Chapter 11
Fungal Oxidoreductases and Humification in Forest Soils

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11.1 Introduction

Humic substances (HS) are ubiquitous and recalcitrant by-products of dead matter hydrolysis and oxidative biotransformation (humification). Their resistance to biodegradation is both a result of structural complexity due to selective preservation of most stable chemical forms during microbial decay (Orlov 1990) and a result of physicochemical protection by interactions with soil minerals (Mikutta et al. 2006). The residence time of HS in soils is 10^2–10^3 years; they comprise up to 90% of soil organic matter (humus), which is the largest carbon reservoir in the biosphere estimated at 1,462–1,548 Pg of C_{org} in the 0–1 m layer excluding litter and charcoal (Batjes 1996). Humification can be thus considered as a key process in Netto Biome production leading to a long-time sink of atmospheric CO_2. About 1/3 (470 Pg) of world soil organic carbon reserves is captured in boreal forests soils and almost half of this amount (224 Pg C) is accumulated in the soils of Russia (Stolbovoi 2006). A better knowledge of humus turnover processes in forests of cold humid climate will allow better predictions of the global carbon dynamics under changing environment. Synthesis, transformation, and mineralization of HS are largely oxidative processes with wood- and soil-inhabiting fungi being a major driving force due to extracellular production of non-specific oxidative enzymes. In this chapter, we provide an over view of the occurrence of oxidoreductases in wood-decomposing,

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soil-inhabiting and symbiotic fungi and attempt to elucidate the role of certain fungal groups in humus synthesis and transformation in soil.

11.2 The Origin of HSs

Natural HS comprise operationally defined material extracted from soils by alkali and further separated into humic acids insoluble at pH < 2 (HA, molecular weight, MW, 5–100 kDa) and acid-soluble fulvic acids (FA, MW 1–10 kDa). Non-extractable residues that bind tightly to soil minerals is defined as humin. Distinctive features of HS are a N content of 1–3% (FA) or 2–6% (HA), a C content of 40–50% (FA) and 50–60% (HA), an aromatic C content of 25–35%, a total acidity of 6–14 mmol(−)/g (FA) and 5–8 mmol(−)/g (HA) and characteristic infrared and UV-Vis spectra (Orlov 1990). Humus formation in forest soils begins in the litter: 93–94% of fresh litter mass is utilized by microbiota on the soil surface with the release of CO_2 as a final product (Glazovskaya 1996); only 6–7% of initial C input is leached down as soluble products of decay or/and undergoes transformation into HSs. HSs are formed from highly heterogeneous material comprising dead matter: modified lignin, polyphenols, melanins, chitin, aliphatic compounds (lipids, waxes), carbohydrates, amino acids, proteins, etc. In forest ecosystems lignin serves as the main source of humus precursors due to its quantitative abundance in plant tissues. By contrast, in tundra soils polyphenols, chitin, and melanins from lignin-free lichens and mosses can be important starting material of HS. It is generally recognized that two major humification pathways co-exist in soils (reviewed by Stevenson 1994). (1) Oxidative biodegradation theory (Waksman 1931; Alexandrova 1980) postulates that initial polymeric material (e.g., lignin) is only partially modified by oxidases yielding HA, which can be then oxidized and depolymerized to FA. Changes in lignin include loss of OCH_3 groups with formation of hydroxyphenols and oxidation of aliphatic side chains to form COOH groups. Further oxidation of phenolic groups to semiquinones and quinones enables incorporation of nitrogenous and other compounds into HS structure via free-radical reactions. (2) Polyphenol theory postulates that low MW phenolic aldehydes and acids released during lignin breakdown (or from other sources) are oxidized to reactive semiquinones and quinones and undergo polymerization in presence of nitrogenous compounds and other soluble precursors. First FA are formed and then HA (Kononova 1966; Flaig 1966). The first pathway should predominate in poorly drained soils and peats, while second pathway should be more typical for mineral horizons of well-aerated forest soils, where soluble polyphenols in leachates from litter are main humus precursors. Irrespective of how they are formed the resulting product (HS) are a polydisperse mixture of N-containing molecules composed of substituted aromatic rings, heterocycles, and aliphatic side chains connected by a variety of linkages and bearing functional groups among which carboxylic and phenolic are most abundant (Stevenson 1994). Differentiation of HS from other alkali-soluble compounds (e.g., lignins and melanins) in the soil extracts is quite problematic. Based on NMR signals in HA extracted from

peat and soil A_h horizon, Kelleher and Simpson (2006) concluded that the vast majority of humic material is a complex mixture of microbial and plant biopolymers present in soil at the time of extraction. This finding does not rule out the formation of HS closely related to the parent biopolymers or existence of distinct chemical categories of HS. Nevertheless, the presence of non-humic matter in HS preparations is highly possible, especially when HS from organic sources (litter, peat, coal) are under investigation.

11.3 Major Fungal Oxidative Enzymes

The major fungal redox enzymes, involved in oxidative transformation of plant debris, are lignin peroxidase (LiP), Mn-dependent peroxidase (MnP), versatile peroxidase (VP), other peroxidases, laccase, and tyrosinase (Table 11.1). The catalytic action of these enzymes can be divided into two principal stages: (1) an enzymatic substrate oxidation with formation of diffusible, reactive intermediates – phenoxy radicals or quinones from phenolic units, aryl radicals from non-phenolic units; (2) non-enzymatic spontaneous reactions of free radicals, initiating substrate transformation. Two opposite processes may occur: substrate polymerization via radical cross-coupling or substrate degradation via bond cleavage, aromatic ring opening, demethylation, demethoxylation, substituents release, etc. The post-enzymatic step largely extends the substrate range of the enzymes.

