Cleavage of the Diketonitrile Derivative of the Herbicide Isoxaflutole by Extracellular Fungal Oxidases

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Isoxaflutole is a herbicide activated in soils and plants to its diketonitrile derivative, the active herbicide principle. The diketonitrile derivative undergoes cleavage to the inactive benzoic acid analogue. In this paper, it is established that an oxidative mechanism implicating two successive reactions in the presence of dimethyldioxirane can chemically initiate the cleavage of the diketonitrile. It is also shown that two white rot strains, *Phanerochaete chrysosporium* and *Trametes versicolor*, are able to convert the diketonitrile to the acid when cultured in liquid media. This main metabolite amounts to 24.6 and 15.1% of initial herbicide content after 12−15 days of culture. Another polar metabolite represents <3.7% of the parent compound amount during the same period. Oxidative enzymes produced by the fungi show a time course similar to that of diketonitrile degradation. Purified laccase (EC 1.10.3.2), in the presence of 2 mM 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) acting as a redox mediator at pH 3 supports the reaction with rates of 0.3−0.4 nmol h^{-1} $unit^{-1}$.

Keywords: *Herbicide; metabolism; dimethyldioxirane; white rot fungi; Phanerochaete chrysosporium; Trametes versicolor; laccases*

INTRODUCTION

Isoxaflutole (IUPAC name: 5-cyclopropyl-1,2-oxazol-4-yl α,α,α-trifluoro-2-mesyl-*p*-tolyl ketone, compound **1**; Figure 1) is a recently developed herbicide for pre- and postemergence control of a wide range of important broadleaf and grass weeds in corn and sugarcane (Luscombe et al., 1995). After herbicide application, susceptible weed species show a bleaching symptomology of newly developed leaves, followed by growth suppression and necrosis prior to plant death, similar to that seen with herbicidal inhibitors of carotenoid biosynthesis. Furthermore, reduction of carotenoid and chlorophyll content is associated with an indirect inhibition of the phytoene desaturase due to the depletion of the cofactor plastoquinone. That depletion is caused by the inhibition of the enzyme 4-hydroxy-phenylpyruvate dioxygenase (Pallett et al., 1998; Viviani et al., 1998). The dioxygenase catalyzes the oxidative decarboxylation of 4-hydroxyphenylpyruvate, forming homogentisate.

In plants and soils, isoxaflutole is rapidly converted to a diketonitrile derivative (DKN, compound **2**; Figure 1) by opening of the isoxazole ring (Viviani et al., 1998). DKN is the active herbicide principle and is a potent inhibitor of the dioxygenase (Pallett et al., 1998). DKN undergoes degradation to the inactive benzoic acid analogue (BZA, compound **3**; Figure 1) in treated plants. The extent of this degradation is a basis for herbicidal selectivity, being more rapid in tolerant plants than in the susceptible species.

Because of their implication in herbicide selectivity (agronomic impact) and breakdown (environmental impact), it is of great importance to identify enzymatic systems involved in the conversion of DKN to BZA. Enzymes responsible for the cleavage of diketone bonds have been poorly characterized to date. Sakai et al. (1986) reported a bacterial *β*-diketone hydrolase (EC 3.7.1.7) involved in the degradation mechanism of poly(vinyl alcohol). The enzyme was also active on aliphatic *β*-diketones and on aromatic *β*-diketones such as 1-phenyl-1,3-butanedione, presenting a framework close to this of DKN. Nevertheless, such a hydrolytic mechanism producing a methyl ketone is not relevant to the formation of BZA.

White rot fungi have been known for many years for their ability to transform various xenobiotics by using their rich enzymatic equipment (Barr and Aust, 1994). These organisms commonly live woody plants, but they can also found in soils. For that reason, their ability to cleave the diketone bond of the herbicide was investigated. We propose in this paper a hypothetical pathway for the oxidative transformation of DKN. Then we report herbicide transformation by two strains of white rot fungi cultured in liquid media and by purified oxidases.

MATERIALS AND METHODS

Chemicals and Reagents. The unlabeled DKN derivative of isoxaflutole, [*ring*-UL-^{14}C]DKN (909 MBq/mmol, radiochemical purity = 96%), and standard of BZA were gifts from Rhône-Poulenc Agro (Lyon, France). NAT 89 was a commercial phospholipid source supplied by Natterman Phospholipid GmbH (Cologne, Germany). All other chemicals and reagents were obtained from Sigma-Aldrich (St-Quentin Fallavier, France), and solvents came from Carlo Erba (Val de Reuil, France).

