Plant and fungal cytochrome P-450s: their role in pesticide transformation

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Plant and Fungal Cytochrome P-450s: Their Role in Pesticide Transformation

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Cytochromes P-450 (P-450s) constitute a large family of oxygenases catalyzing phase I metabolism of various xenobiotics, including pesticides. In most cases, they mediate reactions resulting in detoxification of the parent molecule. Plant P-450s play an important role in herbicide metabolism, by constituting a key factor of herbicide selectivity, and of herbicide resistance recently observed in some weed biotypes. Definitive proof of P-450-mediated pesticide metabolism in filamentous fungi has not yet been obtained. However, indirect evidence supports such an hypothesis. The detoxification and degradation potential of P-450s from plants or microorganisms could be exploited for bioremediation of water, industrial waste and soil contaminated with pesticides. A more comprehensive knowledge of these enzymes, including functional characterization of genes and proteins, remains necessary for an efficient management of P-450 potentialities.

Living organisms are all directly or indirectly exposed to foreign organic molecules (or xenobiotics), called pesticides. In most cases, xenobiotics are absorbed by living cells and undergo biochemical modifications. These modifications may result from several types of phase I reactions, such as hydrolysis, reduction, or oxidation, and from various conjugation reactions. Such reactions participate to different extents in the metabolism of each pesticide. A survey shows that, among the initial steps responsible for the transformation of parent molecules into primary metabolites, the most frequent are oxygenation reactions. In animals, it has been known for many years that such reactions are mainly mediated by P-450s. In plants, evidence for the role of

P-450 in the oxygenation of a pesticide, *i.e.* the *N*-demethylation of the herbicide monuron [*N'*-(4-chlorophenyl)-*N*,*N*-dimethylurea], was obtained at the end of the 1960's (*1*). In the case of filamentous fungi, no data are yet available concerning pesticides.

The purpose of this chapter is to present the main structural features and functions of P-450s, and to exemplify their involvement in the metabolism of pesticides in higher plants and filamentous fungi. As far as possible, data are found in recent reviews listed in the literature section, and are concerned with enzyme structure and phylogeny (*2,3)*, reactions and functions (*4,5,6*), nomenclature (*7*), plant P-450 molecular enzymology (*8,9*), and microbial and fungal P-450s (*10,11,12,13*).

Properties and Functions of Plant and Microbial P-450s: Similarities and Divergences

Properties

P-450s constitute a large family of heme-thiolate proteins, widely distributed among living organisms. In most cases, they function as monooxygenases, by binding and activating molecular oxygen, incorporating one of its atoms into an organic substrate, and reducing the second atom to form water, according to the following reaction:

$$RH + O_2 + NAD(P)H + H^+ \text{--> } ROH + H_2O + NA(D)P^+$$

The result of catalysis, depending on the P-450 protein and its substrate, is in most cases hydroxylation, but epoxidation, heteroatom dealkylation, deamination, isomerization, C-C or C=N cleavage, dimerization, ring formation, dehydration, dehydrogenation or reduction have also been reported.

For most eucaryotic P-450s, a FAD/FMN-dependent NADPH-P-450 reductase is needed to transfer the electrons used for oxygen activation from cytosolic NADPH. In plants and filamentous fungi, as well as in other eucaryotic organisms, P-450s and reductases are usually microsomal membrane-bound proteins, exposed to the cytosol. Nevertheless, soluble forms of P-450s, coupling P-450 and reductase in a single fusion protein, have also been found in bacteria and fungi (*12*). A second type of P-450s has been identified in prokaryotes and animal mitochondria. In addition to a flavoprotein, it needs a small redox iron-sulfur protein (ferredoxin) to transfer electrons from NAD(P)H to the terminal P-450 component. Such P-450s are usually soluble or associated with inner mitochondrial membranes. A third class of P-450s does not require an auxillary redox partner or molecular oxygen to catalyze the rearrangement of hydroperoxides. Some of such plant P-450s seem to be located in the plastids (*14*).

When reduced, P-450s can bind carbon monoxide instead of oxygen, forming a complex that shifts the Soret absorption maximum between 447 and 452 nm. As a result, the enzymatic reaction is blocked, but inhibition can be partly reversed by light, with a maximum of efficiency around 450 nm. A second species absorbing at 420 nm,

that is considered to be an inactive form of P-450, is often simultaneously detected. A shift of the Soret of the oxidized P-450s can also be detected. When a substrate binds the active site, it results in a so-called type I spectrum with a peak near 390 nm and a trough around 420 nm. After addition of inhibitors such as heterocyclic compounds, it results in a type II absorbance change (peak around 430 nm, and trough at 390-400 nm). Such absorption changes are indicative of shifts in the spin equilibrium and redox potential of the cytochrome.

P-450s are encoded by a superfamily of genes. The sequences of more than 500 of them have already been recorded in all living organisms (*7*). They are named and grouped into more than 150 families, according to the amino acid sequences of the deduced proteins. With a few exceptions, based on phylogenetic considerations, protein sequences within a given gene family are >40% identical. When two sequences are more than 55% identical, proteins are designated as members of the same subfamily. Following the prefix CYP, numbers designate families, and letters subfamilies. Plant P-450s correspond to the families CYP71 to CYP99, then CYP701 and above. Fungal P-450s comprise families CYP51 to CYP66.

