-triazines from the growth medium was monitored by gas chromatography using a thermionic specific detector (TSD), and aliquots (3 kl) of extracts were injected. Extraction and analysis protocols have been described previously.[5]
Electron-Impact mass spectral analysis were per- formed on an Ion-Trap instrument (Varian Saturn II) with an ionizing voltage of 70 eV as previously described.[5] Samples were introduced by GC onto a Varian 3400 chromatograph. Confirmation of the identity of the chlorinated transformation products obtained from cultures was achieved by GC-MS analysis by co-chromatography with spectra from commercially avail- able standards, and to a described fragmentation pattern for s-triazines.[19]

Experimental error

Unless otherwise stated in the text, each experiment was done in triplicate and repeated twice. Results are expressed as means. The standard deviation was less than 10% of the mean.

3. Results

Transformation of atrazine and related degradation products in liquid cultures of *Phanerochaete chrysosporium*

The transformation of atrazine and related degradation products was investigated at 25°C. For atrazine, total radioactivity found in the culture medium accounted for up to 92% of the initial radioactivity. In agreement with our previous study,[5] the parent herbicide was preferentially turned into CIAT rather than CEAT, in amounts corresponding to 53% and 9% of the initial atrazine concentration, respectively (data not shown). CEAT and CIAT accumulated in the culture medium. Moreover, assays performed with labelled atrazine and CEAT as substrates showed no conversion

of these compounds by the fungus within eight days at 25°C, since atrazine and CEAT accounted for 97% and 98% of the initial radioactivity, respectively.

Minor metabolites were also detected in the culture medium. They were contaminants of the stock solution of labelled atrazine, and consisted of a mixture of CAAT and OIET. No significant increase in CAAT content was noted in our experiments. By contrast, OIET formation from atrazine increased slightly during the 16-day incubation period.

Biotransformation of OIET by cultures of *P. chrysosporium* was first assayed with ring-labelled compound. Changes in OIET content and in metabolite formation are presented in Fig. 1. OIET content in the culture medium decreased slowly within the first four days of incubation, and more rapidly during the following four to six days, so that only 40% of the initial radioactivity was quantiÐed in the culture medium after 10 days of incubation. A similar pattern occurred for ethyl-labelled OIET. By contrast, whatever the labelled position, OIET content remained constant in the uninoculated sterile controls. Mineralization was never detected. The total radioactivity recovered throughout the experiments was higher than 95% of the initial radioactivity. Mass-balance analysis showed that less than 2% of the radioactivity was associated with the mycelial fraction. The structure of the metabolite derived from OIET transformation was original, since this compound did not co-chromatograph with standards of known N-dealkylated metabolites of hydroxy-, chloro- and methoxy-s-triazines. Results from Fig. 1 also indicate that OIET was not N-deethylated by *P. chrysosporium*. The metabolite was stable under acidic conditions and was insoluble in most organic solvents. Unfortunately, it could not be derivatized for GC-MS analysis and its high thermodegradation during TSP-LC-MS analysis prevented any determination of its structure by this method. Moreover, no structural information was obtained by NMR.

Labelled simazine was poorly transformed within eight days. During this period, 5.4% of the initial herbicide content was converted to CEAT (Table 2). No [^{14}C] carbon dioxide was trapped in incubations and only a small amount of radioactivity (less than 3É0%) remained associated with the mycelial fraction. Biotransformation of unlabelled propazine and terbuthylazine was also established by GC-TSD and GC-MS. Analysis showed that both herbicides underwent transformation corresponding to 44.8 and 95.4% of their initial concentration, respectively (Table 2). N-dealkylation of the herbicides was confirmed since N-dealkylated metabolites were identified by comparison with available standards for retention time and GC-MS analysis. Table 3 summarizes the relative abundance of the most characteristic ions of metabolites detected by GC-MS after eight-day fungal incubations with s-triazines. Mass spectra of the metabolites extracted from cultures were identical to those of commercially available standards. CBAT structure was deduced from a general fragment tion pattern for s-triazines.[19] Results corresponding to atrazine transformation are reported for comparison. Simazine, propazine and terbuthylazine were oxidized

to yield CEAT, CIAT and CBAT, respectively. It is noteworthy that CEAT formation proceeded at similar rates for simazine and atrazine. The same pattern occurred for CIAT formation from atrazine and propazine. No loss of the tert-butyl group of terbuthylazine was detected. *P. chrysosporium* was unable to carry out simultaneously the two-fold N-dealkylation of any parent herbicide. Moreover, it was likely that all the three herbicides, as well as atrazine, underwent a slight hydrolysis in liquid cultures.