11.3.1 Peroxidases

Peroxidases catalyze one-electron substrate oxidation by H_2O_2 with formation of free-radical cation intermediates and H_2O. Fungal peroxidases are quite similar in the structure of their active center and catalytic cycle to plant peroxidases. Enzyme

Table 11.1 Comparative characteristic of fungal phenol oxidases and ligninolytic peroxidases

Property	LiP	VP	MnP	Peroxidase	Laccase	Tyrosinase
Redox potential	1.2–1.5 V	–	~1.1 V	~1.0 V	0.7–0.9 V	0.26–0.35 V
pH optimum	2.5–3.5	–	4.0–4.5	~5.5	4.0–5.0 5.0–6.0	6.0–7.0
pI	3.2–4.0	3.5	~4.5	~3.5	~4.0	4.5–8.5
MW, kDa	38–46	45	38–50	40–45	40–70	30–50
Active center	Fe-protoporphirin IX				4 Cu atoms	2 Cu atoms
Substrate transformation	Depolymerization, mineralization					
				Polymerization		
Main producers	White-rot basidiomycetes					
			Litter-decomposing basidiomycetes, ectomicorrhizae			
				Ascomycetes, lichens		

Literature used: Fakoussa and Hofrichter (1999), Wong (2008), Hammel and Cullen (2008), Mester and Field (1998), Thurston (1994), Baldrian (2006), Makino et al. (1974)

molecules contain a prosthetic heme group-Fe(III) protoporphyrin IX (active center) and two Ca^{2+} ions supporting native structure of the enzyme. The catalytic cycle includes $2e^-$ oxidation of Fe(III) protoporphyrin IX by H_2O_2 to give the radical cation intermediate (compound I) and then two consecutive $1e^-$ reductions of compound I by reducing substrate to give compound II ($1e^-$ reduced enzyme) and the resting enzyme (Welinder 1992).

Lignin-peroxidase (EC 1.11.1.14, diarylpropane:oxygen, hydrogen peroxide oxidoreductase) is unique in its ability to directly oxidize the non-phenolic structures in lignin, which comprise up to 90% of the polymer and have high redox potential (>1.5 V). The major route is *β-O*-4 or C_α–C_β cleavage in the propyl side chain to give benzaldehydes. The enzyme also oxidizes phenolic substrates and aromatic amines to phenoxy radicals. LiP has a specific acidic pH optimum (Table 11.1) and is quite rare and unstable enzyme. The "classic" producer is *Phanerochaete crysosporium* (Tien and Kirk 1983), the enzyme was also described in few other white-rot fungi including *Trametes versicolor*, *Phlebia radiata*, *Bjerkandera adusta*, and *Nematoloma frowardii* (Morgenstern 2008). Expression of LiP in other groups of fungi seems to be limited.

Mn-dependent peroxidase (EC 1.11.1.13, Mn(II):hydrogen peroxide oxidoreductase) catalyses oxidation of Mn^{2+} to Mn^{3+}, which is stabilized by bidentate chelators like oxalate, malonate, tartrate, or lactate and acts as non-specific diffusible oxidant of a variety of phenolic compounds and aromatic amines via phenoxy radical intermediates. MnP is produced almost exclusively by basidiomycetes (Hofrichter 2002).

Versatile peroxidase (VP, EC 1.11.1.16) is a hybrid peroxidase that combines catalytic properties of MnP, LiP, and plant peroxidases and has molecular properties similar to MnP (Camarero et al. 1999). The enzyme catalyses the oxidation of Mn^{2+} to Mn^{3+} resembling MnP; it also oxidizes phenolic substrates and aromatic amines in the absence of Mn^{2+} like plant peroxidases; some VPs (e.g., that of *Pleurotus eryngii*) oxidize non-phenolic compounds like LiP due to presence of an invariant tryptophan residue required for long-range e^- transfer from aromatic donors (Perez-Boada et al. 2005). Production is known in few species of white-rot fungi, e.g., *Bjerkandera* spp., *Pleurotus* spp. (Heinfling et al. 1998), and *Panus tigrinus* (Lisov et al. 2003).

Peroxidase (EC 1.11.1.7) has substrate specificity similar to plant peroxidases and laccase. The enzyme oxidizes variety of phenolic compounds via phenoxy radicals. Production is known in different fungal groups including white rots *Pleurotus ostreatus* (Shin et al. 1997) and *Junghuhnia separabilima* (Vares et al. 1992), litter basidiomycetes from the family *Coprinaceae* (Heinzkill et al. 1998), and deuteromycetes (e.g., *Arthromyces ramosus*, Nakayama and Amachi 1999).

11.3.2 Laccase and Tyrosinase

Laccase and tyrosinase are multicopper phenol oxidases that catalyze oxidation of electron-donor substrates by O_2 with formation of free radicals and reduction of O_2

to H_2O (Solomon et al. 1996). Catalytic cycle of laccase contains several $1e^-$ transfers between the 4 Cu atoms. The T1 "blue" Cu site accepts the electrons from the reducing substrate (four $1e^-$ oxidations) and shuttle them to T2/T3 sites where two $2e^-$ reductions of O2 to H_2O occur. So-called yellow laccases have a modified T1 site and thus lack blue color (Leontievsky et al. 1997a). Tyrosinase has a diamagnetic spin-coupled Cu pair in the active centre. Resting enzyme is a met-form that accepts $2e^-$ from diphenol to give quinone and reduced deoxy-form. Deoxy-form reacts with O_2 to give oxy-form, which is a key intermediate, reacting with a monophenol or diphenol (Sanchez-Ferrer et al. 1995).

Laccase (EC 1.11.1.14, benzendiol: oxygen oxidoreductase) preferable substrates are substituted phenols and aromatic amines which are oxidized to semiquinones. These reactive species can be further oxidized to quinones and/or polymerize to form (in)soluble complexes. Alternatively, the semiquinone may react with O_2 to yield superoxide radical that initiate depolymerization processes in a similar way to MnP via alkyl-phenyl and C_α–C_β cleavage of phenolic oligomers (Guillen et al. 2000). Laccases can also degrade non-phenolic lignin model compounds either directly (yellow laccases; Leontievsky et al. 1997a) or in presence of natural or synthetic redox mediators (Eggert et al. 1996). Laccase is almost ubiquitous in white-rot and litter-decomposing basidiomycetes, widespread in ascomycetes and deuteromycetes (Baldrian 2006) and was found in some taxa of micorrhizal fungi (Burke and Cairney 2002) and lichens (Laufer et al. 2006a; Zavarzina and Zavarzin 2006).