The first plant P-450 gene, CYP71A1, was cloned at the beginning of the 1990's. There was then an explosion in the rate at which genes encoding plant P-450s have been identified. More than 200 genes have been registered in plants, 40 in fungi (including yeast), and 90 in prokaryotes. The apoprotein sequences (45 to 60 kDa) are highly variable, but their three-dimensional structures seem to be somewhat similar. The most highly conserved part of all P-450s is the core surrounding the heme prosthetic group. The proximal side of the heme is formed of a loop including the cysteine residue (serving as the sixth ligand of the iron) and the eleven most highly conserved residues of the protein. Other conserved elements, found on the distal side, are responsible for oxygen binding and activation, and for the transfer of the protons to the activated oxygen. Most P-450s seem to be anchored to membranes primarily via a 30 to 50 amino acids N-terminal segment. Information on targeting, insertion and topography of fungal, and especially plant P-450s remains sparse.

Knowledge concerning the regulation of plant and fungal P-450s is only starting to accumulate. In plants, expression generally seems to follow a developmentally regulated tissue-specific pattern. Physicochemical (light, osmotic stress, wounding), physiological (infection, ageing, hormones) factors, or xenobiotics (agrochemicals, ethanol, or drugs like phenobarbital or aminopyrine) were reported to induce plant P-450s. In many cases, expression is very low or undetectable in the absence of induction. Plant or fungal P-450 enzymes can be inhibited by mechanism-based inactivators (heme or apoprotein alkylating agents, like 1-aminobenzotriazole (ABT) or acetylenic substrate analogues), heterocyclic molecules (imidazole, pyrimidine, triazole derivatives) or methylenedioxy compounds (*e.g.* piperonyl butoxide (PBO) or piperonylic acid). Some of these compounds inhibit a broad range of enzymes, while others seem to be more selective. Selective induction and inhibition can be used to differentiate between P-450s involved in specific oxygenation reactions.

Plant and fungal P-450 systems so far remain less understood than their mammalian homologues, because these proteins are typically present in low abundance and are often unstable during purification. In general, P-450 amounts in

non-induced plants, fungi and bacteria are less than 0.1 nmole mg^{-1} microsomal proteins, and represent only the one-tenth the amount naturally expressed in mammalian liver.

Functions

Most of the plant P-450 genes recently isolated have not been attributed with a function, either *in vitro* or *in vivo*. The natural substrates reported so far are plant specific secondary metabolites, like phenylpropanoids, isoprenoids, alkaloids, plant growth regulators, amino acid derivatives, or natural compounds also present in fungi and animals such as sterols or fatty acids. Some plant P-450s are also capable, probably fortuitously, of metabolizing xenobiotics. Data accumulated in the last ten years, indicate that they constitute the major oxidative pathway involved in herbicide metabolism. In fungi, the involvement of P-450 systems in many complex bioconversions processes (metabolism of alkanes, synthesis of sterols and antibiotics, reduction of nitrogen oxide) has been demonstrated in recent years. Among xenobiotics, only polycyclic aromatic hydrocarbon (benzo[a]pyrene) have been identified as substrates of fungal P-450s. Bacterial P-450s, metabolizing herbicides such as sulfonylureas, carbamates or triazines, have also been reported.

Role of Plant P-450s in Herbicide Metabolism and Selectivity

Metabolic detoxification is one of the main mechanisms of herbicide selectivity, and is responsible in many cases for the tolerance of major crops and resistant weeds. It is now well established that P-450-catalyzed reactions are often at the origin of this phenomenon (*15*,*16*,*17*). Evidence indicates qualitative and quantitative differences in P-450 contents from plant to plant. For that reason, species and cultivars exhibit differences in herbicide metabolism, as well as differential enzymatic induction or inhibition.

Phenylureas provide the best-documented example of herbicide metabolism in higher plants. Monuron dealkylation in cotton (*Gossypium hirsutum*) was one of the first P-450-dependent reactions to be characterized as early as 1969 (*1*). Different reactions associated with P-450-dependent chlortoluron [*N'*-(3-chloro-4-methylphenyl)-*N*,*N*-dimethylurea] metabolism were later reported in many plants, including both crops and weeds. In addition, the only genes of herbicide-metabolizing plant P-450s characterized so far, are all involved in phenylurea detoxification.

Metabolism of Chlortoluron in Plants

Chlorotoluron is active against a number of monocots and dicots, and is principally detoxified by hydroxylation of the ring-methyl (CPUH), or *N*-demethylation

(CPUDM). The ring-methyl hydroxylated and the di-*N*-demethylated products are non-phytotoxic. The polar metabolites can be partly conjugated to glucose, resulting in detoxification.