Synthesis of Putative Transformation Products of DKN. NMR data (^{1}H, 300 MHz; ^{13}C, 75.5 MHz) were recorded on a Varian Gemini 300 instrument (Les Ulis, France). All NMR spectra were recorded in deuteriochloroform ($CDCl_3$). Chemical shifts are reported in *δ* (parts per million) relative to $CHCl_3$ ($CDCl_3$) as internal reference: 7.27 ppm for ^{1}H (77.14

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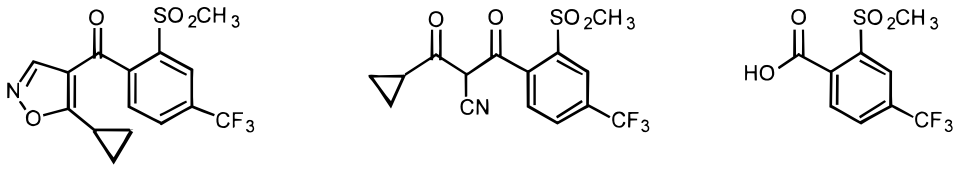

1 2 3

Figure 1. Structures of isoxaflutole (**1**), diketonitrile (DKN; **2**) and benzoic acid (BZA; **3**).

ppm for ^{13}C). Coupling constants (J) are given in hertz (Hz). Multiplicities are recorded as s (singlet), d (doublet), t (triplet), q (quadruplet), and m (multiplet) and br (broad). Mass spectra (MS) were obtained on a Nermag (Poissy, France) R10-10C by direct insertion probe or linked to a Varian 3300 GC. Ionization was obtained either by electronic impact (EI) or by chemical ionization with ammonia (CI, NH_3). Infrared spectra (IR) were obtained on a Nicolet Avatar 320 FT-IR (Trappes, France) and are reported in terms of frequency of absorption (ν, cm^{-1}).

To a 0 °C solution of diketone **2** (69 mg, 0.19 mmol) in CH_2Cl_2 (3 mL) was added an acetone solution (0.01 M) of dimethyldioxirane (3 mL, 0.30 mmol) prepared by the well-formulated protocol of Adam et al. (1991). The resultant solution was stirred for 5 min at 0 °C, dried ($MgSO_4$), filtered, and evaporated under reduced pressure. The crude product (69 mg) was immediately dissolved in $CDCl_3$ for NMR analysis. We observed the presence of epoxide **4** and lactone **5** (33:66; the ratio was determined by NMR integration of signals of methyl groups). After NMR analysis, $CDCl_3$ was removed under reduced pressure and the residue kept at room temperature overnight; NMR analysis of the residue showed only the presence of lactone **5**.

The solution of diketone **2** in CH_2Cl_2 and dimethyldioxirane was stirred overnight at room temperature and treated as above for direct oxidation to the aromatic acid BZA **3** and cyclopropanic acid **6**. These compounds **3** and **6** were characterized by esterification with diazomethane to give ester **7** (35 mg) and **8** (2 mg) separated by chromatography on silica gel (30 AcOEt:70 cyclohexane).

4: ^1H NMR δ 8.25 (s, 1 H), 8.00 (d, 1 H, $J = 7.5$ Hz), 7.55 (d, 1 H, $J = 7.5$ Hz), 3.20 (s, 3 H), 2.85 (m, 1 H), 1.50−1.20 (m, 4 H); ^{13}C NMR (partial) δ 197.3 (s), 81.3 (s), 45.5 (q), 17.3 (d), 16.4 (t), 15.7 (t).

5: ^1H NMR δ 8.40 (s, 1 H), 8.05 (s, 2 H), 6.25 (s, 1 H), 3.35 (s, 3 H), 2.30 (m, 1 H), 1.40−1.20 (m, 4 H); ^{13}C NMR (partial, no aromatic carbons) δ 194.7 (s), 163.8 (s), 134.8 (m), 112.3 (s), 67.0 (d), 45.0 (q), 17.9 (d), 14.3 (t), 14.0 (t); DEI MS, m/z (%) 376 ($MH^{+\bullet}$) (0.5), 356 (2), 280 (1), 251 (30), 69 (100); DCI (NH_3) MS, m/z (%) 393 ($M + NH_4^+$) (100), 377 ($M + 2\ H^+$) (10), 286 (8); IR ($CDCl_3$, cm^{-1}) 3170, 3014, 2929, 2250, 1760, 1735, 1672, 1324, 1157.

