

Functional Ecology of Ectomycorrhizal Fungi

Peroxidases, Decomposition, Spatial Community Patterns

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Cover: Sporocarp putatively formed by *Cortinarius delibutus* Fr. with hyphal mats in organic matter in a subarctic birch forest near Abisko, Lapland, Sweden.

(photo: Karina Clemmensen)

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Abstract

Boreal forest ecosystems constitute a globally important carbon (C) sink, due to accumulation of complex organic matter, persistent to decomposition. Nitrogen (N) is immobilized in these complex compounds and, thereby, unavailable to the plant community. Fungal peroxidases (ClassII) are oxidative enzymes, predominantly studied in white-rot wood decomposers and known to efficiently mineralize phenolic complexes, such as lignin, to CO₂. Peroxidase activity is also commonly measured in forest soil, where typical white-rotters are absent and ectomycorrhizal fungi predominate. Peroxidase activity is known to increase under low inorganic N availability. The aim of this study was to explore the ectomycorrhizal decomposer potential in boreal forest ecosystems. The central hypothesis is that ectomycorrhizal fungi produce ClassII peroxidases to mobilize N, bound to phenolic complexes in boreal forest litter and humus.

Genes coding for ClassII peroxidases were found to be widely spread among ectomycorrhizal taxa, particularly within the genus of *Cortinarius*. Gene transcription of peroxidases in forest humus could be linked directly to the species *Cortinarius semisanguineus*. In a field experiment, colorimetric enzyme assays showed a halving of peroxidase activity in short-term response to N-amendment. In non-treated control samples, *Cortinarius* species and other rhizomorph forming ectomycorrhizal fungi were co-localized with peroxidase activity hotspots. Ectomycorrhizal *Cortinarius* species may, thus, be key players in N-acquisition, from organic macromolecules, and central decomposers of complex organic matter in boreal forest ecosystems.

Root-associated fungi, including ectomycorrhizal ones, were able to compete with free-living saprotrophs for colonization of litter. However, they were less efficient decomposers than specialized litter saprotrophs.

It is concluded that some mycorrhizal fungi may release C while foraging for N. They may also indirectly act to preserve soil C by suppressing more efficient saprotrophic decomposers. The findings highlight ectomycorrhizal fungi as central regulators of C dynamics in boreal forests. Ectomycorrhizal symbiosis, thus, constitutes a direct link between above-ground photosynthesis and below-ground decomposition.

Keywords: Boreal forest ecosystem, ectomycorrhizal fungi, ClassII peroxidases, soil organic matter, decomposition, nitrogen acquisition, carbon sequestration.

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Dedication

Für meine Familie, insbesondere Leni Bödeker (1910 – 2012)

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I I.T.M. Bödeker, C. M. R. Nygren, A. F. S. Taylor, Å. Olson, B. D. Lindahl. (2009). ClassII peroxidase-encoding genes are present in a phylogenetically wide range of ectomycorrhizal fungi. *The ISME Journal* 3(12), 1387-1395.
- II I. T. M. Bödeker, K. E. Clemmensen, W. deBoer, Å. Olson, B. D. Lindahl Ectomycorrhizal fungi drive enzymatic oxidation in humus layers of northern forest ecosystems with low nitrogen availability. (Manuscript).
- III I. T. M. Bödeker, Å. Olson, B. D. Lindahl, K. E. Clemmensen. Impact of substrate quality, vertical placement and fungal community composition for organic matter decomposition in boreal forest. (Manuscript).

Paper I is reproduced with the permission of the publishers.

The contribution of Inga Bödeker to the papers included in this thesis was as follows:

- I Design of primers and molecular work in the laboratory with advice from supervisors. Analysis of data and writing of manuscript together with the supervisors.
- II Participating in the experimental design and development of new methods. Implementation of field study, assisted by supervisors. All laboratory work. Analysis of data and writing of manuscript together with the supervisors.
- III Initiated the study and planned the experimental design. All laboratory work. Analysis of data and writing of manuscript together with the supervisors.