Chlortoluron provides a very good example of metabolism associated with herbicide selectivity between weeds and crops. In the tolerant winter wheat (*Triticum aestivum*), the half-life of chlortoluron is less than 24 hours. The main metabolite is the non-phytotoxic *ring*-methyl hydroxylated derivative. In the susceptible weed blackgrass (*Alopecurus myosuroides)*, the main metabolite is the mono-*N*-demethylated compound. Phytotoxicity of this metabolite has a half-life greater than 24 hours. In the tolerant weed Persion speedwell (*Veronica persica)*, on the other hand, the herbicide has a half-life of only 6 hours, and is converted to the non-phytotoxic di-*N*-demethylated product.

Identification of the enzymes responsible for the oxidation reactions in wheat was first attempted using *in vivo* experiments. First evidence was Gaillardon *et al.*, who showed that administration of PBO and ABT increased the toxicity of chlortoluron (*18*). ABT strongly inhibited both CPUH and CPUDM activities (*19*). This finding was later extended to other tolerant species, but *N*-demethylation of chlortoluron was little affected by ABT in Persion speedwell (*20)*.

Metabolism of Chlortoluron in Cell Cultures

Similar experiments were performed with cell cultures. CPUDM was more active in wheat cells than in the whole plant. ABT, and the plant growth retardants tetcyclacis [5-(4-chlorophenyl)-3,4,5,9,10-pentaaza-*te*-teracyclo-5,4,1,$0^{2,6}$,$0^{8,11}$-dodeca-3,9-diene] and paclobutrazol [(2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pentan-3-ol], inhibited herbicide transformation (*21*). By contrast, metabolism was increased after pretreatment of cells with 2,4-D [(2,4-dichlorophenoxy)acetic acid], the herbicide safener cyometrinil, [(Z)-α[(cyanomethoxy)-imino]benzene acetonitrile] or the imidazole fungicide prochloraz [*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]imidazole-1-carboxamide] (*21*). 2,4-D and cyometrinil also stimulated chlortoluron metabolism in Persion speedwell cells, by inducing expression of a new CPUH activity (F. Cabanne, unpublished results).

Metabolism of Chlortoluron in Microsomal Preparations

All *in vivo* results strongly suggested that oxidative transformation of chlortoluron could be mediated by P-450s. A first proof *in vitro* was obtained for CPUDM activity with microsomes isolated from Jerusalem artichoke tubers (*Helianthus tuberosus*) (*22*). Definitive proof for CPUH activity arose from assays with microsomal preparations isolated from wheat (*23*). Metabolism of chlortoluron was also detected in microsomes from Persion speedwell (N. Polge, unpublished results). A higher efficiency of tuber slices and cell cultures versus etiolated seedlings was due to the fact that they could be pretreated with inducers prior to the preparation of microsomes

(*22*,*24*). In all species, both CPUH and CPUDM exhibited properties of P-450-mediated activities. In wheat and Jerusalem artichoke, spectral evidences also confirmed P-450 involvement in chlortoluron metabolism. Reduced carbon monoxide spectra showed that microsomes isolated from wheat cells grown for 48 h in the presence of 300 µM 2,4-D, contained about 250 pmol P-450 per mg microsomal protein (Figure 1A). A type I spectrum was obtained by incubating such microsomes with chlortoluron, indicating binding of the herbicide to the catalytic site of P-450 (Figure 1B). In addition, the fungicide procloraz, inhibiting chlortoluron oxidation in wheat microsomes, induced the formation of type II spectra when applied at 100 to 400 µM, which suggested its binding to P-450 heme iron (Figure 1C). Microsomal preparations from wheat were also able to *N*-demethylate the phenylurea herbicides isoproturon [*N*'-(4-isopropylphenyl)-*N*,*N*-dimethyl-urea] and diuron [*N*'-(3,4-dichlorophenyl)-*N*,*N*-dimethyl-urea] (*25*).

In wheat, P-450 effectors differently affected CPUH and CPUDM. First, inhibitors such as heterocyclic molecules and methylenedioxy compounds did not inhibit the activities to the same extent. Only CPUH was very sensitive to ABT. On the other hand, CPUDM was more strongly stimulated than CPUH when cells were treated with P-450 inducers. Finally, only CPUDM was active with organic hydroperoxides as electron donors. Thus, at least two P-450s are probably involved in the metabolism of chlortoluron in wheat. Similar conclusions were drawn concerning CPUH and CPUDM activities from Persion speedwell.

Interspecific differences were also evidenced by different sensitivities towards carbon monoxide, tetcyclacis and ABT (Table I). In both wheat and Persion speedwell, enzymatic systems responsible for chlortoluron metabolism exhibited an induction pattern similar to laurate hydroxylase, used as physiological P-450 marker. In wheat, the activities were, however, clearly distinguished by using specific inhibitors (*26*).

Following the pioneering work performed with phenylureas, evidence for the involvement of P-450s in the metabolism of pesticides belonging to other major classes of herbicides (sulfonylureas, alloxy-phenoxyalkanonates, chloroacetanilides, imidazolinones, bentazon, flumetsulam) have been obtained in major crops (wheat, corn: *Zea mays*, barley: *Hordeum vulgare*, sorghum: *Sorghum bicolor*) or weeds.

