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White-rots, chlorine and the environment – a tale of many twists

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ABSTRACT

White-rot fungi possess a unique oxidative mechanism by which the recalcitrant lignin component of wood is mineralised. The activity of lignin-degrading enzymes, chiefly lignin and manganese peroxidases, depends on several small organic molecules. Some of these (e.g. chloroanisyl alcohols) are chloroaromatics and may act as environmental pollutants in the forest soil, whereas the synthesis of others (e.g. veratryl alcohol) requires chloromethane. Certain white-rot genera, notably *Phellinus* and *Inonotus*, release excess quantities of chloromethane into the atmosphere where it acts as a greenhouse gas. On the other hand, their powerful ligninolytic system enables white-rot fungi to degrade a wide range of man-made environmental pollutants, including recalcitrant chloroaromatics such as DDT, PCP, 2,4-D and 2,4,5-T. This review describes the multifarious interactions of white-rot fungi with their environment *via* the chlorine cycle.

1. White-rots and brown-rots

Wood is formed when the protoplasts of plant cells, initially separated by the middle lamella and the primary wall, lay down a thick secondary wall and eventually die. The main polymers of both the middle lamella and the primary wall – pectin and cellulose, respectively – consist of chains of repeated sugar units which are assembled in a regular fashion by means of specific enzymes (Fig. 1). A further important cell wall polymer, hemicellulose, consists of a number of different sugar moieties, but these, like pectin and cellulose, are linked in a more or less regular way which renders them accessible to cleavage by hydrolytic enzymes. Further, the sugars, once released, are readily assimilated and utilised by most living organisms. In contrast, the subunits of lignin, the material making up the secondary wall, consist of phenolic substances which are difficult to degrade. Furthermore, during lignin biosynthesis these recalcitrant building blocks are cross-linked in a random fashion by way of free radical reactions. Lignin therefore has a complex, non-repetitive three-dimensional structure which renders it resistant to attack by hydrolytic enzymes. In fact, the only organisms capable of mineralising lignin into water and carbon dioxide are a select group of basidiomycetes, the so-called white-rot fungi (Fig. 2). However, even white-rots cannot live on lignin alone but require other, more easily utilised carbon sources to sustain lignin degradation which is thus said to be co-metabolic. Other fungi attacking wood, notably brown-rotting and soft-rotting species, are more or less confined to degrading pectin, cellulose and hemicellulose, leaving behind the lignin which assumes a distinctly brown colour especially when oxidised (Figs 2 and 3). Since the structural coherence is lost with the disintegration of the middle lamella and primary walls, wood attacked by brown-rots cracks, crumbles and ultimately becomes incorporated into