In-vitro transformation of atrazine by purified peroxidases

Transformation of atrazine was assayed with purified LiPs and MnPs exhibiting high levels of veratryl alcohol and vanillylacetone peroxidase activities, corresponding to 109.0 and 145.0 nkat ml^{-1}, respectively (Table 4). The results clearly establish that purified LiPs and MnPs, alone or in mixture, were unable to carry out the N-dealkylation(s) of the herbicide. Nevertheless, incubation medium initiated hydrolysis of atrazine to OIET, which is a minor reaction in fungal cultures. That occurred in controls at pH 3 in presence of hydro- gen peroxide, and chemical hydrolysis was reduced when the enzyme was added.

Localization of the enzymatic systems involved in atrazine transformation

The effects of incubation conditions on fungal growth and atrazine transformation were studied in liquid cultures. At 25°C under ambient air atmosphere, steady growth was observed for 8-10 days (Fig. 2(a)), then biomass remained constant for the following 10 days. When cultured at 15°C, the fungal growth presented a lag period (eight days) corresponding to a slow germination rate of the spores. Then the biomass increased up to 20 mg dry weight at the end of the experiment (20 days). Increasing temperature to 25¡C between days 6 and 8 resulted in shortening the lag period. Then the growth rate was similar to that observed for cultures maintained at 15°C, and the final biomass was 32 mg dry weight.

Atrazine disappearance from the medium depended on temperature (Fig. 2(b)). The decrease in atrazine content was very rapid at 25°C, and only 25% of the initial herbicide concentration remained at the end of the experiment. At 15°C, atrazine decrease was slow during the first 8-10 days, and then more rapid. About 40% of the initial herbicide was quantified in the culture medium after 20 days. Maximal atrazine disappearance from the medium occurred when the fungus was grown at 15°C with a two-day period at 25°C. In that case, the medium contained only 9% of the initial atrazine at day 20, whilst the fungus did not reach its stationary phase. Atrazine content remained constant in the uninoculated sterile controls.

Atrazine was then incubated with extracellular fluid and fungal biomass. When separated from seven-day- old cultures, the extracellular fluid alone did not support any N-dealkylation of the

herbicide within 24- or 48-h incubations. Conversely, the washed mycelium was able to transform atrazine when resuspended in boiled fluid in the presence of atrazine, cycloheximide and phenyl methyl sulfonyl fluoride according to described protocols.[20,21] CIAT was detected in the cultures with amounts corresponding to 2.2 and 7.1% of the initial radioactivity after the same incubation periods.

Possible involvement of P450 monooxygenases in atrazine N-dealkylation by *Phanerochaete chrysosporium*

Microsomes isolated from seven-day-old cultures contained cytochrome P450 (Fig. 3). Amounts of 88.6 pmol (mg microsomal protein)$^{-1}$ or 797.4 pmol (litre culture)$^{-1}$ have been calculated from the reduced carbon monoxide difference spectra, which exhibited maxima at 452 nm. They showed also an important peak at 420 nm, possibly due to cytochrome P420, an inactive form of P450. Nevertheless, we were unable to obtain a type I binding spectrum with atrazine.

Microsomes were also assayed for enzymatic activities. NADPH-cytochrome c reductase, typically used as a marker of endoplasmic reticulum, gave specific activities of 24.5 nmol min^{-1} m^{-1}. We failed to detect significant N-dealkylation of atrazine during incubations with microsomal proteins.

Possible involvement of P450 in atrazine transformation was finally assayed through indirect experiments with P450 effectors. When added to the cultures at the beginning of the experiments, ABT exerted inhibiting effects on atrazine transformation with respect to effector concentration, but only after four days of incubation (Fig. 4). Residual atrazine in the culture fluid accounted for 28.6% of the initial radioactivity in controls after a 12-day culture period, whereas it was 68.0% in the presence of 10-^{3} M ABT. The remaining decrease observed in that case was due to atrazine penetration in the fungal biomass. ABT had no effect on fungal biomass.