7: ^1H NMR δ 8.40 (s, 1 H), 7.95 (d, 1 H, $J = 7.5$ Hz), 7.85 (d, 1 H, $J = 7.5$ Hz), 4.00 (s, 3 H), 3.40 (s, 3 H); ^{13}C NMR (partial, no aromatic carbons) δ 166.5 (s), 134.0 (m), 53.7 (q), 45.0 (q); GC analysis (AT-5, 0.32 mm i.d. × 30 m, 180−300 °C, 10 °C min^{-1}), retention time = 2.12 min; EI MS, m/z (%) 282 ($M^{+\bullet}$) (2), 267 (20), 251 (100), 235 (2), 220 (10), 203 (12), 188 (10), 172 (10), 160 (15), 145 (20).

8: ^1H NMR δ 3.90 (s, 3 H), 2.75 (m, 1 H), 1.30−1.10 (m, 4 H); GC analysis (AT-5, 0.32 mm i.d. × 30 m, 50 °C (30 s), 300 °C, 10 °C min^{-1}), retention time = 4.20 min; EI MS, m/z (%) 128 ($M^{+\bullet}$) (5), 69 (100), 59 (10), 41 (50).

Fungal Strains and Culture Conditions. *Phanerochaete chrysosporium* (BKM-F-1767, ATCC 24725) and *Trametes versicolor* were from the culture collections of the Unité de Phytopharmacie et Médiateurs Chimiques (INRA, Versailles, France). The strains were maintained at 37 °C on solid medium of malt (20 g L^{-1}), agar (16 g L^{-1}), and yeast extract (1 g L^{-1}).

We distributed 10-mL fractions of culture media in 150-mL Erlemeyer flasks for in vivo degradation studies (*P. chrysosporium* and *T. versicolor*), 200 mL in Roux flasks for biomass production (*P. chrysosporium* and *T. versicolor*), and 5 L in a bioreactor for laccase production (*T. versicolor*).

We used for *P. chrysosporium* cultures a medium previously described (Mougin et al., 1994). The medium was inoculated with conidiospores (10^8 spores/L of medium). For *T. versicolor* cultures, glycerol was replaced by maltose (20 g L^{-1}), bactopeptone was added (2 g L^{-1}), and NAT 89 was omitted (Lesage-Meesen et al., 1996). Cultures were inoculated by three to five agar plugs (5 mm) of mycelium taken from the solid medium plates.

The air-lift bioreactor used for laccase production was inoculated with pounded mats and performed as previously described (Jolivalt et al., 1999). It contained 5 L of mineral culture medium (Collins et al., 1996). We buffered the medium to pH 5 with 0.29 g L^{-1} of dimethylsuccinic acid. The bioreactor was inoculated with a suspension of mycelium obtained by pounding two mats of *T. versicolor* in 100 mL of sterile water. Laccase production was stimulated by supplementing the medium with 20 mM 2,5-xylidine.

Incubation Conditions for in Vivo Degradation Studies. For DKN degradation studies, the medium (10 mL) was supplemented with 30 μM DKN consisting of a mixture of unlabeled and labeled (2.5 kBq) pesticides dissolved in acetone (50 μL). The Erlenmeyer flasks were enclosed in 1-L 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 dark at 25 °C. Every 3 days, the cultures were flushed with ambient air and sodium hydroxide solutions were replaced. Uninoculated sterile controls were submitted to a similar incubation design.

Enzyme Purification. Purified lignin peroxidases (LiPs) and manganese-dependent peroxidases (MnPs) were obtained from *P. chrysosporium* cultures. These enzymes were generous gifts of Dr. M. Asther (INRA, Unité de Biotechnologie des Champignons Filamenteux, Marseille-Luminy, France). Purified laccases (Lacs) were obtained from *T. versicolor* cultures as previously described (Jolivalt et al., 1999). The purified enzyme was stored at −20 °C in 25% w/v glycerol.