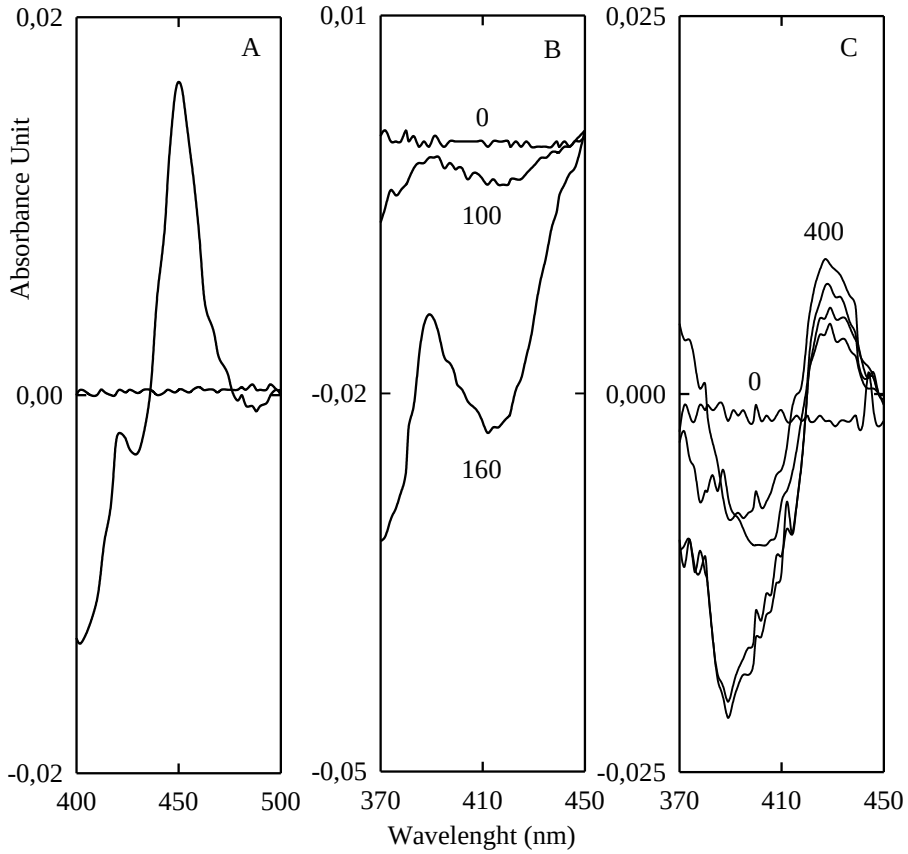

Figure 1. Difference spectra of wheat cell microsomes. A, reduced carbon monoxide spectrum; B, chlorotoluron-enzyme type I binding spectrum (herbicide concentrations were 0, 100 and 160 µM); C, procloraz-enzyme type II binding spectrum (final concentrations of fungicide ranged from 100 to 400 µM). (Reproduced from reference 25. Copyright 1992 Elsevier).

Table I. Inhibition of Chlorotoluron Metabolism by P-450 Inhibitors in Wheat and Persion speedwell

Inhibitors		% of inhibition			
		Wheat		*Speedwell*	
		CPUH	CPUDM	CPUH	CPUDM
carbon monoxide		50	35	75	98
tetcyclacis	10 µM	20	10	74	45
	100 µM	60	40	99	80
ABT	10 µM	74	57	0	0
	100 µM	100	100	35	7

P-450 Genes Involved in Phenylurea Metabolism

Paradoxically, the first gene shown to encode a P-450 protein metabolizing phenylureas, and to increase plant tolerance to chlortoluron was the benzo[a]pyrene activating CYP1A1 from rat liver (*27*). This P-450 displayed both CPUH and CPUDM activities, but their turnovers were quite low. Recently, the genes of two other phenylurea metabolizing P-450s were isolated from higher plants (*28*,*29*). The gene of the *H. tuberosus* P-450 previously reported to catalyze chlortoluron *N*-demethylation, was isolated on the basis of its inducibility by aminopyrine (*28)*. CYP76B1 expression in yeast confirmed that it had chlortoluron di-*N*-demethylase, but no CPUH activity. CPUDM catalytic efficiency was surprisingly high and similar to that observed with physiological substrates. CYP76B1 also catalyzed the *O*-dealkylation of various 7-alkoxy-coumarins and 7-alkoxyphenoxazones, like CYP1A1, but had no benzo[a]pyrene oxygenase activity. Another gene, CYP71A10 was isolated from soybean (*29*). CYP71A10 has properties more similar to CYP1A1 with regard to phenylurea metabolism, and catalyzes both CPUH and CPUDM activities with turnover rates much lower than that of CYP76B1. Both P-450s metabolize a broad range of phenylureas and are capable of increasing phenylurea tolerance of susceptible plants.

Role of Plant P-450s in Herbicide Cross-Resistance in weeds

As exemplified by the case of chlortoluron, the level of tolerance conferred by P-450-mediated metabolism of herbicides can be quite high in crops, but also in weeds. Herbicide resistance in weeds is in general more frequently related to mutation of the target site than to enhanced metabolism. Nevertheless, cases of weed resistance resulting from increased P-450 metabolism following intensive use of herbicides have been reported in Australian biotypes of rigid ryegrass (*Lolium rigidum)* (*30*) and

European biotypes of blackgrass (*31*,*32*) in the ten last years. The nature of the metabolites formed, and the effects of known P-450 inhibitors, gave strong evidence of the involvement of P-450 in the resistance of these weeds. Despite intensive efforts, definitive proof could never be established *in vitro* using microsomal preparations of rigid ryegrass, although direct evidence was obtained in blackgrass.