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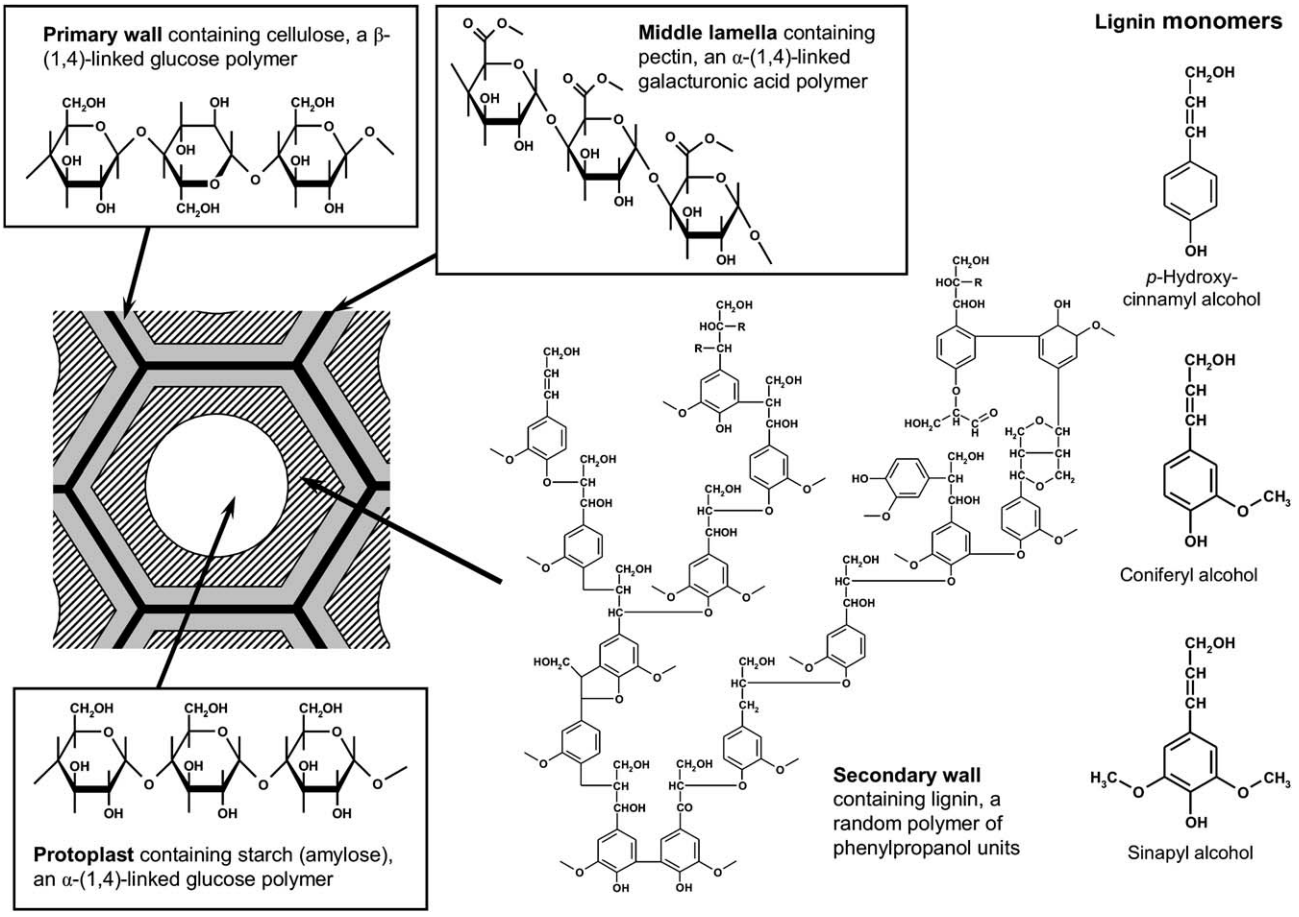

Fig. 1 – Carbon-containing polymers and their localisation in plant tissue. Lignin consists of a complex polymer of the three phenylpropanoid units shown, which may be linked in numerous different combinations. The example drawn here has been modified from Adler (1977). Hemicelluloses, heterogeneous chains made up of glucose, xylose, arabinose and mannose, are localised in the primary and secondary cell walls (not shown). Reprinted from Webster and Weber (in press), with permission by Cambridge University Press.

humus. Although the methods by which brown-rot fungi decay wood are in themselves remarkable (see Green & Highley 1997), the present review will focus on white-rots because of the unsuspected and potentially far-reaching impact of their activities on the environment. These are directly related to the unique mechanism of white-rot attack on lignin by means of oxidative enzymes.

Fig. 2 – Beech wood colonised by the white-rot fungus *Trametes hirsuta* (left) and by an unidentified brown-rot fungus (right).

2. The mechanism of lignin degradation

Lignin is degraded by way of free radical intermediates similar to those involved in its synthesis. In order to avoid a futile depolymerisation-repolymerisation cycle, the concerted action of several enzymes is required to break down lignin. Much of the initial work on lignin degradation, including the discovery of the major lignin-degrading enzymes and development of theories on their mode of action, was carried out with *Phanerochaete chrysosporium*, the most thoroughly studied of all white-rot fungi (Glenn *et al.* 1983; Tien & Kirk 1984). The initial attack on lignin is mediated by lignin peroxidases (LiP) or manganese peroxidases (MnP), and either or both of them are produced by most white-rots (de Jong *et al.* 1994a; Heinzkill & Messner 1997). It is unlikely that there is any direct contact between these enzymes and their substrate since the pore size of lignin would be too small to permit the diffusion of

Fig. 3 – Humification of a log of spruce (*Picea abies*) by the brown-rot fungus *Fomitopsis pinicola*.

ligninolytic enzymes into the wood. Further, on theoretical grounds it is difficult to conceive how the reaction centre of any enzyme could spatially accommodate the highly variable structure of lignin (see Fig. 1). In fact, there is now good evidence that small diffusible molecules act as redox charge carriers between the peroxidase enzymes and their final substrate.