Enzymatic Assays. Lignin peroxidase (LiP; EC1.11.1.14) activity in the extracellular fluid was determined by the rate of oxidation of veratryl alcohol ($\epsilon_{310} = 9300\ M^{-1}\ cm^{-1}$) as described by Tien and Kirk (1984). The assay was conducted at 30 °C in 1 mL of a solution containing 2 mM veratryl alcohol in 100 mM phosphate−citrate buffer (pH 3). Manganese peroxidase (MnP; EC 1.11.1.13) activity was also determined spectrophotometrically according to the method of Paszczynski et al. (1986) with vanillylacetone ($\epsilon_{334} = 18300\ M^{-1}\ cm^{-1}$) as a substrate, at 30 °C. The reaction mixture (1 mL) contained 0.1 mM vanillylacetone and 0.1 mM $MnSO_4$. Both reactions were started by adding hydrogen peroxide. Laccase (Lac; EC 1.10.3.2) activity was measured as the oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; $\epsilon_{420} = 36000\ M^{-1}\ cm^{-1}$) according to the method of Wolfenden and Wilson (1982). The assay was conducted at 30 °C in 1 mL of a solution containing 1 mM ABTS in 100 mM phosphate−citrate buffer (pH 3). Enzymatic activities were expressed in units per milliliter (units mL^{-1}).

DKN transformation by purified oxidases was assayed in 100 mM phosphate−citrate buffer (pH 3 or 5). The solution (1 mL final) contained 10 μM DKN (including 1.8 kBq of labeled herbicide) dissolved in 20 μL of acetone. It was incubated at 30 °C under stirring. Enzymatic transformation of DKN is expressed in nanomoles per hour per unit. A solution of

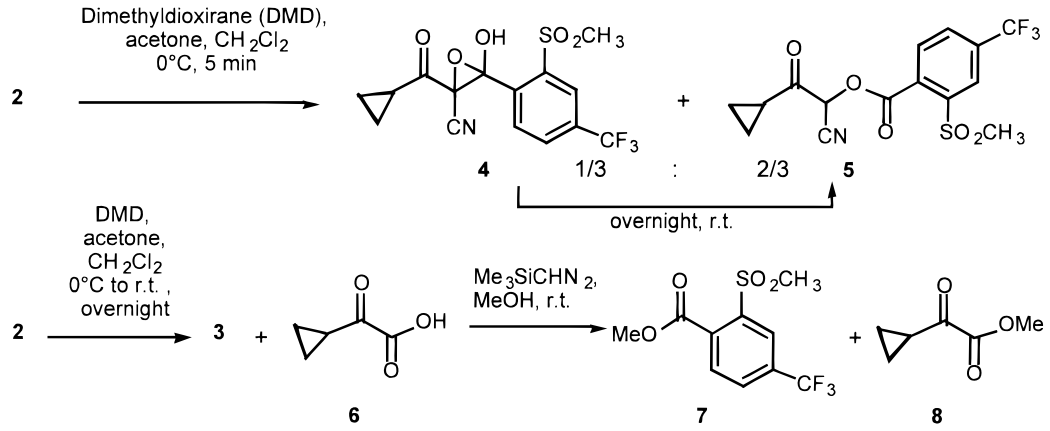

Figure 2. Hypothetical pathway for DKN chemical cleavage.

hydrogen peroxide (15 mM) was continuously added (20 μL h^{-1}) during incubations with peroxidases. Controls (where at the least one component was omitted) were also performed.

Analytical Procedures for Herbicide and Transformation Products. Fractions of the filtered aqueous media resulting from in vivo degradation studies were concentrated on a C_{18} guard column MCH-10 (3 cm × 4 mm i.d.; Varian, Les Ulis, France) at a flow rate of 1 mL min^{-1} with an isocratic pump (Mougin et al., 1994). The guard column, on-line interfaced with the HPLC through an electric valve, was used to load the sample for analysis. Elution of DKN and transformation products was then achieved from the guard column onto the analytical column TSK ODS-80TM (25 cm × 4.6 mm i.d.; Varian) with a pump delivering a solvent system composed of water/acetonitrile/H_3PO_4 (99:1:0.25 v/v/v) at 1 mL min^{-1} for 3 min. During the next 15 min, the solvent mixture was linearly brought to 0:100:0.25 (v/v/v). These solvent conditions were then held for 12 min. Radioactivity (radiochemical detector) and A_{254} (UV detector) of the column eluate were monitored. Radioactivity remaining in the fungal biomass was assessed by digestion of filtered mycelia with 2 mL of Optisolve solubilizer (EG&G, Evry, France) for 5 h. Finally, mixtures were supplemented with 10 mL of Optiphase 3 (EG&G), and liquid scintillation counting was achieved 24 h later. Labeled carbon dioxide evolved from the cultures was determined by liquid scintillation counting of 500-μL aliquots of the sodium hydroxide solutions.