Tyrosinase (EC 1.14.18.1, monophenol, o-diphenol: oxygen oxidoreductase) oxidizes some mono- and diphenols but substitution in the aromatic ring decreases enzyme activity. The enzyme catalyses two concomitant reactions: o-hydroxylation of monophenols yielding o-diphenols (monophenolase or cresolase activity) and $2e^-$ oxidation of o-diphenols to o-quinones (diphenolase or catecholase activity). Highly reactive quinones undergo spontaneous coupling to form mixed melanins and heterogeneous polymers (Selinheimo et al. 2007). Tyrosinases were found in basidiomycetes such as *Neurospora crassa*, *Agaricus bisporus*, *Trametes* spp, *Pycnoporus* spp, ascomycetes e.g., *Aspergillus* spp, *Trichoderma* spp (Selinheimo et al. 2007), in micorrhizal fungi (Burke and Cairney 2002), and lichens (Laufer et al. 2006b; Zavarzina and Zavarzin 2006).

11.4 Humification Activities of Fungi in Wood and Soil

HSs are formed in soils by sequential degradative and synthetic processes. The extents to which wood, soil and litter-decomposing fungi participate in each of these processes depend on their enzymes production patterns. Fungal phenol oxidases differ largely by the redox potential and hence by the oxidative power (Table 11.1). High redox potential ligninolytic peroxidases (LiP, MnP, VP) preferentially catalyze degradation of lignin and polymeric phenolic substrates to CO_2 and small soluble fragments (Leonowicz et al. 1999). Non-specific peroxidases and

laccase can cause both degradation/polymerization reactions of polyphenols depending on initial substrate MW and environmental conditions. Depolymerization is favored at acidic pH, high oxygen supply, and if initial enzyme substrate is polymeric (Yaropolov et al. 1994; Rabinovich et al. 2004). Low redox potential of tyrosinase suggests that this enzyme does not participate in degradative processes and is involved exclusively in polymerization (Ghosh and Mukherjee 1998). It can be therefore suggested that humification activities of fungi possessing ligninolytic peroxidases (white-rot and litter-decomposing basidiomycetes) should be associated mainly with soluble precursor and FA production via degradation of lignin and humic acids. Fungi producing peroxidases, neutral laccases, and tyrosinases (ascomycetes) should be responsible for synthesis of humic acids.

11.4.1 Wood Decomposers

Wood decomposers do not directly participate in humification processes in soils; however, soluble degradation products (FA-like compounds) can be leached down to the soil with atmospheric depositions, while insoluble products (HA-like compounds) can enter soil at the final stages of wood decay. The initial fungal substrate in wood is lignocellulose (Fig 11.1a). It is generally recognized that enzymes of wood-decomposing basidiomycetes (LiP, MnP, laccase) are too large to diffuse through sound wood and that small non-proteinaceous species initiate the decay (Hammel et al. 2002). These include ·OH radicals, superoxide radicals (O^{2-}), and ferryl ions (Fe^{4+}), derived from Fenton reaction (brown-rot decay); veratryl alcohol cation radical, oxalic acid, or Mn^{3+} (white-rot decay).

11.4.1.1 Soft-rot Ascomycetes: Production of Large Soluble Fragments

Soft-rot ascomycetes are active in the outer layers of wood, altering its mechanical properties and causing wood "softening," resulting in spongy texture of the wood surface (Schwarze 2007). Xylariaceaeous ascomycetes, e.g., *Daldinia*, *Hypoxylon*, *Kretzschmaria*, and *Xylaria* are found in standing trees and rotting wood (Schwarze 2007), while microscopic fungi grow on moist wood in contact with soil (see Sect. 5.2). Although ascomycetes preferentially decompose cellulose and hemicellulose, some species have the ability to partially degrade lignin due to laccase activity (type II soft rot). For instance, *Xylaria* spp selectively delignified litter producing bleached areas on fallen leaves (Osono 2007). Unlike the white-rot fungi, which mainly produce low MW decomposition products, wood-colonizing ascomycetes can produce large water-soluble lignocellulose fragments which can serve as HS precursors in soil. Formation of soluble products with MW of 3, 30, and 200 kDa as a result of bond cleavage between lignin and hemicellulose by hydrolytic enzymes was shown for *Xylaria polymorpha* growing on beech wood (Liers et al. 2006). The same fungus produced water- and dioxin-insoluble products from synthetic lignin in presence of laccase. As soft-rot fungi typically start the fungal

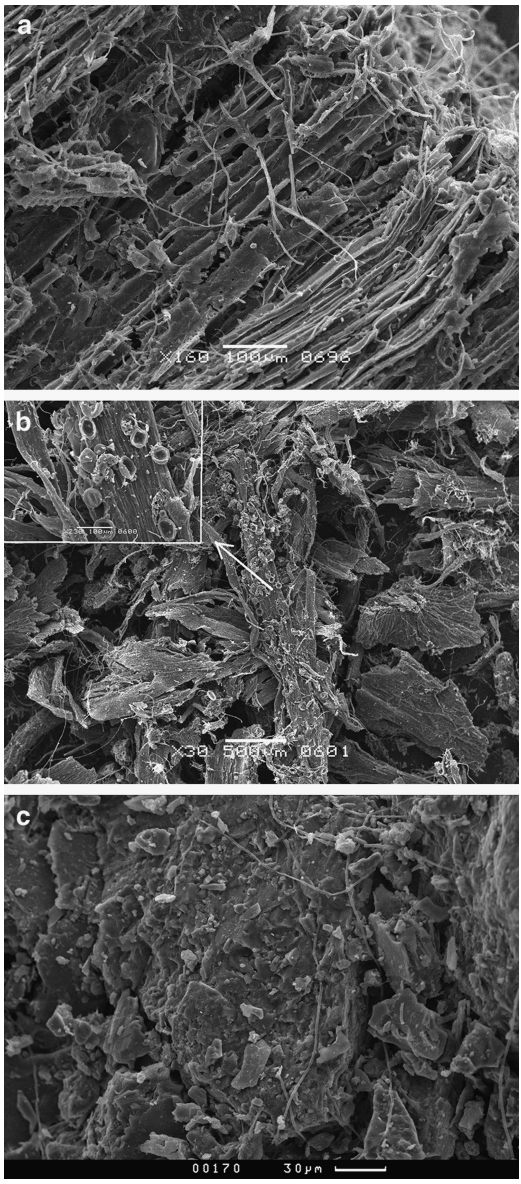

Fig. 11.1 SEM images of decomposing aspen wood (**a**), leaf litter (**b**), and mineral soil particles (**c**) showing difference in organic matter physical state in soil and demonstrating colonization of wood, litter, and soil by fungi (photo by Dr. A.M. Kuznetsova, Biological Faculty, Moscow State University)

succession on moist decaying wood (Rabinovich et al. 2001); they can be considered as "pioneer" humification agents, responsible for release of soluble HA precursors (hydrolytic activity) and their re-polymerization by laccase into HA-like products.