The development of increased metabolism sometimes results in cross-resistance to several herbicides from different classes and leads to multiple resistant weed populations (*30*). Cross-resistance may also result from the cross-pollination of plants with resistant target sites, and plants with increased metabolism. The mechanism of acquisition of metabolism-dependent herbicide resistance in weeds is not yet understood.

Role of Plant P-450s in Fungicide Metabolism in the Host Plant

Fenpropimorph [(±)-*cis*-4-[3-(4-*tert*-butylphenyl)-2-methylpropyl]-2,6-dimethyl morpholine] is a derivative acting as a sterol biosynthesis inhibitor (SBI), that has been widely used during recent decades as a systemic fungicide against powdery mildew and rust diseases of cereals. This fungitoxic molecule, however, was also found to interfere with the biosynthesis of the sterols of the host plant by replacing Δ^5-sterols by Δ^8-sterols and cyclopropylsterols *(33)*. This accumulation of abnormal sterols is due to the inhibition of two target enzymes: the cycloeucalenol obtusifoliol isomerase (COI) and Δ^8->Δ^7-sterol-isomerase. This section reports new experimental results that concerned interactions of fenpropimorph and agrochemicals with wheat plants.

Experimental

To study the effects of agrochemicals on sterol profiles, wheat seedlings were germinated in vermiculite in the dark at 20°C, and then watered daily with solutions containing fenpropimorph, ABT or naphthalic anhydride [naphthalene-1,8-dicarboxylic anhydride] (each at 2 mg L^{-1}) over 10 days. Then, shoots and roots were measured. Sterols were extracted and analyzed as reported (*34*). To evaluate the effects of agrochemicals on wheat P-450 content, seeds were soaked and germinated on paper immerged in water containing the chemicals, for 5 days in the dark at 25°C. Approximately 40 g of etiolated seedlings were harvested and used for the preparation of microsomes (*24*). Cytochromes were measured according to Omura and Sato (*35*). [ring-^{14}C]Fenpropimorph was incubated with microsomes using published protocols (*24*), prior medium extraction for TLC and GC-MS analysis.

Combined Effects of Fenpropimorph and Agrochemicals on Sterol Profiles

With respect to untreated controls, seedlings treated with fenpropimorph accumulated abnormal sterols (Δ^8-sterols and cyclopropylsterols) in leaves and roots, whereas the Δ^5-sterols initially present declined to very low levels (Table II). The modification of the sterol profiles was correlated with an increase in abnormal sterols from less than 10% to 85.7% in leaves and 95.9% in roots. ABT and naphthalic anhydride alone increased the amounts of Δ^8-sterols to 30%, by reducing the levels of Δ^5-sterols (not shown).

When compared to sterol profile in fenpropimorph treated wheat, this of seedlings treated with the mixture fenpropimorph/ABT accumulated precursors in roots, which were essentially cyclopropylsterols (87.9%). ABT activity led to a synergistic effect on fenpropimorph action in roots. As the first target of fenpropimorph on sterol biosynthesis enzymes is the COI, the synergistic effect of ABT may result in a complete inhibition of this enzyme.

The combination of fenpropimorph and naphthalic anhydride led to a significant change in sterol biosynthesis mainly in leaves (Table II). Total amounts of Δ^8-sterols and cyclopropylsterols decreased from 85.7% in leaves treated by fenpropimorph alone to 60.2% in presence of naphthalic anhydride. On the other hand, we observed an increase in the amount of total sterols from 14.3 to 39.8% in leaves and from 4.1 to 10.9% in roots. This suggests that naphthalic anhydride, an inducer of plant P-450s, may either activate fenpropimorph detoxification, or stimulate a P-450 enzyme involved in sterol biosynthesis, such as the 14α-demethylase.

Table II: Sterol Distribution in Wheat Seedlings after 10-Day Treatments with Fenpropimorph (Fen), or Fenpropimorph + ABT, Fenpropimorph + Naphthalic Anhydride (NA), in Leaves (L) and Roots (R)

Sterol distribution	*Control*		*Fen.*		*Fen. + ABT*		*Fen. + NA*	
(% of total sterol)	L	R	L	R	L	R	L	R
Δ^5 sterols	94.0	91.3	13.3	2.8	10.5	7.0	37.5	8.5
Δ^7 sterols	1.7	1.4	1.0	1.3	1.0	0.4	3.2	2.4
Δ^8 sterols	1.7	1.9	46.8	31.3	40.9	5.0	29.2	25.1
Cyclopropylsterols	2.4	4.4	38.9	58	47.5	87.9	31.0	64.0
Normal sterols	95.7	92.7	14.3	4.1	11.5	7.4	39.8	10.9
Abnormal sterols	4.3	7.3	85.7	95.9	88.5	92.5	60.2	89.1

Effects of Fenpropimorph with Agrochemicals on Wheat P-450 Content

Seedlings treated with fenpropimorph alone (0.05% by weight of seeds) showed a 1.7-fold increase in microsomal P-450 content from 113 to 196 pmoles mg^{-1} protein (Table III). When fenpropimorph was combined with naphthalic anhydride or

clofibrate, the P-450 level was to 300 pmoles mg^{-1}. In mixture with fenpropimorph, the effectors PBO, ABT and tetcyclacis slightly decreased P-450 contents to 130-160 pmoles mg^{-1}, but high amounts of P-420 were detected. Inducing effects may be blocked by the last three compounds, which can bind to or inactivate the neo-formed enzymes (*36*). We thus may not detect any strong inducing effect.