A consensus reaction cycle of LiP is summarised in Fig. 5, based on several reviews (de Jong *et al.* 1994a; Heinzkill & Messner 1997; ten Have & Teunissen 2001). The enzyme consists of a single glycosylated polypeptide (38-43 kDa) and a prosthetic group (iron protoporphyrin IX) which is embedded deep within the polypeptide chain, accessible only through a narrow pore (Piontek *et al.* 2001). LiP becomes highly oxidised by losing two electrons when hydrogen peroxide (H_2O_2) is reduced to water. The enzyme returns from this highly oxidised state (LiP I) to the ground state by two steps, gaining one electron in each step by the oxidation of a reductant (e.g. veratryl alcohol) into a cation radical. The two veratryl alcohol cation radicals thus formed leave the enzyme and may oxidise lignin directly or pass on their charge to other molecules acting as redox charge carriers. Either way, veratryl alcohol returns to its reduced ground state and is thus recycled. Veratryl alcohol is produced in abundance by most white-rot fungi (de Jong *et al.* 1994a), underlining its important function as an initial substrate of lignin peroxidases.

The conversion of LiP to LiP I requires an H_2O_2-producing enzyme system. Several such systems exist, with the physiologically most important ones likely to be the extracellular enzymes glucose oxidase, glyoxal oxidase and aryl alcohol oxidase (de Jong *et al.* 1994a). The latter system is of relevance in the present context because it uses chlorinated anisyl alcohols as substrates, and these are potential environmental pollutants (see below).

Manganese peroxidase (MnP) is closely related to LiP in terms of protein structure and sequence (Canales *et al.* 1998) and also in its general catalytic cycle (see Fig. 5), except that the reductant is Mn^{2+} instead of veratryl alcohol. The oxidised redox charge carrier (Mn^{3+}) is stabilised by certain organic acids such as malonate or oxalate, and these are thought to mediate its diffusion from the enzyme to the substrate where a one-electron oxidation of lignin converts Mn^{3+} back to Mn^{2+} (de Jong *et al.* 1994a). As with LiP, there may be further diffusible molecules carrying the redox charge from the enzyme to the lignin substrate. Those white-rots which produce both MnP and LiP often show differential production under various environmental conditions; for example, in *P. chrysosporium* the transcription of MnP is stimulated by the presence of manganese whereas that of LiP may be suppressed (Perez & Jeffries 1992). Both enzymes are usually produced as a range of related isoforms, especially under conditions of carbon starvation (Leisola *et al.* 1987; Kirk & Cullen 1998). An unusual 'hybrid' peroxidase has been isolated from *Bjerkandera* (Mester & Field 1998); this is capable of oxidising both Mn^{2+} and veratryl alcohol, probably using the same catalytic centre.

A third group of enzymes capable of attacking lignin are the laccases, ubiquitous copper-containing glycoproteins which are larger than the peroxidases (60-80 kDa). Laccases perform four one-electron oxidations by reducing O_2 to H_2O. They can attack phenolic substrates directly and may therefore be involved in the breakdown of smaller lignin fragments. However, they can also oxidise mediator molecules which may then diffuse into lignin and oxidise it. In fact, lignin breakdown products themselves may act as such redox charge transfer molecules (ten Have & Teunissen 2001). Although the precise role of laccases in lignin degradation is still unknown, they most probably have one (see Eggert *et al.* 1997), and they are produced by most if not all white-rot fungi.