11.4.1.2 Brown-rot Fungi: Formation of Humic Acids from Partially Oxidized Lignin

Brown-rot fungi comprise about 6% of all described wood decay fungi and most of them belong to the *Polyporaceae*. They are predominantly associated with conifers (gymnospermous species) and distributed mostly in northern and southern temperate regions (Schwarze 2007). Representatives comprise *Poria placenta*, *Gloeophyllum trabeum*, and *Coniophora puteana*. Brown-rot fungi effectively depolymerize and metabolize cellulose and hemicelluloses, without altering lignin significantly. Lignin is only partially oxidized, giving the decayed wood the characteristic reddish brown color. The hydroxyl radicals generated by Fenton system ($Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + \cdot OH + H_2O$) are considered to initiate destructive process (Goodell 2003). The ·OH radicals are unable to catalyse cleavage of β-1 and β-*O*-4 bonds in lignin and degradation is limited to demethylation, demethoxylation, aromatic ring hydroxylation, oxidation of initially formed catechol groups, and side-chain oxidation. Participation of lignin-degrading enzymes in oxidative process remains poorly understood. A few reports indicate the presence of extracellular laccase (Lee et al. 2004), LiP (Dey et al. 1991), and MnP (Szklarz et al. 1989) in brown-rots. Intracellular laccase released during hyphae autolysis can cause slight decrease (up to 10%) in lignin content (Rabinovich et al 2004). However the role of laccase in brown-rot decay needs further clarification. The brown-colored modified lignin is enriched in phenolic and carboxylic groups and depleted in methoxyl groups (Kirk 1975), thus approaching HS by the functional groups content and physicochemical properties. High MW HA, formed from polymeric oxidized lignin, are accumulated in decayed wood; absence of destructive ligninolytic peroxidases favors this process (Rypacek and Rypackova 1975). Humified products of decay can easily enter the soil since cubical remnants of brown-rotted wood are abundant on and in the forest floor.

11.4.1.3 White-rot Fungi: Production of Small Soluble Polyphenols (Structural Units) and FAs

White-rot fungi are unique in their ability to completely degrade lignin. They are more frequently found on angiosperm than on gymnosperm wood and can cause simultaneous destruction of lignin, cellulose, and hemicellulose (*Trametes versicolor*, *Phanerochaete chrysosporium*, and *Phlebia radiata*) or selective delignification (*Pleurotus spp*.) giving rise to cellulose-enriched white wood material (Schwarze 2007). The ligninolytic enzyme system consists mainly of MnP, LiP, and laccase. Enzyme production patterns differ between the species. Many white-rot fungi produce either all of the three enzymes (*Nematoloma frowardii*, *Trametes versicolor*) or a combination of any two from them, e.g., LiP and MnP (*Phanerochaete chrysosporium*), MnP (VP), and laccase (*Panus tigrinus*) (Hatakka 1994). In few species (e.g., *Picnoporus cynnabarinus*), solely laccase is produced (Eggert et al. 1996). Production of ligninolytic peroxidases is triggered by starvation in N, C, or S resulting in

switching of fungus to secondary metabolism and idiophasic growth. Laccase is mostly a substrate-inducible enzyme, although constitutive expression of laccase can occur (e.g., Koroleva-Skorobogat'ko et al. 1998).

Degradation of lignin and related compounds by white-rot fungi is cometabolic event and occurs only in presence of easily metabolizable carbon source, e.g., glucose. White-rot fungi typically decompose lignin in an acidic medium, producing mainly low MW fulvic acid–like products and CO_2 (Leonowicz et al. 1999). MnP is considered as a key degradative enzyme (Hofrichter 2002), followed by LiP – powerful oxidant of non-phenolic units. The precise role of laccase in ligninolysis remains controversial. Suggestions that laccase acts in synergy with MnP during degradation of macromolecular phenolic substrates (Galliano et al. 1991; Schlosser and Hofer 2002) are contradicted by reports on in vitro depolymerization of lignin (Maltseva et al. 1991) and soil HAs (Zavarzina et al. 2004) by blue laccase in absence of mediators or other enzymes. It seems likely, however, that efficient depolymerizing activity of laccases in vivo require presence of redox mediators. *Picnoporus cinnabarinus* which excretes solely laccase degraded lignin in comparable rate to *P. chrysosporium* due to production of metabolite 3-hydroxyanthranilate (Eggert et al. 1996). Yellow laccases of white-rot fungi, produced exclusively under solid-state fermentation conditions, were found to directly oxidize non-phenolic units in lignin probably due to presence of lignin-generated modifier in their structure that functions as electron-transfer mediator (Leontievsky et al. 1997b, 1999). There is an opinion that laccases in white-rot fungi have polymerizing function and are required for detoxification of low MW lignin breakdown products (Thurston 1994); HA can be formed as a result. Indeed, *Coriolus hirsutus* and/or *Cerrena maxima* grown on oat straw produced humic acid–like substances as a result of laccase activity; HAs were polymeric (23 kDa) and resembled soil HA by elemental composition and spectroscopic data (Yavmetdinov et al. 2003).