Abbreviations

| | |
|------------------------------|--|
| ANOVA | Analysis of Variance |
| ATP | Adenosine Triphosphate |
| C | Carbon |
| CA | Correspondence Analysis |
| CCA | Canonical (or constrained) Correspondence Analysis |
| CII | Other ClassII peroxidases, not belonging to LiP, MnP or VP |
| DNA | Deoxyribonucleic Acid |
| cDNA | copy DNA |
| DOC | Dissolved Organic Carbon |
| F-layer | Organic soil layer of fragmented litter |
| GLM | General Linear Model |
| H | Humus |
| ITS | Internal Transcribed Spacer |
| L1 | Litter 1, defined as freshly abscised litter |
| L2 | Litter 2, defined partly decomposed litter |
| LiP | Lignin Peroxidase |
| Mn | Manganese |
| MnP | Manganese Peroxidase |
| N | Nitrogen |
| NH ₄ ⁺ | Ammonium |
| NH ₄ -N | Nitrogen present as ammonium |
| P | Phosphorous |
| PCR | Polymerase Chain Reaction |
| RFLP | Restriction Fragment Length Polymorphism |
| RNA | Ribonucleic Acid |
| mRNA | messenger RNA |
| RT-PCR | Real Time PCR |
| TRFLP | Terminal RFLP |
| VP | Versatile Peroxidase |
| VA | Veratryl Alcohol |

1 The Boreal Forest Ecosystem

The boreal forest is one of the largest biomes on earth, covering much of Russia, Finland, Sweden, inland Norway, a large part of Canada, and Alaska at a latitude of 50 - 65° degrees north. It covers about 17% of the total land surface (Figure 1; Rodin *et al.*, 1975; Bonan, 1989). The climate is cold with annual mean temperatures varying from 5°C to -5°C (Whittaker, 1975). The dominant tree species are coniferous trees like pine and spruce and the understory vegetation is relatively poor in diversity mainly consisting of ericaceous shrubs, lichens, and mosses. The boreal forest biome only contains 3% of the world's vascular tree species.

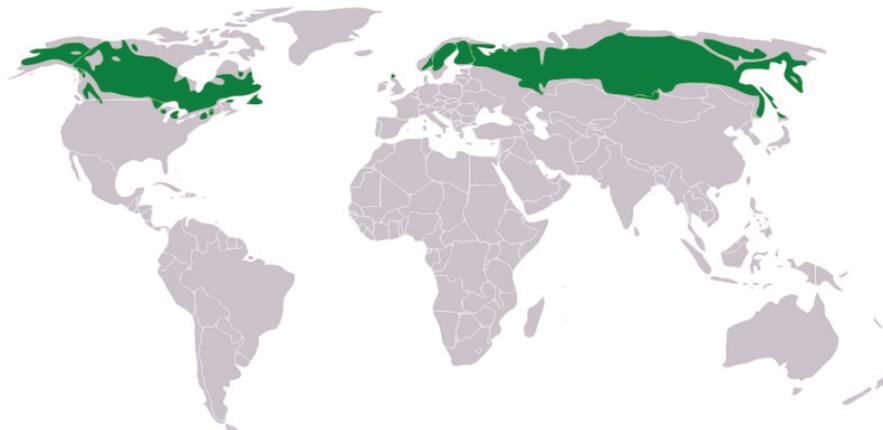


Figure 1. The boreal forest biome stretches over Eurasia and North America and is one of the largest biomes on earth. Picture taken and adapted from:
http://en.wikipedia.org/wiki/Boreal_Forest

In Fennoscandia, the dominant tree species are Scots pine (*Pinus sylvestris* (L.) H. Karst), Norway spruce (*Picea abies* L.), and birch (*Betula* sp.). In the northern subarctic areas of this biome, pine and spruce are replaced by alpine birch, which forms the natural northern tree line at these high latitudes. The needles and leaves from the dominating plant species have a high content of polyphenolic secondary metabolites, which serve as protection against the harsh climatic conditions. In fallen litter, these complex compounds are difficult to decompose (Aerts, 1995), resulting in low carbon (C) and nutrient accessibility in this needle litter compared to leaf litter from temperate, deciduous forests (Fog, 1988).