3. Chloromethane production by white-rot fungi

Methylation, e.g. the conversion of a hydroxyl (-OH) group into a methoxy ($-OCH_3$) group, is frequently performed as a late biosynthetic step by fungi as well as many other organisms. We have already encountered examples of such *O*-methylated metabolites in the shape of chloroanisyl alcohols and veratryl alcohol (see Fig. 5). The most widespread donor of methyl groups in biological systems is *S*-adenosylmethionine (SAM). Interestingly, chloromethane (CH_3Cl) may also act as a methyl donor and appears to be crucial in the biosynthesis of veratryl alcohol (Harper *et al.* 1990) as well as methyl esters of a range or organic acids (Fig. 6). It can be speculated that chloromethane is used for methylations of these very common metabolites because its production is less expensive in energetic terms than that of SAM (Harper 2000). Although chloromethane is now known to be produced by several white-rot fungi, its presence initially passed unnoticed because in most species its production is so tightly coupled with its consumption that detectable quantities of chloromethane are not released by laboratory cultures. Excessive chloromethane production was, however, observed in members of the Hymenochaetaceae which may volatilise a substantial proportion of the chloride ion pool present in their substrate (Harper *et al.* 1988). A particularly striking case is *Phellinus pomaceus* (syn. *P. tuberculosus*), common on rosaceous trees (Fig. 4), which may release over 90 % of chloride ions from the substrate as chloromethane (Harper 1985). This species possesses a uniquely efficient methyl chloride transferase system (Saxena *et al.* 1998).

Fig. 4 – Dead branch of an old plum tree colonised by the white-rot *Phellinus pomaceus*, the main fungal contributor to atmospheric chloromethane pollution.

With about 5 million tonnes released annually worldwide, chloromethane is the most abundant halocarbon gas in the outer atmosphere (stratosphere) and contributes significantly to the destruction of the ozone layer. Unlike other greenhouse gases such as the chlorofluorocarbons (CFCs), chloromethane appears to originate mainly from natural rather than human activities, even though the relative importance of these natural sources is only just beginning to be understood. According to recent estimates summarised by Keppler *et al.* (2005), up to 2.5 million tonnes of chloromethane *per annum* may be released by complex reactions of chloride with the methyl groups of plant cell wall constituents such as pectin and, to a lesser extent, lignin (see Fig. 1). Pectin and lignin demethylation may take place during the burning of biomass and as biotic and abiotic decomposition processes in the soil (Hamilton *et al.* 2003). Salt marshes are a further significant source of chloromethane emission (Rhew *et al.* 2000). The fungal chloromethane contribution has been estimated at around 150,000 tonnes *per annum* (Watling & Harper 1998). This may appear low in comparison but it is nonetheless remarkable in apparently being due almost entirely to activities by the white-rot genera *Phellinus* and, to a lesser extent, *Inonotus* (Watling & Harper 1998). It will be interesting to see in which way the abiotic and biotic mechanisms of chloromethane release respond to climatic change.

4. Chloroaromatic substances from white-rot fungi

Among non-gaseous chlorinated metabolites, the most ubiquitous and ecologically significant compounds are probably