Table III : Effect of Treatment of Seedlings with Agrochemicals On Microsomal P-450 Content

Treatment	*Amount** (%)	*P-450*** (pmoles mg^{-1})
Control (water)	0	113
Fenpropimorph	0.05	196
Fen. + PBO	0.05+ 0.05	160
Fen. + naphthalic an.	0.05 + 0.1	302
Fen. + ABT	0.05 + 0.05	130
Fen. + tetcyclacis	0.05 + 0.025	138
Fen. + clofibrate	0.05 + 0.05	340

NOTE: * by weight of seeds, ** SD does not exceed 12% of value.

Involvement of P-450 in Fenpropimorph Metabolism

Fenpropimorph was metabolized to an oxygenated compound by microsomal fractions isolated from wheat seedlings. The mass spectrum of the metabolite fitted with an oxidized derivative either being hydroxylated on the *tert*-butyl group or N-oxidized (Figure 2). The reaction required NADPH as cofactor, and was partly inhibited by carbon monoxide. The enzymatic activity was low (93 pmoles mg^{-1} h^{-1}), but increased 2-times (180 pmoles mg^{-1} h^{-1}) when seedlings were pretreated with naphthalic anhydride whether the fungicide was present or not. The fungicide oxidation was thus assumed to be P-450-mediated in wheat.

These results confirm that agrochemicals acting as P-450 effectors can modify the metabolism of a pesticide, and may lead to toxic effects on the host plant. These experiments also suggest that these agrochemicals can modulate the overall metabolism of a plant, by increasing or inhibiting physiological activities, thereby affecting plant growth.

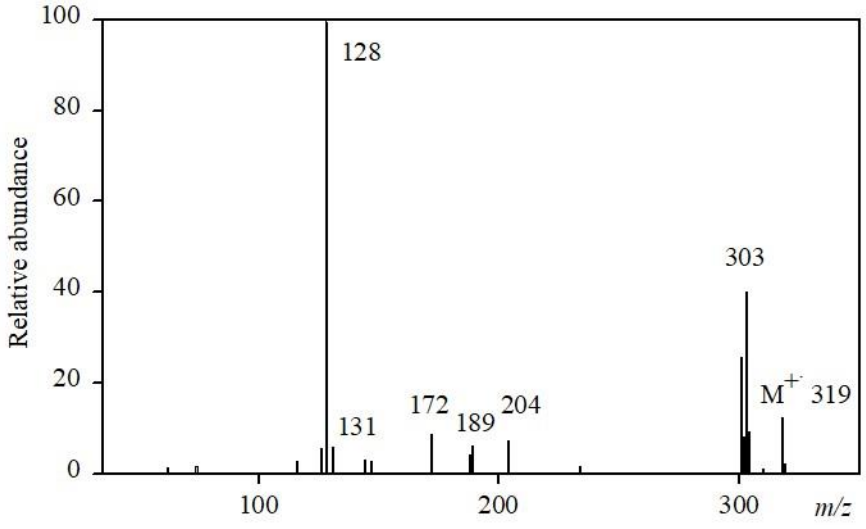

Figure 2. GC-EI-MS spectrum of the oxygenated metabolite formed from fenpropimorph by wheat microsomal preparations.

Fungal P-450s: Tools for Bioremediation

Remediation of water and soils contaminated with organic pollutants is a becoming a major challenge because of widespread pollution and its detrimental implication in human health. As a general rule, removing the contaminants (decontamination) is preferred to sequestering them (stabilization). Many organisms, including plants and microorganisms, can be used for that purpose. We have studied filamentous fungi as tools for the bioremediation of soils contaminated with pesticides or polycyclic aromatic hydrocarbons. The major advantage of such organisms is their direct contact with the pollutants in the solid, liquid and vapor phase of the soil, avoiding problems of uptake and translocation occurring with plants. Moreover, fungi can transform a wide range of pollutants, with a good resistance to possible toxic effects of the biocides. Fungi usually release most of the metabolites, inducing a synergism with the indigenous microflora present in their vicinity.

Evidence of P-450s in Filamentous Fungi

Our model is the ligninolytic strain *Phanerochaete chrysosporium*. This basidiomycete has been studied for many years for its production of exocellular oxidases, namely lignin and manganese-dependent peroxidases. Recently, we have confirmed the presence of intracellular enzymes in the fungus grown in liquid cultures. A reduced P-450-CO spectrum was obtained using microsomal preparations prepared from *P. chrysosporium*. Unfortunately, the amount of P-450 was low (89 pmol mg^{-1} microsomal proteins), and we failed to associate xenobiotic metabolism to this preparation *(37)*. An explanation could be found in the high level of P-420, indicative of partial degradation of the enzyme during the isolation process.