In the boreal forest, photosynthesis rates are higher than decomposition rates, which allow higher C fluxes to belowground pools than C effluxes caused by decomposition. The decomposition rate is low, due to the cold climate and the high litter recalcitrance (Myneni *et al.*, 2001). Four to five year old organic material, derived from needles and mosses, is often still possible to identify visually. As a result, large C pools accumulate belowground together with organically bound nutrients such as nitrogen (N) and phosphorous (P) (Northup *et al.*, 1995). Due to the huge land cover and soil C accumulation, the boreal forest biome acts as a global C sink and has thus plays a major role on the global C budget and dynamics. In order to predict future development of atmospheric CO₂ levels and their consequences, understanding these dynamics is of great importance.

Another inherent property of boreal forest soils is the vertically stratified profile of the organic horizon into different layers. The litter layer at the top consists of freshly fallen and partly decomposed litter, together with mosses. This layer transitions into a layer of well decomposed, fragmented litter (F-layer), which can be interwoven with dense mats of fungal mycelium. The plant-derived organic material here is approximately 0 to 10 years old, reaching from freshly fallen litter down to the fragmented litter layer. The humus layer is situated below this and consists of organic matter with no or only very little mineral particles. The material is condensed with a darker color. Here, fine roots from trees and shrubs are abundant. The organic matter in this layer generally has an age of at least 15 years and is situated above the mineral layer (Lindahl *et al.*, 2007).

In forest soils, C-dynamics are primarily driven by saprotrophic decomposition of dead organic material and biotrophic utilization of recently fixed photosynthesis products into the rhizosphere. Photosynthetically fixed C is delivered via the roots to the root-associated fungal (and bacterial) community. A large part of the newly fixed, root-derived C is stored within living and, perhaps most important, dead fungal mycelium (Clemmensen *et al.*,

2012). Decomposition is regulated by the C and nutrient demands of the soil microbial community. During the course of litter decomposition, easily available C bound in easily degradable cellulose, as well as easily available nutrients are first removed from freshly fallen litter by the microbial decomposer community. The most limiting nutrient in boreal forest ecosystems is N and therefore, the C and N cycles are intimately related with each other (Boberg, 2009). Traditionally, it has been assumed that nutrients are mineralized during the decomposition process, wherein organically bound N is available in excess of microbial demand and is therefore released as ammonium (NH_4^+). Plants and microbes are able to directly take up this mineral form of N. However, litter-decomposing fungi only mineralize N when their N status is saturated and C becomes the limiting factor to growth, and this rarely occurs in boreal forest litter (Boberg *et al.*, 2010).

During decomposition, large fractions of N are bound to residual organic complexes (Knicker, 2004; Berg & Staaf, 1980; Berg & McClaugherty, 2003; Schmidt-Rohr *et al.*, 2004) and thereby become inaccessible to direct plant uptake. Natural inputs of mineral N by atmospheric N-deposition are very low in boreal forest ecosystems and if present, inorganic N will immediately be taken up by soil organisms (Nadelhoffer *et al.*, 1999). Thus, mineral N in form of NH_4^+ is typically only scarcely available in typical boreal forests (Nordin *et al.*, 2001).

Generally, in soils with high concentrations of lignin derivatives, N is likely to be bound directly to the aromatic structures and is therefore not available, neither to the plant community, nor to most microorganisms, which have a limited capacity to degrade phenolic compounds (Northup *et al.*, 1995; Schmidt-Rohr *et al.*, 2004). Soil pH serves as an indicator of N availability: when, as is often the case in boreal forest soils, the pH is low (≤ 4), soluble, inorganic N is absent (Giesler *et al.*, 1998). In the litter layer, the C:N ratio decreases with the increasing age of the organic matter (Lindahl *et al.*, 2007; Hilli *et al.*, 2008). Increases in absolute amount of N have also been observed (Berg *et al.*, 1982), presumably due to import by litter colonizing fungi (Boberg, 2009). Thus, while the actual abundance of N is high, it is not primarily present in plant available forms, and this N limitation of tree growth limits ecosystem productivity.

2 Functional Groups of Fungi in Forest Soils

Decomposition and nutrient cycling in boreal forest soils is mainly driven by fungi. Fungi are heterotrophic organisms that use organic C as their energy source for maintenance and biomass production. Freshly fallen litter and dead wood fulfills this need for saprotrophic needle and wood decomposing taxa that are dependent on C acquisition through decomposition of organic material. Biotrophic fungi, in contrast, interact with a host organism, either in a mutualistic or parasitic way, with the host organism acting as the main C source. Fungi also require N and mineral nutrients, which are either removed from the substrate during decomposition, taken from the soil solution, or from the host organism in a parasitic lifestyle.