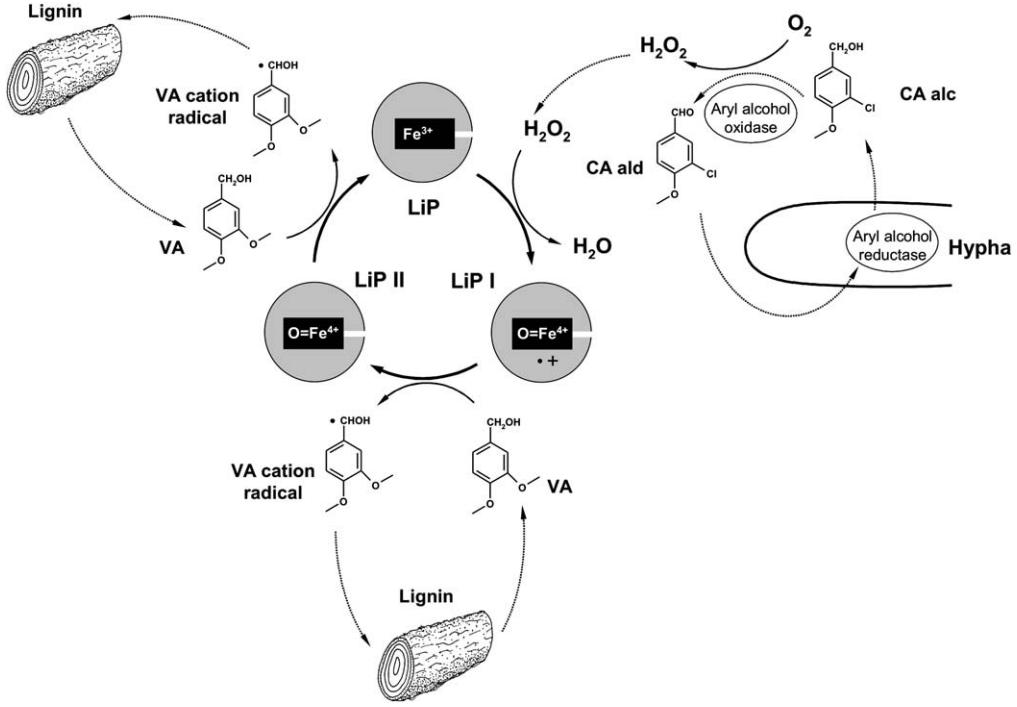

Fig. 5 – Reaction cycle of lignin peroxidase. The reduction of H_2O_2 to H_2O withdraws two electrons from the ground-state enzyme (LiP), one from the ferric ion to give the ferryl ion (Fe^{4+}) and the other from the protoporphyrin group itself which is converted to the porphyrin cation radical. This results in the highly oxidised LiP I state which catalyses two separate one-electron oxidations of a reductant, e.g. veratryl alcohol (VA), becoming reduced *via* the LiP II state to the ground state. The VA cation radical is regenerated when it withdraws one electron from lignin itself or from other molecules serving as redox charge carriers (not shown). H_2O_2 can be generated by several means, including the oxidation of 3-chloro-anisylalcohol (CA alc) to give its corresponding aldehyde (CA ald). Reprinted from Webster and Weber (in press), with permission by Cambridge University Press.

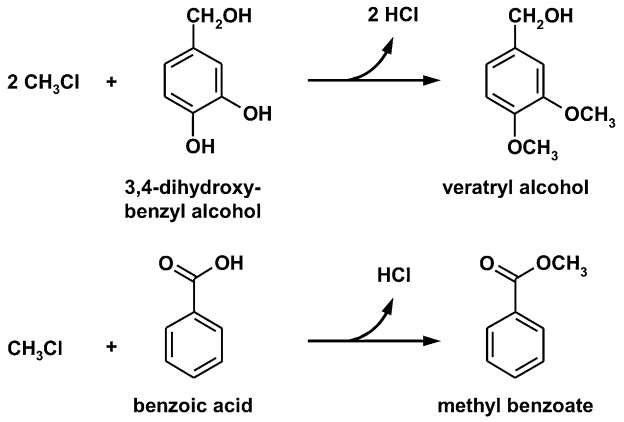

Fig. 6 – The physiological role of chloromethane as a methyl donor in methylations of hydroxy (top) and carboxy groups (bottom). Based on Harper (2000).

those involved in generating H_2O_2 for the activation of LiP and MnP (see Fig. 5), *i.e.* anisyl alcohols/aldehydes and similar chloroaromatics. These substances are produced by many basidiomycetes in substantial amounts (Hautzel & Anke 1990; Pfefferle *et al.* 1990; de Jong & Field 1997). In laboratory cultures, concentrations of over 100 mg l^{-1} are not uncommon, and production often coincides with the expression of ligninolytic enzymes (Teunissen 1999). In line with their function in lignin degradation, chlorinated anisyl metabolites can be detected in rotting wood and forest litter especially in the vicinity of fruit-bodies e.g. of *Hypholoma*, *Pholiota*, *Bjerkandera* and *Lepista* spp. (de Jong *et al.* 1994b). *Hypholoma* spp. excrete 3,5-dichloroanisyl alcohol and its corresponding aldehyde into woody substrates at concentrations up to 180 mg kg^{-1} (dry weight), and forest litter in the vicinity of *Lepista nuda* may contain up to 73 mg kg^{-1} of these compounds. Total concentrations of adsorbable organohalogens (AOX) may be as high as 350 mg organic chlorine kg^{-1} soil (Asplund & Grimvall 1991).