The role of white-rot fungi in the synthesis of HA seem to be limited, because under natural conditions FA and not HA accumulate in white-rotted wood and only small amounts of HA are produced on lignified plant material in laboratory experiments. Rypacek and Rypackova (1975) suggested that this can be a result of HA degradation by ligninolytic peroxidases. Indeed, many white-rot fungi were found to decolorize (up to 80%) and depolymerize HA of different origin (reviewed by Grinhut et al. 2007). The decolorization of HA is considered to be caused by splitting of double bonds, resulting in the breakdown of macromolecular mesomeric system and dissipation of the brown color (Fakoussa and Frost 1999). Humic acids with low aromatic C and high carbohydrate C contents (e.g., from litters, low rank coal) are closer by their structure to lignin – original substrate of ligninolytic enzymes – and are therefore more susceptible for oxidative attack than highly oxidized and aromatic soil HA (Almendros and Dorado 1999; Yanagi et al. 2002). For instance, lignite HA were effectively decolorized by laccase in submerged cultures of *P. cinnabarinus* (58% bleaching; Temp et al. 1999) or *Trametes versicolor* (80% bleaching; Fakoussa and Frost 1999), while HA from sod-podzolic soil lost 45% of its initial color under action of *P. tigrinus* laccase *in vivo* (our unpublished data).

11.4.2 Litter and Soil-inhabiting Fungi

Humification in litter and mineral soil horizons differ from that in wood in terms of nature of organic matter and enzymes involved. It was found that laccases, followed by MnP, are dominant redox enzymes in forest soils (Rosenbrock et al. 1995; Snajdr et al. 2008), while activities of LiP and VP have never been reported so far. Fungal substrates in litter are represented by particulate organic matter, consisting of plant debris (leaves, needles, branches), animal remains, root fragments, fungal hyphae, etc. (Fig 11.1b). This organic material is highly heterogeneous but not that compact as lignocellulose in wood that facilitates direct enzymatic attack. Enzymes in litter (MnP, laccase) represent active pool associated mostly with decomposition and mineralization processes (Allison 2006). In underlying soil layers, 90% of organic matter exist as coatings on mineral grains (Fig 11.1c) largely inaccessible to enzymatic attack. Fungi should thus preferentially utilize soluble substrates leaching down from decaying plant litter or excreted by roots. Many of these compounds are potentially toxic polyphenols which are polymerized into HS by laccases, peroxidases, or tyrosinases, immobilized on mineral supports.

11.4.2.1 Microfungi: Lignocellulose and Humus Solubilization, Synthesis of Melanins, and HS

Soil mycelial fungi (micromycetes) are largely ascomycetes, deuteromycetes, and zygomycetes that colonize wood in contact with soil, litter, and also soil up to 1 m depth. They comprise important group of soil microbial community and predominate over other fungal groups on the early stages of litter decomposition (Mirchink 1976; Lindahl et al. 2007). The role of soil microfungi in humification has for long time been attributed to the intracellular production of the high MW polyphenol-like brown pigment melanin (Kang and Felbeck 1965; Kononova 1966; Martin and Haider 1969; Valmaseda et al. 1989). Melanins have certain similarities with humic acids in terms of irregular aromatic structure, stochastic mechanism of synthesis, behavior in solvents, and some physicochemical properties (Zaprometova et al. 1971). This led to assumption that the bulk of unaltered fungal melanins, released upon the cell wall lysis, can form the stable humic acid fraction in soils (Zviagintsev and Mirchink 1986). Recent work has demonstrated that melanins are less resistant to biodegradation than soil HA and before contributing to stable humus fractions undergo oxidative transformations leading to depolymerization, increase in O-content, and optical density (Zavgorodnyaya et al. 2002).

Extracellular humification activity of micromycetes received far less attention than that of basidiomycetes. Soil microfungi are best known for production of cellulase–hemicellulase systems and comprise 60–90% of cellulose degrading microbial population in soils. Representative genera showing high biodiversity are *Aspergillus*, *Chaetomium*, *Ceratocystis*, *Phialophora*, *Trichoderma*, *Fusarium*, *Penicillium*, *Rhizoctonia*, and *Mortierella* (Rabinovich et al. 2001). Although carbohydrates are preferable substrate, representatives of these genera were found to mineralize 5–10%

and solubilize 15–20% of synthetic and wood-derived lignin during *primary growth* (Haider and Trojanowski 1975; Rodriguez et al. 1997; Kluczek-Turpeinen et al. 2003). Moreover, many species including the deuteromycete *Paecylomices inflatus* (Kluczek-Turpeinen et al. 2005), the ascomycetes *Thrichoderma* spp., and *Penicillium* spp. (Laborda et al. 1999), species of *Alternaria*, *Clonostachys*, *Phoma*, and *Paecilomyces* (Řezáčová et al. 2006) as well as *Acremonium*, *Botrytis*, *Chaetomium*, and *Rhizoctonia* (Gramss et al. 1999) were able to cause partial mineralization (5%), solubilization (6–25%), decolorization (2–30%), and depolymerization of HAs from litters and soil. The deuteromycete *Chalara longipes* isolated from spruce needle litter caused even 75% bleaching of humus extract from O_F litter layer (Koukol et al. 2004). Solubilizing activity is typical for ascomycetes and most likely involves microbial alkaline substances and a synergistic effect of cellulases and hemicellulases (Holker et al. 1999). As a result, some species (e.g., *Trichoderma atroviride*) can grow on HA or coals using them as a sole carbon source (Gramss et al 1999; Silva-Stenico et al. 2007). Oxidative activity is believed to be attributed to production of laccase (Kluczek-Turpeinen et al 2003, 2005), peroxidase (Haider and Trojanowski 1975), peroxidase and tyrosinase (Koukol et al. 2004), phenoloxidase and/or MnP (Laborda et al. 1999; Řezáčová et al. 2006), or combination of laccase, tyrosinase, and peroxidase as in *Botrytis cinerea* (Gramss et al. 1999). The direct involvement of these enzymes in degradation of phenolic compounds remains to be proven, because some species showed bleaching activity in absence of oxidases (Gramss et al. 1999). The extent to which phenoloxidases and peroxidases are produced by microfungi is also poorly understood. Generally, production of ligninolytic peroxidases is rare: LiP-like enzymes were detected so far in *Chrysonilia sitophila* (Duran et al. 1987) and *Penicillium decumbens* (Yang et al. 2005) and MnP-like activity was reported in *Fusarium solani* (Saparrat et al. 2000), *Trichoderma* sp, *Penicillium* sp (Laborda et al. 1999), and some others (Řezáčová et al. 2006).