Involvement of P-450s in Lindane Transformation

P. chrysosporium metabolized the insecticide lindane [1α,2α,3β,4α,5α,6β-hexachlorocyclohexane] to polar compounds (*i.e.* tetrachlorocyclohexene epoxide and tetracyclohexenol) and carbon dioxide (*38*). The same metabolites are formed by microsomal fractions from rat liver. ABT treatment of *P. chrysosporium* cultures drastically reduced pesticide metabolism, inhibiting the formation of all metabolites as well as final carbon dioxide release. On the other hand, phenobarbital treatment did not modify lindane mineralization, but increased the oxidation of tetrachlorocyclohexene to tetrachlorocyclohexene epoxide and tetracyclohexenol. These indirect effects are consistent with the involvement of P-450s in one or more steps of lindane metabolism.

Soil Bioremediation

Biotransformation of lindane was investigated in sterilized and non-sterilized soils, each supplemented or not with *P. chrysosporium* (*39*). After spore inoculation of the sterilized soil, the fungal biomass increased rapidly during the first week, then at a lower rate during 8 weeks. Only limited fungal development occurred in non-sterile soils. Lindane mineralization, as a result of indigenous microflora activity, was 2-fold increased in the presence of the fungus (Table IV). As extractable amounts of residual lindane were quite similar in soils, with or without fungal addition, *P. chrysosporium* modified insecticide degradation pathway, by increasing the conversion to carbon dioxyde of volatile metabolites formed from lindane transformation by the indigenous microflora.

Table IV. Mass-Balance Analysis of ^{14}C in Soil Cultures after 9-Week Incubations

Treatment	*Fraction* (% of initial radioactivity)				
	CO_2	Extractable	Non-extractable	Volatile	Total
Sterile,non inoculated	0.0	77.7	5.2	n.d.	82.9
Sterile, inoculated	0.7	74.3	6.4	n.d.	81.4
Non-sterile,non inoculated	21.6	9.7	18.4	2.6	52.4
Non-sterile, inoculated	49.1	9.1	14.4	n.d.	72.6

NOTE: n.d.: not detected
SOURCE: Reproduced from reference 39. Copyright 1997 Elsevier.

These results confirm that the bioremediation of soils may be achieved using a bioaugmentation approach with filamentous fungi, established in a synergistic relationship with the indigenous microbial community. Nevertheless, a good efficiency of fungi is expected only when pollutants exhibit sufficient water solubility and bioavailability.

Some of the filamentous fungi are able to degrade an extremely diverse range of persistent or toxic environmental pollutants. That ability is often due to the production of several families of exocellular enzymes, such as peroxidases or laccases. The extracellular systems (well known in white-rot fungi) use non-specific mechanisms, and are regulated by nutrients. Consequently, they can degrade a chemical present at concentrations reduced to levels too low for effective enzyme induction.

Concluding Remarks

P-450s are clearly involved in pesticide metabolism in plants and fungi, but the genes encoding P-450 proteins responsible for this metabolism are only at the initial stage of characterization. It is already clear that genes with redundant functions will be obtained. The isolation and functional characterization of more genes is needed to be able to determine their relative importance for herbicide metabolism and selectivity in different plant species, and their potential for bioremediation. So far, there are only indirect indications that some plant P-450s may metabolize a broad range of herbicides. Only a recent preliminary report seems to confirm this with an isolated recombinant enzyme (*40*). Isolation of the genes of such herbicide metabolizing P-450s is one of the keys for understanding the mechanism(s) of weed cross-resistance, and regulation of herbicide metabolism.

As an alternative to the isolation of existing genes, it is possible to envisage directed or random genetic engineering of already available genes of plants or fungi for specific pesticide or pollutant metabolism. The available genes can also be engineered for improved catalytic activity.

Over-expression of native and modified genes in plants or microorganisms, together with suitable P-450-reductases, should be useful for increasing resistance of susceptible crops, or removal of organic pollutants. Manipulation of P-450 enzymes is thus a promising long-term challenge.