These findings have several implications. Most importantly, they have led to the realisation that environmental pollution with organohalogens, traditionally thought to be due principally to human activities, is also an ongoing natural process. Since the AOX concentrations in environmental samples may exceed the regulatory limits for chloroaromatics set by many countries, a strict application of these laws would require even pristine forest soils to be considered polluted habitats in need of remediation! Secondly, low levels of chlorinated anisyl metabolites have been detected in fruit-bodies of basidiomycetes such as *Lepista nuda* (de Jong *et al.* 1994b). This species is considered edible, but the impact of its chlorinated metabolites on human consumption remains to be investigated. Thirdly, 3,5-dichloroanisyl alcohol has been shown to inhibit fungal chitin synthase activity (Pfefferle *et al.* 1990), and the concentrations required for inhibition are in the same order of magnitude as those found in forest soil. Therefore, in addition to being involved in lignin degradation this compound might hold a second benefit to white-rot fungi as a chemical defence of their substrate against other fungi.

5. Biological activities of halogenated secondary metabolites

Chloromethane and compounds involved in H_2O_2 generation for LiP and MnP activity are not the only halogenated fungal metabolites, and neither are white-rot basidiomycetes their only producers. In fact, approximately 200 different halogenated metabolites are known from fungi (de Jong & Field 1997; Gribble 2003), and new ones are being discovered continuously (e.g. Rether *et al.* 2005). To give an extreme example, some *Mollisia* spp. (ascomycetes) produce secondary metabolites in which the number of chlorine atoms exceeds that of carbon atoms (Nakanishi *et al.* 1989).

Chlorine is the most abundant halide in fungal organohalogens. Some brominated compounds are also known, but fluorinated or iodinated metabolites are rather rare. In laboratory cultures, addition of bromide to the medium can induce a shift from chlorination to bromination of secondary metabolites, or may result in metabolites carrying both bromine and chlorine residues (Anke *et al.* 1995; Stadler *et al.* 1995). Many of these compounds exhibit strong antimicrobial, cytotoxic and nematicidal activities, and they may also inhibit chitin or melanin biosynthesis in ascomycetes and streptomycetes (Pfefferle *et al.* 1990; Thines *et al.* 1995).

Although the natural functions of most halogenated metabolites are still unknown, it is interesting to note that a chlorine substitution on an aromatic ring usually enhances the biological activity of the molecule. For example, the mycotoxin ochratoxin A is considerably more toxic than its dechlorinated form, ochratoxin B (Cole & Cox 1981). Analogues of the antifungal strobilurins carrying a chlorine substitution on the aromatic ring possess higher antifungal activities than their unsubstituted parent compounds (Anke *et al.* 1988). Chlorine substituents also greatly enhance the inhibition of chitin synthase by strobilurins, and a similar effect has been described for anisyl alcohol and its 3,5-dichloro derivative (Pfefferle *et al.* 1990). Furthermore, halogenated melleins are more strongly nematicidal than unhalogenated parent compounds (Anke *et al.* 1995). However, in some cases chlorination may have a differential effect on various biological activities (Köpcke *et al.* 1999), or none at all (Eilbert *et al.* 2000).

6. Degradation of chloroaromatics by white-rot fungi

When discussing the potential of white-rot fungi to pollute the environment with chloroaromatics, we should note at least in passing that these substances are also degraded in the soil by a diverse array of micro-organisms and catalytic mechanisms (Harper 2000; Castro 2003). Indeed, even a proportion of the chloromethane released by *Phellinus* and *Inonotus* spp. may be consumed by soil microbes before escaping into the atmosphere (Moore *et al.* 2005). Further, white-rot basidiomycetes themselves have an immense potential as degraders of hazardous substances. Some of the most serious anthropogenic environmental pollutants are chloroaromatics, e.g. the herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T