According to Martin and Haider (1971), microfungi play a significant role in the synthesis of HS in soil. Polymerization activity is mostly associated with laccases, which are widespread in microfungi (Baldrian 2006). Rabinovich et al. (2004) suggested that unlike acidic laccases of white-rot fungi, laccases of the majority of soil micromycetes are predisposed for substrate polymerization, rather than depolymerization due to their more neutral pH optimum (pH 6.0–7.0). Although pH of most forest soils is acidic, some microfungi are able to adjust pH of their microenvironment to neutral values (Stepanova et al. 2003; Kluczek-Turpeinen et al. 2007). Thus, microfungi can be those laccase-producing species responsible for synthesis of HA in litter and mineral soil horizons. Solubilizing activity associated with hydrolytic enzymes can be important prerequisite for lignin and HS modification in litter by more efficient ligninolytic systems of basidiomycetes.

11.4.2.2 Saprotrophic Basidiomycetes: Production of the "White-Rot" Humus and FA-like Compounds

Ligninolytic fungi comprise as much as 10% of the entire fungal decomposer communities in forest litters (Osono 2007): mycelia of these fungi are often concentrated

in the interface between the freshly fallen leaves in L layer and near-humus materials in the F layer. Saprotrophic basidiomycetes colonize litter on the later stages of decomposition than ascomycetes and can cause substantial loss of recalcitrant compounds including lignin, HS, tannins, melanins with production of CO_2, soluble fragments, and bleached humus. Representatives of about 20 genera were reported as lignin decomposers: many of them possess MnP activity in addition to laccase, while LiP activity has not been found so far (Steffen et al. 2000; Osono 2007). The most active ligninolytic species belong to the genera *Agrocybe*, *Clitocybe*, *Collybia*, *Marasmius*, *Mycena*, and *Stropharia*. Delignification activity of litter-decomposing basidiomycetes resembles that of white-rot fungi in wood being a cometabolic event (Steffen et al. 2007). Although saprotrophic basidiomycetes decompose lignin at half the rate of white-rot fungi (Gramss et al. 1999; Steffen et al. 2000), they can produce substantial amounts of FA-like compounds (0.9 kDa) from insoluble litter material (Steffen et al. 2002) and can cause decarboxylation (up to 50%) and degradation of HA to CO_2 and lower molecular mass compounds (Rabinovich et al. 2001). A considerable decrease in 30–50 kDa fraction of litter-derived HA and formation of products with mean MWs of 1.0–2.0 kDa were observed in cultures of *Gymnopus* sp., *Hypholoma fasciculare*, *Rhodocollybia butyracea* (Valaskova et al. 2007), and *Collybia dryophila* (Steffen et al. 2002). MnP was considered as a key enzyme in the process. It is important to note that HA depolymerization effect observed in above-mentioned studies need careful interpretation because it could indicate the frequent cleavage of lignin polymer present as admixture in alkali extracts from litters rather than HS degradation (Sect. 2).

11.4.3 Symbiotic Fungi

Ectomycorrhiza (ECM) and lichens comprise two groups of symbiotic fungi that are common and abundant in northern forest ecosystems. These fungi obtain all or most of their carbon from the photosynthetic partner, and their saprotrophic activity appears to be limited. Despite this fact, both ECM (e.g., Taylor et al. 2004) and lichens (e.g., Dahlman et al. 2004) are able to assimilate exogenous C by taking up simple organic compounds (glucose, amino acids). Many ECM fungi are known for their abilities to metabolize lignocellulose, hemicellulose and polyphenols (Read and Perez-Moreno 2003). Thus, access to a photosynthate does not preclude facultative saprotrophy, which might be alternative foraging strategy during periods of low photosynthate supply or during massive mycelial production when supplementary resources for growth are needed (Talbot et al. 2008). Among the enzymes involved in organic matter transformation, activities of laccases and tyrosinases have been found in some taxa of ECM and lichens. Irrespective of whether these enzymes are used for saprotrophy-related activities or not, once released or leached into the soil they have a potential to participate in humus synthesis or degradation.

11.4.3.1 Ectomycorrhiza

Ectomycorrhiza is a symbiotic extracellular association of a fungus (usually basidiomycete) with plant fine roots. It is typically formed between the roots of woody plants belonging to *Pinaceaea*, *Betulaceae*, and others. In forest soils ECM are distributed primarily in fragmented litter, humus horizon and mineral soil, being spatially separated from saprotrophs which strongly dominate in the fresh and partially decomposed litter layers rich in labile C (Lindahl et al. 2007). Such prevalence of ECM in specific parts of the soil profile is likely to reflect preferential exploitation of substrates of a particular quality as reflected by their C:N ratios (Read and Perez-Moreno 2003). ECM fungi are considered as nutrient-mobilizing components of the soil fungal community and their hyphae function in the adsorption and translocation of N, P and water to the host plant. Most of N and P in forest soils are present in the colloidal organic material surrounding roots e.g., lignocellulose cell wall structures of dead plant material, HSs, proteins, protein–tannin complexes and HSs. It was found that many ECM fungal taxa can mobilize nutrients from these organic sources via production of lytic enzymes (cellulases, xylanases, proteases, polyphenol oxidases) causing some decrease in lignocellulose and humus in soil (Cairney and Burke 1994; Bending and Read 1996a, b, 1997; Read and Perez-Moreno 2003). When grown on tannic acid as the sole carbon source ECM fungi utilized carbon contained in the substrate and released dark-colored reactive quinone-like compounds (HS precursors) as a by-product (Bending and Read 1996a). Decomposition of lignin and HSs by ECM is limited in comparison with cellulose, hemicellulose and hydrolysable phenols (Durall et al. 1994; Bending and Read 1997; Read and Perez-Moreno 2003). This can be explained by apparent lack in ECM fungi of ligninolytic peroxidases, needed for effective HS and lignin breakdown. There is no convincing evidence for LiP genes in ECM (Cairney et al. 2003) and extracellular production of MnP was confirmed so far only in Tylospora fibrillosa (Chambers et al. 1999). The extents to which ECM fungi produce laccases and tyrosinases and their role in polyphenol transformation is not completely known. Gene fragments with high similarity to laccase from wood-rots were found in ECM species from the genera *Amanita*, *Cortinarius*, *Hebeloma*, *Lactarius*, *Paxillus*, *Piloderma*, *Russula*, *Tylospora*, and *Xerocomus* (Chen et al. 2003). Laccase gene sequences attributed to ECM fungi comprised almost half (45.5%) of investigated laccase genes in forest soil (Luis et al. 2005). However, upregulation of genes is not necessarily mean production of the enzyme. While activities of polyphenol oxidases were detected in many ECM using axenic mycelia, non-sterile mycelia, sporocarp tissue, or ECM root tips, oxidation of the laccase substrate syringaldazine was rare, suggesting that ECM fungi produce tyrosinase rather than laccase (Burke and Cairney 2002). Production of laccase has been confirmed only in few species, e.g., *Cantharellus cibarius* (Ng and Wang 2004) and *Thelephora terestris* (Kanunfre and Zancan 1998). It has been suggested that laccases of ECM might be involved in depolymerization of lignins, release of N from insoluble protein-tannin complexes and HS degradation (Bending and Read 1996, 1997; Gramss et al. 1999). However, an alternative hypothesis suggests