Inducers (2,4-dichlorophenoxyacetic acid and phenobarbital) had no significant effect on atrazine transformation in liquid cultures of *P. chrysosporium*.

4. Discussion,

In a previous paper,[5] we reported the biological N- dealkylation of the herbicide atrazine in liquid cultures of *P. chrysosporium*. It was obtained by using culture conditions optimized for an optimal LDS production.[9,16,22] These conditions were primarily strong oxygenation of the culture at the beginning of the experiments, adapted levels of nutrients and a culture temperature of 37°C. By contrast, it has been reported that BKM F-1767 strain was unable to transform atrazine in liquid cultures.[6] The present paper confirms our previous results[5] and proves that the same strain grown under our culture conditions is able to transform the herbicide.

P. chrysosporium is also able to transform several s- triazine compounds, including a transformation product of atrazine and other related herbicides. Mono-N-dealkylation appears to be the first and main step in the chlorinated-s-triazine degradation pathway by the fungus in accordance with previous results.[5] The resulting dealkylated metabolites were not further converted by the fungus, as is usual for fungi,[23] in contrast to bacteria which are capable of further degradation and mineralization. OIET was the main degradation product found in soils treated with atrazine, and it resulted mainly from a soil-catalysed chemical process leading to hydrolytic dechlorination. That reaction is promoted by acidic and alkaline conditions.[24] *P. chrysosporium* actively transformed OIET to an unknown compound that accumulated in the culture medium. Unfortunately, we failed to determine its chemical structure. Results show that the presence of both alkyl groups and of chlorine at the 2-position are necessary for the mono N-dealkylation of atrazine by *P. chrysosporium*. Consequently, formation of desethylhydroxyatrazine (OIAT) previously reported in liquid cultures5 should result from hydrolysis of CIAT. Experiments with terbutylazine, atrazine and simazine also show that the removal of the ethyl side chain is the preferential reaction, and might depend on the mass of the second alkyl group. In other words, compounds with a high-mass group linked to one amino substituent are expected to undergo a higher N-dealkylation affecting the other chain. The symmetric compounds propazine and simazine were also degraded at a slower rate than atrazine. Similar results have been reported with bacteria.[25]

Our in-vitro assays performed with purified LiPs and/or MnPs exhibiting high specific activities, as well as those previously reported6 confirm that the enzymes, alone or in mixture, are ineffective in transforming the herbicide atrazine, even when the latter is N-dealkylated by the whole culture. Moreover, the recovery of radio- activity up to 99% is not consistent with a possible mineralization of atrazine by these purified enzymes. Taken together, the results rule out a peroxidase-mediated N-dealkylation of atrazine by *P. chrysosporium*, whilst herbicide hydrolysis appears to be chemically catalysed in liquid medium under acidic conditions by the presence of a strong oxidizing reagent.

In *P. chrysosporium* liquid cultures, enzymes other than peroxidases might be implicated in the metabolism of various xenobiotics,[20,26] but they have rarely been characterized. Enzymatic systems typically involved in xenobiotic metabolism in living organisms (for example P450s) might also participate in xenobiotic metabolism in *P. chrysosporium* cultures. It has been recently demonstrated that P450 was involved in PAH hydroxylation.[2] Comparison between mycelium growth and atrazine degradation shows that transformation of the latter occurs during the fungal growing phase, and that the length of the growing phase appears more important for pesticide transformation than final biomass amount. Moreover, our results show that atrazine transformation is associated with the mycelium, rather than with the extracellular fluid. Cytochromes P450 are also present in our cultures. Unfortunately, we failed to obtain atrazine N-dealkylation in vitro, probably