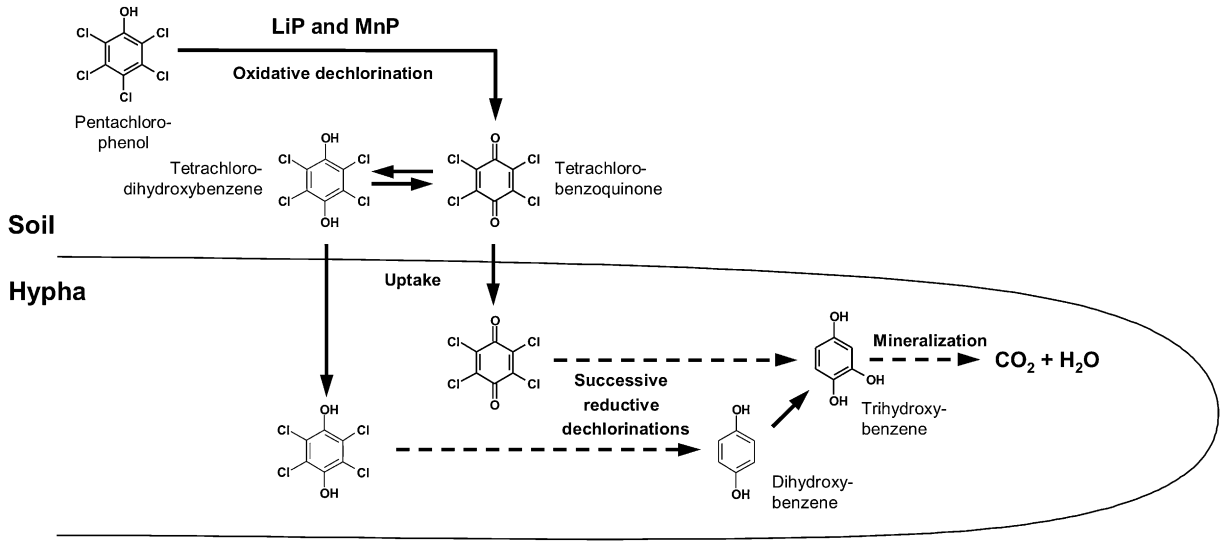

Fig. 7 – Biodegradation of the xenobiotic pentachlorophenol (PCP) by the concerted action of different enzyme systems in the white-rot fungus *Phanerochaete chrysosporium*. Oxidative removal of the first chlorine atom is catalysed by extracellular LiP and/or MnP, followed by uptake of the resulting tetrachloro derivatives and their successive reductive intracellular dechlorination. The resulting trihydroxybenzene ring can then be broken up by an intracellular dioxygenase enzyme, followed by mineralisation. Data from Reddy and Gold (2000).

(2,4,5-trichlorophenoxyacetic acid, 'Agent Orange'), the insecticide DDT (dichloro-diphenyl-trichloromethane), and the wood preservative PCP (pentachlorophenol). LiP, MnP and laccases as well as several non-ligninolytic enzyme systems, alone or in combination, have been implicated in the attack on such xenobiotics which may become biotransformed, mineralised or polymerised (Rabinovich *et al.* 2004; Tortella *et al.* 2005). The issue of bioremediation of environmental pollutants by white-rot fungi is beyond the scope of our review, and we can only give an example here to indicate ways in which various enzyme systems might interact in the degradation of xenobiotics (Kremer *et al.* 1992; Reddy & Gold 2000). In this scheme (Fig. 7), PCP undergoes an initial extracellular oxidative dechlorination mediated by LiP or MnP to give tetrachlorobenzoquinone; this or its conversion product tetrachlorodihydroxybenzene is subjected to successive reductive intracellular dechlorinations until, eventually, trihydroxybenzene is produced as the last aromatic compound prior to mineralisation by ring cleavage and release of CO_2 and H_2O.

7. Conclusions

Attempts at understanding the sources of environmental pollution with chlorine-containing substances have led to the realisation that there are, in fact, numerous natural processes by which chlorine becomes actively and reversibly incorporated into organic molecules. The biochemistry of chlorine seems to be particularly highly evolved in the white-rot fungi which use this element as a methyl donor, as an activating group in antibiotic compounds, and in molecules contributing to the H_2O_2-regenerating system essential for the functioning of ligninolytic peroxidases. These oxidative enzymes in turn make significant contributions to the biochemical versatility for which white-rot fungi are noted, especially concerning the degradation of recalcitrant xenobiotics. The soil is a diverse ecosystem, and exchanges of chloromethane as well as chloroaromatics and chloride must take place between white-rot fungi and other soil-borne micro-organisms. These therefore form part of the worldwide 'chlorine cycle' whose outlines are beginning to take shape.

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