high possibility of non-enzymatic oxidation of phenolic substrates in ECM by radicals derived from Fenton reagent (Cairney and Burke 1998). ECM fungi may thus contribute to partial degradation of lignin by the way similar to that of brown rots (Sect. 11.4.1.2.) and can be involved in the HA synthesis rather than degradation. HSs can be also formed as by-products during detoxification of host-defence compounds by laccases and tyrosinases of ECM fungi (Cairney and Burke 2002). Further studies are needed to define the role of ECM fungi in humification.

11.4.3.2 Lichens

Lichens represent bi- or tripartite associations of a fungus (usually ascomycete) with green algae and/or cyanobacteria. They grow on stone, wood, or soil (epilythic, epiphytic, and epigeyic species, respectively) and are characterized by a variety of morphological and chemical adaptations for surviving stressful conditions and for fast restoration of metabolic activity (Beckett et al. 2008; Kranner et al. 2008). Lichens have long been recognized as important agents of soil formation due to their weathering action on rocks (Chen et al. 2000). Being dominant in soil cover of tundra and boreal forest ecosystems, lichens also serve as considerable source of mortmass for humification. Since lichens lack lignin, polymeric HS precursors should be mainly chitin and melanins. Lichens also produce considerable amounts of water-soluble low MW phenolic compounds that can serve as monomeric HS precursors upon leaching from the lichen thalli with rain water. Recent finding of laccases and tyrosinases in lichens of different taxonomic and substrate groups (Laufer et al. 2006a, b; Zavarzina and Zavarzin 2006) opens new perspectives in investigation of pedogenetic role of these symbiotic organisms. Lichens may play important role in humus formation processes as not only the source of the organic compounds for humification, but also producers of enzymes that can synthesize or degrade HS. Representatives of the order *Peltigerales* (genera *Peltigera*, *Solorina*, *Nephroma*), which mostly belong to fast-growing epiphytic and epigeyic species in wet microenvironments, were found to be the most active producers of laccase and tyrosinase. One order lower laccase activities and minor peroxidase activities were detected in more xerophytic lichens from the order *Lecanorales* (genera *Cladonia*, *Cetraria*, *Stereocaulon*). Laccases in lichens are constitutively expressed and stimulated by desiccation and wounding (Laufer et al. 2006a). Most of laccase activity in studied peltigerous lichens was located intracellularly or in the loosely and hydrophobically bound cell wall fractions, while a greater proportion of tyrosinases occurred intracellularly (Laufer et al. 2006b). Approximately 5–10% of the total extractable laccase activity could be washed out from the intact thalli of *Peltigerous* lichens by distilled water, suggesting possibility of their involvement in extracellular processes of polyphenols transformation (Zavarzina and Zavarzin 2006).

While lichen tyrosinases (e.g., in *Peltigera malacea*, *P. rufescens*, and *Pseudocyphellaria aurata*) had typical MW of about 60 kDa (Laufer et al. 2006b), lichen laccases were found to be unusually large. In the study of Laufer et al. (2009), active laccase isoforms in concentrated water extracts from 13 lichen species belonging to