because of inactivation/degradation of the enzymes during microsomal preparation, as attested by the presence of high levels of P420. An indirect approach was used with P450 effectors, namely 1-aminobenzotriazole, phenobarbital and 2,4-dichlorophenoxyacetic acid. Similar studies have shown the involvement of P450 in the transformation of other pesticides by *P. chrysosporium*, such as endosulfan[3] or lindane.[4] Amino- benzotriazole has also been shown to decrease simazine N-dealkylation in *Lolium rigidum* Gaud.,[27] whereas phenobarbital is known to increase P450 amounts and atrazine metabolism in rat liver microsomes.[28] In our experiments, ABT inhibited atrazine transformation whereas inducers had no significant effects. This apparent contradiction might have several causes. Firstly, phenobarbital and 2,4-dichlorophenoxyacetic acid might undergo a rapid transformation by the fungus, preventing any effects. Secondly, the inducers are highly specific for selected P450 gene families and might induce P450 enzymes not involved in atrazine transformation. Accordingly, results do not rule out a P450 involvement in atrazine N-dealkylation, as already reported in bacteria,[29] higher plants[30] and vertebrates.[28] Thus, the complete mechanisms of atrazine transformation by *P. chrysosporium* are not yet resolved.

P. chrysosporium thus appears to be efficient in transforming chlorinated s-triazines to mono-N-dealkylated metabolites. At least in liquid cultures, that reaction might be the first step for a complete biodegradation process involving bacteria capable of using N-dealkylated products as nitrogen source.31 We are currently attempting to isolate active enzymes responsible for atrazine N-dealkylation.

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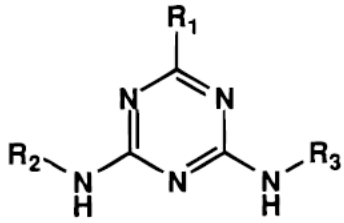
Table 1. Functional groups, common names and abbreviations for s-triazine herbicides and related transformation products



Functional group				
R1	*R2*	*R3*	*Common name*	*Abbreviation*
Cl	C_2H_5	iso C_3H_7	Atrazine	
Cl	H	iso C_3H_7	Deethylatrazine	CIAT
Cl	C_2H_5	H	Deisopropylatrazine	CEAT
Cl	H	H	Deethyldeisopropylatrazine	CAAT
Cl	iso C_3H_7	iso C_3H_7	Propazine	
Cl	C_2H_5	C_2H_5	Simazine	
Cl	C_2H_5	*t*-C_4H_9	Terbuthylazine	
Cl	H	*t*-C_4H_9	Deethylterbuthylazine	CBAT
OH	C_2H5	iso C_3H_7	Hydroxyatrazine	OIET

Table 2. Metabolite formation in *Phanerochaete chrysosporium* cultures after eight-day-incubations with s-triazines

	Metabolite Formation[a] *(% of initial content)*		
Herbicide compounds	*CEAT*	*CIAT*	*CBAT*
Simazine	5·4	n.a.	n.a.
Atrazine	6·6	40·0	n.a.
Propazine	n.a.	44·8	n.a
Terbuthylazine	0·0	n.a.	95·4

[a] n.a.: metabolite formation not applicable.

Table 3. Relative abundance of the characteristics mass ions of the chlorinated metabolites produced from s-triazines by *Phanerochaete chrysosporium* cultures

Compounds	*Characteristic mass ions*, m/z (*with relative intensity*)
CEAT	68 (61), 110 (38), 145 (80), 147 (31), 158 (76), 160 (29), 173 [M^+] (100), 175 (35)
CIAT	68 (22), 110 (9), 145 (19), 147 (6), 172 (100), 173 (8), 174 (37), 187 [M^+] (45), 189 (15)
CBAT	68 (27), 110 (16), 145 (28), 147 (10), 186 (100), 188 (31), 201 [M^+] (21), 203 (7)

Table 4. In-vitro assays of atrazine transformation by purified lignin (LiP) and manganese-dependent (MnP) peroxidases

Incubation conditions	*LiP activity* (*nkat* ml^{-1})	*MnP activity* (*nkat* ml^{-1})	*Atrazine transformation (% of initial radioactivity)*[a] *OEIT*	*CEAT*	*CIAT*
pH 3					
Complete medium for LiPs	109		5·5	0·2	0
without enzyme			10·1	1·4	0
without H_2O_2			3·8	0	0
+ MnPs	47		2·7	0·3	0·2
pH 5					
Complete medium for MnPs		145	0	0·2	0
without enzyme			0·3	0	0
without H_2O_2			0	0	0
+ LiPs		109	0	0.1	0

[a] Values are corrected with respect to the radiochemical purity of labeled atrazine, which is 96·4%.