Literature Cited

1. Frear, D. S.; Swanson, H. R.; Tanaka, F. S. *Phytochemistry* **1969**, *8*, 2157-2169.
2. Hasemann, C. A.; Kurumbail, R. G., Boddupalli, S. S.; Peterson, J. A.; Deisenhofer, J. *J. Current Biol.* **1995**, *2*, 41-62.
3. Durst, F.; Nelson D. R. *Drug Metab. Drug Interact*. **1995**, *12*, 189-206.
4. Halkier, B. A. *Phytochemistry* **1996**, *43*, 1-21.
5. Mansuy, D. *Comp. Biochem. Physiol. C.* **1998**, *121*, 5-14.
6. Schuler, M. A. *Crit. Rev. Plant. Sci.* **1996**, *15*, 235-284.
7. URL http//drnelson.utmem.edu/homepage.html.
8. Maughan, J. A.; Nugent, J. H. A.; Clark, I. M.; Westlake, A. C. G.; Hallahan, D. L. *Brighton Crop Prot. Conf. Weeds*; BCPC: Farnham, UK, 1997; Vol. 2, *7A-4*, 597-604.
9. Chapple, C. *Annu. Rev. Plant Physiol. Mol Biol*. **1998**, *49*, 311-343.
10. Sariaslani, F. S. *Adv. Appl. Microbiol*. **1991**, *36*, 133-178.
11. Vanden Bossche, H.; Koymans, L. *Mycoses* **1998**, *41*, 32-38.
12. Van den Brink, H. J. M.; van Gorcom, R. F. M.; van den Hondel A. M. J. J.; Punt, P. J. *Fungal Gen. Biol*. **1998**, *23*, 1-17.
13. O'Keefe, D. P., *Drug Metab. Drug Interact*. **1995**, *113*, 755-763.
14. Blee, E.; Joyard, J. *Plant Physiol*. **1996**, *110*, 445-454.
15. Werck-Reichhart, D. *Proc. Brighton Crop Prot. Conf. Weeds*; BCPC: Farnham, UK, 1995; Vol. 3, *7B-1*, 813-821.

16. Barrett, M. *Drug Metab. Drug Interact*. **1995**, *12*, 299-316.
17. Frear, S. *Drug Metab. Drug Interact.* **1995**, *12*, 329-358.
18. Gaillardon, P.; Cabanne, F.; Scalla, R.; Durst, F. *Weed Res*. **1985**, *25*, 397-402.
19. Cabanne, F.; Huby, D.; Gaillardon, P.; Scalla R.; Durst, F. *Pestic. Biochem. Physiol*. **1987**, *28*, 371-380.
20. Gonneau, M.; Pasquette, B.; Cabanne, F.; Scalla, R. *Weed Res*. **1988**, *28*, 19-25.
21. Canivenc, M.-C.; Cagnac, B.; Cabanne, F.; Scalla, R. *Plant Physiol. Biochem*. **1989**, *27*, 1-10.
22. Fonné, R. Ph.D. thesis, University Louis Pasteur, Strasbourg, France, 1985.
23. Mougin, C.; Cabanne F.; Canivenc, M.-C.; Scalla, R. *Plant Sci*. **1990**, *66*, 195-203.
24. Mougin, C.; Polge, N.; Scalla, R.; Cabanne, F. *Pestic. Biochem. Physiol*. **1991**, *40*, 1-11.
25. Mougin, C.; Cabanne, F.; Scalla, R. *Plant Physiol. Biochem*. **1992**, *30*, 769-778.
26. Helvig, C.; Tardif, F. J.; Seyer, A.; Powles, S. B.; Mioskowski, C.; Durst, F.; Salaün, J. P. *Pestic. Biochem. Physiol*. **1996**, *54*, 161-171.
27. Shiota, N.; Inui, H.; Okhawa, H. *Pesticide Biochem. Physiol*. **1996**, *54*, 190-198.
28. Robineau, T.; Batard, Y.; Nedelkina, S.; Cabello-Hurtado, F.; LeRet, M.; Sorokine, O.; Didierjean, L.; Werck-Reichhart, D. *Plant Physiol*. **1998**, *118*, 1049-1056.
29. Siminsky, B.; Corbin, F. T.; Ward, E. R.; Fleischmann T. J.; Dewey, R. E. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 1750-1755.
30. Kemp, M. S.; Moss, S. R.; Thomas, T. H. In *Managing resistance to agrochemicals*; Green, M. B., Le Baron, H. M., Moberg, W. K., Eds.; ACS Symposium Series 421; ACS: Washington, DC, 1990, pp 377-393.
31. Preston, C.; Tardif, F. J.; Christopher, J. T.; Powles, S. B. *Pestic. Biochem. Physiol*. **1996**, *54*, 123-134..
32. Menedez, J.; De Prado, R. *Physiol. Plant*. **1997**, *99*, 97-104.
33. Corio-Costet, M.-F.; Benveniste, P. *Pestic. Sci*. **1988**, *22*, 343-357.
34. Corio-Costet, M.-F.; El Achouri, M.; Lanot, R.; Charlet, M.; Benveniste, P.; Hoffmann, J. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 643-647.
35. Omura, T.; Sato, R. *J. Biol. Chem*. **1964**, *239*, 2370-2385.
36. Franklin, M. R. *Pharmacol. Ther. A* **1977**, *2*, 227-245.
37. Mougin, C.; Laugero, C.; Asther, M.; Chaplain, V. *Pestic. Sci*. **1997**, *49*, 169-177.
38. Mougin, C.; Pericaud, C.; Malosse, C.; Laugero, C.; Asther, M. *Pestic. Sci*. **1996**, *47*, 51-59.
39. Mougin, C.; Pericaud, C.; Dubroca, J.; Asther, M. *Soil Biol. Biochem*. **1997**, *29*, 9-10.
40. Barrett, M.; Avdiushko, S.; Young, A.; Polge, N.; Poneleit, C. *WSSA Abstracts* **1999**, *39*, 249.