The two predominant fungal phyla in boreal forest ecosystems are Ascomycetes and Basidiomycetes. Both groups cover a wide range of ecological functions, reaching from saprotrophs to parasitic pathogens to mutualistic symbionts. The key functional groups for C and N cycling in forest ecosystems are litter and wood-decomposing saprotrophs and root associated mutualistic fungi (biotrophs). The latter live more or less in a well-defined symbiosis with the plants. In a boreal forest ecosystem, these two functional groups are usually spatially separated within the stratified soil profile. Saprotrophic fungi dominate the litter layer, where they have the opportunity to obtain more easily available C by decomposing relatively fresh needle litter compared to further down in the soil profile, where the C accessibility decreases in older, well decomposed organic matter. Down in the humus layer, where fine-roots are present, symbiotic root-associated fungi predominate. Here, they have the advantage of obtaining C as simple sugars via the root system. It has traditionally been assumed that symbiotic root-associates take up N and other nutrients in inorganic form, in order to support themselves and

their host plants. However, the details of N re-allocation from the humus layer remain barely understood.

2.1 Saprotrophic Fungi as Litter Decomposers

Wood and litter decomposing fungi employ a range of strategies to decompose organic matter. Wood degraders cause either so called white-rot, or brown-rot of dead wood. White-rot fungi possess a very efficient oxidative system to degrade lignin, which is chemically complex and restricts access to cellulose. Brown-rot fungi, in contrast, remove mainly cellulose and their oxidative enzymatic repertoire is not as efficient as that of white-rot basidiomycetes. Many litter colonizing basidiomycetes are efficient degraders of needle litter (Boberg, 2009). Boreal litter decomposing fungi must have a well-developed enzymatic system to be able to obtain C and nutrients from the chemically complex litter, which is relatively rich in lignin and tannins (Berg & McClaugherty, 2003).

As litter decomposers, free-living saprotrophs dominate the litter layer in boreal forest ecosystems, presumably by suppressing ingrowth of root-associated biotrophs (Lindahl *et al.*, 2007). Many basidiomycetes form rhizomorphic mycelia. With the help of these chords, many litter decomposers can extend their organismal size up to a decimeter scale, which allows them to transport resources over a larger distance between heterogeneous substrates. Thereby, N can be re-allocated to freshly colonized litter, which minimizes N losses in decomposition to the soil solution (Boberg, 2009). This fungal trait is an adaptation for persistence in an N limited ecosystem like the boreal forest.

2.2 Ectomycorrhizal Fungi

A mycorrhiza is a mutualistic symbiosis between a fungus and plant in which the fungal partner is physically attached to the roots of a vascular plant. Literally, the word mycorrhiza (Greek. $\mu\kappa\acute{o}\varsigma$, mykós, "fungus" and $\rho\iota\zeta\alpha$, riza, "roots") means "fungus-root". This symbiosis was first described by Frank (1885). There are different forms of mycorrhization, which can be intracellular in arbuscular and ericoid mycorrhiza, extracellular, as in ectomycorrhiza, or in an intermediate form of ect-endo mycorrhiza (also referred to as arbutoid mycorrhiza), which occurs for certain ericoid host plants. Different phylogenetic groups of fungi have developed specific forms of mycorrhization. Arbuscular mycorrhizae are uniquely formed by Glomeromycetes, whereas ericoid mycorrhizae are mostly formed by Ascomycetes. Ectomycorrhizae are primarily formed by Agaricomycetes, although numerous examples exist

within the Ascomycota and Zygomycota (Rinaldi *et al.*, 2008; Tedersoo *et al.*, 2010). Ectomycorrhiza is also the most common mycorrhizal association with woody shrubs and trees in boreal forest ecosystems. The fungi provide nutrients and water to their host plants and obtain photosynthetically fixed C from their hosts in return (Smith & Read, 2008). This C is their main energy source and most ectomycorrhizal fungi are not able to live on alternate C sources in nature. Nutrients are taken up from the soil environment, via the extra-radical mycelium. Ectomycorrhizal fungi dominate the humus layer in boreal forest ecosystems (O'Brien *et al.*, 2005; Lindahl *et al.*, 2007), where their direct link to the plant roots enables them to prevent establishment of free-living saprotrophs (Lindahl *et al.*, 2001)