Fig. 1. Amounts of (●) [ring-U-^{14}C]OIET, (○)[ethyl-1-^{14}C]OIET and (▲,△) corresponding transformation products in cultures of *Phanerochaete chrysosporium* at 25°C.

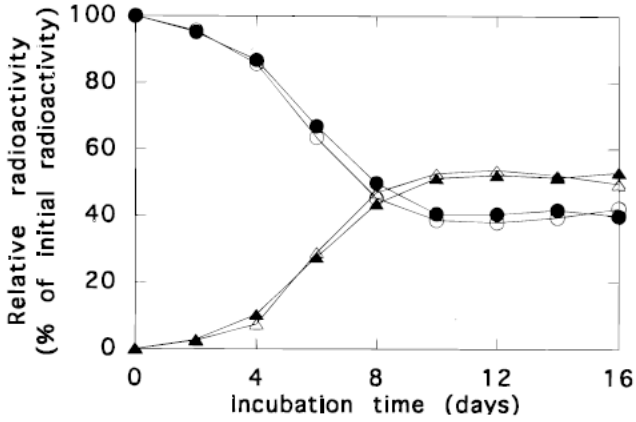


Fig. 2. (a) Mycelial formation by *Phanerochaete chrysosporium* in relation to (b) atrazine content in the growth medium with respect to culture temperature (●) 25°C, (O) 15°C, (▲) 15°C with a two-day period at 25°C between days 6 and 8.

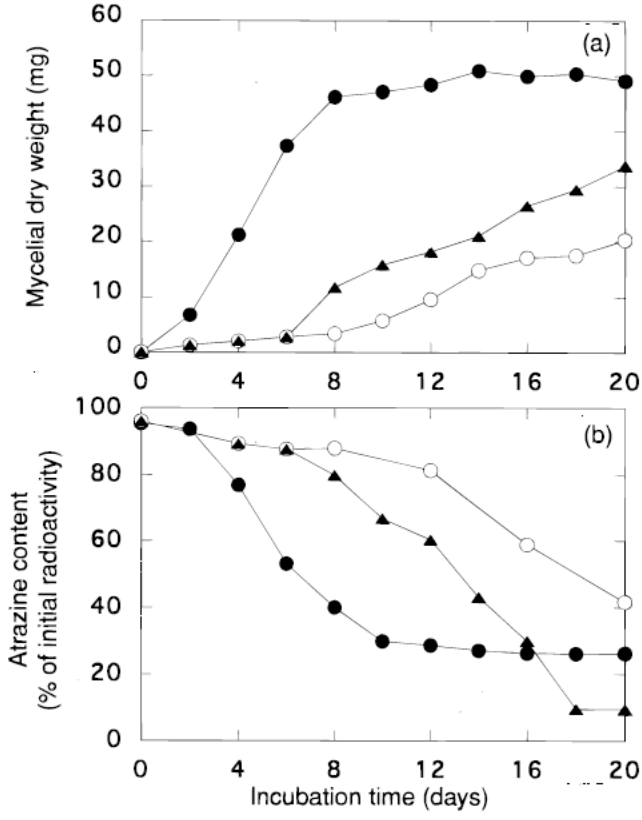


Fig. 3. Reduced carbon monoxide difference spectra of microsomal P450 from *Phanerochaete chrysosporium*.

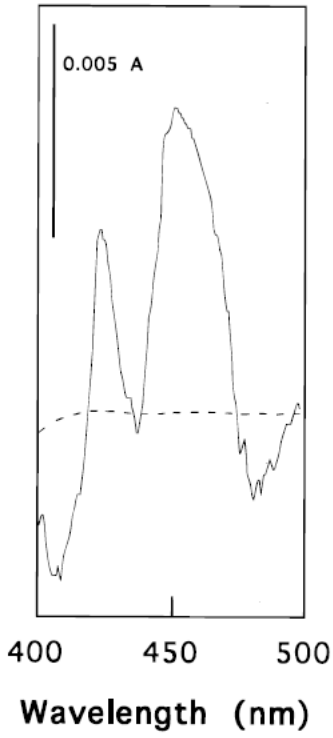


Fig. 4. Effect of aminobenzotriazole (ABT) on atrazine content in the growth medium. (●), Controls; (○), 10^{-5} M; (▲), 10^{-4} M and (△), 10^{-3} M ABT.