Degradation studies with purified enzymes were analyzed by injecting 100-μL aliquots of the incubation media through the HPLC with the chromatographic conditions described above.

Experimental Design. Each experiment was done in triplicate. Results are expressed as means ± SD.

RESULTS

Hypothetical Pathway for DKN Cleavage. We have searched for a pathway for chemical DKN transformation to the BZA metabolite. In the proposed pathway, the parent compound **2** undergoes two successive reactions in the presence of the oxidant dimethyldioxirane to release the acid (Figure 2). After 5 min, it gives a mixture of two compounds: the epoxide **4** and the lactone **5**. Compound **4**, unstable, is rapidly converted into **5**. After a night of contact, the lactone **5** is cleaved to BZA **3** and compound **6**, with a release of cyanide. Both metabolites **3** and **6** were then derivatized into **7** and **8** for structural analysis. We have shown that an oxidative mechanism can chemically transform DKN to BZA. The pathway is consistent with the possible involvement of fungal oxidases in herbicide cleavage.

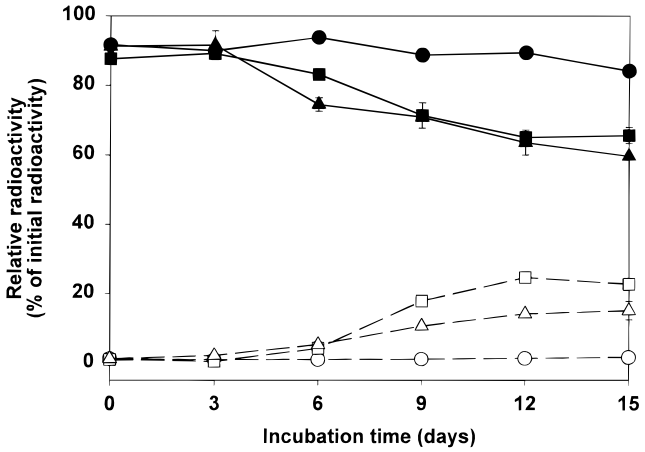

Figure 3. DKN depletion (solid symbols) and BZA formation (open symbols) in uninoculated controls (●) and in cultures inoculated with *P. chrysosporium* (■) and *T. versicolor* (▲).

In Vivo Degradation of the DKN Derivative by the Two Fungal Strains. Cultures of *P. chrysosporium* and *T. versicolor* were incubated with [*ring*-UL-^{14}C]-DKN for 15 days. After that period, the amounts of radioactivity in the medium corresponded to 98.0% of initial radioactivity in the uninoculated controls, to 92.8% with *P. chrysosporium*, and to 81.2% with *T. versicolor*. No herbicide mineralization was detected with any of the fungal strains. Determining the radioactivity trapped by the fungal biomass gave always recoveries of between 92.4 and 97.2% of initial amounts. The amounts of DKN (RT = 16.5 min) in the medium decreased slightly from 91.7 to 84.1% in the uninoculated control (Figure 3). In the presence of *P. chrysosporium* and *T. versicolor*, they were only 65.6 and 59.6% after 15 days. With *P. chrysosporium*, the decrease occurred after a 3-day lag phase, whereas it was already started at this time with *T. versicolor*. With both fungal strains, the DKN decrease corresponded to the formation of two polar metabolites. One of them, the most abundant (M_1, RT = 15.5 min), amounted to 24.6% of initial radioactivivty in the presence of *P. chrysosporium* and to 15.1% with *T. versicolor*. M_1 was detected in low amounts (<1.6%) in the uninoculated controls. The second (M_2, RT = 14.0 min) represented always <3.7% of initial radioactivity.