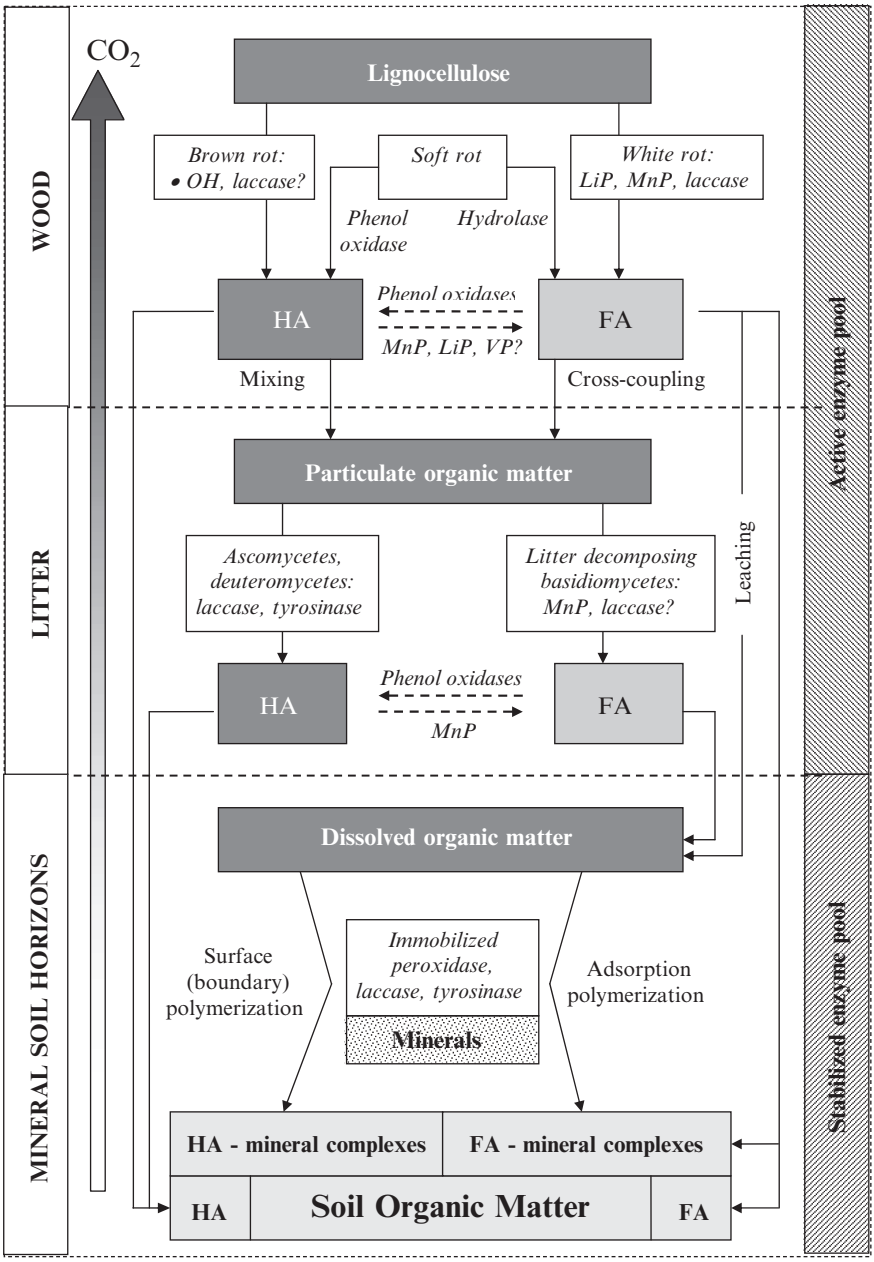

Fig. 11.2 The possible role of fungi and their oxidoreductases in humification process

the suborder *Peltigerineae* had MWs between 135 and 200 kDa, while in 7 lichen species laccases were even larger, 300–350 kDa (e.g., >350 kDa in *P. praetextata*). Lisov et al. (2007) have purified and characterized blue laccase from *Solorina crocea* and yellow laccase from *Peltigera aphthosa* with MWs of 175 kDa and 165 kDa, respectively. The enzymes were typical laccases by their substrate specificity and catalytic properties. In addition to homodimeric laccase forms, monomeric "small" laccases were detected. In *S. crocea* and *P. aphthosa*, "small" laccases had MWs of 45 kDa and 55 kDa, respectively, and consisted of two isoenzymes.

The role of lichen phenol oxidases in humification is unknown. In our preliminary studies we have found that purified laccases from *P. aphthosa* and *Solorina crocea* caused partial depolymerization of soil HA *in vitro*. However, given that washed-out laccase activities are commonly low, involvement of lichen laccases in organic matter degradation in nature is questionable unless lichens produce some metabolites which act as redox mediators. Z. Laufer have found that leachates from lichens were less effective in dye decolorization than intact thalli and that classic laccase mediators speeded up decolorization (personal communication with Prof. RP Beckett). If consider possible effects of laccase alone, the polymerizing activity of leached-out enzyme is more probable, which in turn suggests the possible involvement of lichens in synthesis of HSs. Soil-stabilizing species tightly bound to the mineral substrate, such as *Solorina crocea*, are particularly interesting with this respect. Laccases of such species may be immobilized on mineral grains upon release from the lichen thalli and initiate surface polymerization of water-soluble HS precursors with formation of stable organo-mineral adsorption complexes. Indeed, dark-colored organic coatings (cutanes) are commonly observed on the soil particles or rock fragments under lichen thalli. Further investigations are needed to define the role of lichen enzymes in functioning of the symbiosis and humification.

11.5 Conclusions

The role of fungi in humification processes can be summarized as follows (Fig. 11.2). (1) Humification in wood represents solid-state fermentation of lignocellulose. Soft-rot ascomycetes are pioneers on the surface of wood, while basidiomycetes continue the succession and penetrate in deeper layers by the aid of small non-enzymatic species. Ligninolytic peroxidases (MnP and LiP) of the white-rot fungi cause mineralization of lignin and its breakdown to soluble products (FA-like compounds). Brown-rot fungi and soft-rot fungi cause partial oxidation of lignin with formation of high MW humic acids. Laccases, peroxidases, and OH radicals from Fenton reaction are the oxidants responsible. Soft-rot ascomycetes also produce large soluble lignocellulose fragments by synergistic action of hydrolases. (2) Humification in litter represents transformation of particulate organic matter, which is subjected to direct enzymatic attack. Acidic laccases and MnP of

saprotrophic basidiomycetes are considered as main degradative enzymes which decompose complex organic matter to soluble FA-like fragments and CO_2. Microfungi are mainly responsible for synthesis of HA via partial oxidation of lignocellulose, re-polymerization of low MW polyphenols or production of melanins. Laccases and tyrosinases are the main enzymes involved in this process. Humic colloids formed in wood and litter can undergo slow mineralization or oxidation with release of soluble products. MnP and LiP of white-rot fungi are mainly responsible for this process in wood, while MnP of saprotrophic fungi can bleach, depolymerize, and solubilize HA in litters. Soluble products of plant debris and humus decomposition can be re-polymerized *in situ* by phenol oxidases or can be washed down the soil profile. (3) Soluble compounds become the substrate for laccases, peroxidases, and tyrosinases immobilized on inorganic or organo-mineral supports in humus horizon; microfungi, ectomicorhhizae, and lichens are producers of these enzymes. Two possible reactions may occur: oxidative self-coupling in aqueous phase adjacent to soil particles with adsorption/cross-coupling of polymeric products on the support surface ("precipitative polymerization"); adsorption of substrates on solid particles with formation of high MW product directly on the support surface ("surface polymerization"). Both reactions result in irreversible binding of organic matter and formation of organic coatings on mineral grains. Such humus–mineral complexes form the bulk of solid matrix in humus horizons and comprise the most stable fraction of C_{org} in soils.

The extents to which certain fungal groups participate in humification processes in soil need to be further defined. Future studies should concentrate on enzyme producers other than white-rot fungi, which have been most intensively studied over last three decades. White-rot fungi do not colonize soil under natural conditions and thus their contribution to humus synthesis and degradation appear to be limited. Humification activities of microfungi and symbiotic fungi are largely overlooked, and considerable gaps exist in our knowledge of phenol oxidase enzymology in these fungi.

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