Profiles of extracellular oxidase production by *P. chrysosporium* and *T. versicolor* are shown in Figure 4for 15 days. Both LiPs and MnPs were already detected

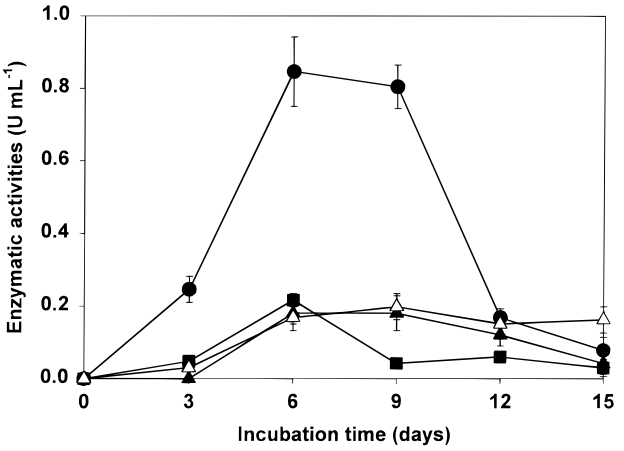

Figure 4. Extracellular enzyme production in cultures of *P. chrysosporium* (●, LiPs; ■, MnPs; ▲, Lacs) and *T. versicolor* (△, Lacs).

after 3 days of culture. They exhibited typical production profiles obtained for *P. chrysosporium* under these incubation conditions (Mougin et al., 1996). The LiPs exhibited the highest activity, with maximal values (~0.84 units mL^{-1}) between 6 and 9 days of incubation. MnP production was more reduced. In contrast, Lac was not produced by this strain at day 3 and showed an activity of 0.12−0.18 units mL^{-1} between days 6 and 12. Only Lac production was measured in *T. versicolor* cultures. Production began after 3 days to maintain its level until the end of the experiment. Activity values were similar to these reported for *P. chrysosporium*. All three oxidative enzymes show a time course similar to that of DKN degradation.

We tried to identify the metabolites formed from the DKN derivative by the fungi. For that purpose, medium fractions were pooled and concentrated on the guard column, prior to HPLC analysis. The peaks were then collected during elution. M_1 cochromatographied with an authentic standard of BZA analogue. After solvent extraction and derivatization, GC-MS analysis confirmed its identity. M_2 could not be extracted by organic solvents, and its structure was not checked by GC-MS.

Degradation of the DKN Derivative by Fungi-Purified Enzymes. The DKN derivative was incubated with fungal extracellular oxidases, without or with their specific redox mediator. Both LiPs and MnPs presenting high specific activities were unable to catalyze the enzymatic transformation of DKN, with or without mediators, during 12-h incubations (data not shown). Only Lacs transformed the herbicide, when incubated with 1 mM ABTS. Three labeled metabolites were formed. One of them cochromatographied with an authentic standard of BZA analogue during HPLC analysis. After solvent extraction and derivatization, GC-MS analysis confirmed its identity as BZA. Another compound presented the same retention time as compound M_2 formed in vivo. The third was a still more polar compound.

The reaction was then studied under different incubation conditions with Lacs purified from *T. versicolor* cultures. That strain was preferentially used for enzyme production because it secreted mainly Lacs in the extracellular fluid in the presence of 2,5-xylidine. The DKN was cleaved at its higher rate (~0.3−0.4 nmol h^{-1} $unit^{-1}$) in the presence of ABTS at acidic pH 3 (Table 1). High incubation temperatures (30−50 °C) allowed the higher enzymatic activities. The rates also depended on ABTS concentration, being maximal with values >1 mM mediator. Compounds such as exogenous (1-hydroxybenzotriazole, 1-HBT) or physiological (3-hydroxyanthranilic acid, 3-HAA; Eggert et al., 1996) mediators were not able to support the reaction.

Table 1. Laccase Activity toward DKN Derivative versus Various Incubation Conditions[a]

incubation conditions		enzymatic activity (nmol h^{-1} $unit^{-1}$)
controls	without Lac	0.05
	without ABTS	0.02
pH	3	0.36
	5	0.08
	7	0.06
temp (°C)	20	0.16
	25	0.28
	30	0.43
	40	0.45
	50	0.44
	60	0.29
ABTS concn (mM)	0	0.02
	0.1	0.08
	0.2	0.13
	0.5	0.23
	1.0	0.36
	2.0	0.46
mediator (1.0 mM)	ABTS	0.28
	3-HAA	0.03
	1-HBT	0.04

[a] Results are expressed as means of triplicates. The standard deviation is <10% of the mean in all cases. ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); 3-HAA, 3-hydroxyanthranilic acid; 1-HBT, 1-hydroxybenzotriazole.

DISCUSSION

Fungal metabolism of xenobiotics by white rot fungi has been studied for many years. In most cases, they have been seen as efficient degraders (Barr and Aust, 1994). However, among the bulk of existing chemical compounds metabolized, a few data are available concerning the mechanisms involved in herbicide metabolism by these filamentous fungi. In the present paper, we study the transformation of the active principle of the new herbicide isoxaflutole, namely its ketonitrile (DKN) derivative, by two well-known white rot fungi.

P. chrysosporium and *T. versicolor* were shown to cleave the diketone in liquid cultures, in producing the BZA also formed in tolerant plants (Viviani et al., 1998). *P. chrysosporium* was more efficient than *T. versicolor*. Nevertheless, herbicide metabolism occurred mainly at the end of the fungal growing phase and seemed to be correlated with the production of extracellular oxidases, such as laccases, by the two strains. A similar situation was recently reported for several PAHs metabolized by fungi producing oxidative enzymes (Gramss et al., 1999).

Enzymes involved in the diketone cleavage remain poorly characterized to date. Sakai et al. (1986) reported a bacterial β-diketone hydrolase (EC 3.7.1.7) involved in the degradation mechanism of poly(vinyl alcohol). Nevertheless, such a hydrolytic mechanism was not relevant to the formation of BZA because a methyl ketone should be formed instead of the acid. Moreover, attempts to isolate a form of β-diketone hydrolase acting on DKN among microorganisms were unsuccessful (J.-C. Thomas, Rhône-Poulenc, personal communication).

Because of the apparent correlation stated above and because of the high and nonspecific oxidizing potential of white rot fungi, we looked for oxidative extracellular enzymes produced by *P. chrysosporium* and *T. versicolor*. In dependence on their ionization potential (IP), substrates can be directly oxidized by peroxidases or laccases or by several enzyme/mediator systems such as laccase/ABTS (Collins et al., 1996). The initial one-electron oxidation of xenobiotics is typical of wood-decay fungi such as white rot. Although the reaction often leads to the formation of quinones, intermediates such as radical cations and hydroxylated intermediates are possibly appearing. On the other hand, chemicals (mainly methyl-, methoxy-, chloro-, and bromophenols) can be submitted to oxidative coupling by laccases, leading to polymeric products of increasing complexity (Gianfreda et al., 1999).

Although the main role of fungal laccase in nature is to depolymerize lignin, examples of bond cleavage among xenobiotics are not abundant. Chivukula et al. (1995) reported the oxidation of phenolic azo dyes by laccase from *Pyricularia oryzae* to benzoquinone. The reaction proceeded by the production of a carbonium ion in which the charge is localized on the phenolic ring carbon bearing the azo linkage and cleavage of the carbon−nitrogen bond. In a similar way, we reported more recently the cleavage of *N*′,*N*′-dimethyl-*N*-(4-hydroxyphenyl)urea (a metabolite of phenylurea herbicide) to *p*-benzoquinone by laccases of *T. versicolor* (Jolivalt et al., 1999). In this paper, we describe a new laccase-mediated pesticide cleavage. Nevertheless, results from our study with purified enzymes establish that a direct attack of the laccase never occurred. Only ABTS acted as a potent redox mediator. As the herbicide was also transformed in vivo, this implies the production and use by the fungus of an unidentified redox mediator. Laccase production can be 6-fold increased in vivo by treatment of *Trametes* cultures with 2,5-xylidine, without any significant diketonitrile cleavage increase (data not shown). This result seems to indicate that the production of the redox mediator is the limiting step in the reaction. On the other hand, we cannot exclude the involvement of other oxidases (i.e., intracellular oxygenases) in DKN breakdown, although we failed to obtain any reaction with intact mycelia or microsomal fractions (unpublished results).

The hypothetical pathway for DKN chemical oxidation to BZA implies the loss of the cyano group of the molecule. In living organisms (bacteria, fungi, and higher plants), the nitrile is converted into the corresponding acid, with a possible release of ammonia as a byproduct (Harper, 1977; Stalker and McBride, 1987). The acid can then be submitted to oxidative decarboxylation to give phenolic compounds (Buckland et al., 1973).

In summary, this work shows that fungal laccases are able to cleave diketone compounds. Nevertheless, the reaction is not due to a direct action of the enzyme on the chemical but requires the presence of a redox mediator acting as a diffusible electron carrier. In vivo, identity of fungal endogenous mediators remains to be determined. Moreover, our results should not prevent the involvement of other unknown enzymatic systems in the cleavage of the diketonitrile derivative of the herbicide isoxaflutole. For all of these reasons, no attempts at cloning the enzymes involved in the metabolic pathway of the herbicide will be started.

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