Morphologically, ectomycorrhizae have been characterized by three structural traits: a mantle of fungal tissue that encases the root tip, secondly, a net of hyphae penetrating the root and thereby surrounding epidermal and cortical root cells, which is called the Hartig's net, and thirdly, a system of emanating hyphae, the extra-radical mycelium. This extra radical mycelium acts as the direct connection between the symbiotic plant and the microsites in the soil environment. According to differences in color, shape, and structure of the mantle and emanating hyphae, ectomycorrhiza can be assigned to different morphotypes (Agerer, 1987-2002; Agerer *et al.*, 2001). These morphotypes help to characterize ectomycorrhizal fungi at a genus level. They can be classified into exploration types based on structural differences of the extra-radical mycelium, their most important distinguishing property (Agerer, 2001). Highly developed extra-radical mycelia, rhizomorphs, enable ectomycorrhizal fungi to expand and to transport C, nutrients and water over longer distances (Brownlee *et al.*, 1983; Agerer, 1991). Nutrient and water uptake occurs at the rhizomorphs' advancing mycelial front, consisting of solitary hyphae with a hydrophilic surface (Unestam & Sun, 1995; Cairney & Burke, 1996; Raidl, 1997). In contrast, the proximal parts of the rhizomorphs are hydrophobic, serving as transport "tubes". Several ectomycorrhizal fungi dominating boreal forest soils belong to rhizomorph forming genera such as *Cortinarius*, *Piloderma*, *Suillus*, *Leccinum*, *Boletus*, and some *Tricholoma* species (Agerer, 2001). Hobbie and Agerer (2009) found elevated $\delta^{15}\text{N}$ values in ectomycorrhizal species forming rhizomorphs, such as *Suillus* sp. and *Cortinarius* sp., especially in N limited boreal forest ecosystems. They conclude that these species take up N from predominantly organic sources, than in mineral form. Furthermore, Genney *et al.* (2006) observed that root-tips colonized by a *Cortinarius* sp. was located further down in the soil profile, whereas the extra-radical mycelium primarily was located in the F-layer. This is another indication that rhizomorph forming ectomycorrhizal fungi utilize

their extra-radical mycelium to forage for nutrients in solid litter substrates with better substrate quality.

Ectomycorrhizal fungi have often been regarded as extensions of the plant's root system, aiding its symbiotic host in mineral nutrient uptake by increasing the assimilating surface area. Even though ectomycorrhizal fungi have traditionally been thought to be unable to break down organic macromolecules, their decomposer potential has been the subject of great deal of research spending many decades (Frank, 1894; Melin & Nilsson, 1953; Entry *et al.*, 1991; Read, 1991; Lindahl *et al.*, 2002; Read & Pérez-Moreno, 2003; Koide, 2008; Talbot *et al.*, 2008). The ability to degrade organic matter would enable mycorrhizal fungi to obtain N from chemically complex organic matter, rather than being restricted to soluble mineralized nutrients. In the mid 1980's Trojanowski *et al.* (1984) observed the release of $^{14}\text{CO}_2$ from radiolabeled lignin, holocellulose, lignocellulose, and coniferyl alcohol (Haselwandter *et al.*, 1990) in the presence of mycorrhizal fungi. Mobilization of N and P from well degraded litter by the ectomycorrhizal fungus *Paxillus involutus* through the production of extracellular enzymes, and further relocation of nutrients to a host pine seedling, was also demonstrated in soil microcosms by Bending & Read (1995). They also showed that ericoid and some ectomycorrhizal fungi were able to clear tannic acid – protein complexes, where the N from proteins was recalcitrantly bound to tannic-compounds (Bending & Read, 1996). In the humus layer where root-associated fungi (including ectomycorrhizal fungi) dominate, the C:N ratio increases with the age of the organic material (Lindahl *et al.*, 2007; Hilli *et al.*, 2008). Carbon, fixed by photosynthesis, is allocated to the roots and incorporated into the ectomycorrhizal mycelium. Part of this carbon is stored within the humus as fungal necromass (Clemmensen *et al.*, 2012), and therefore the C content increases with time. At the same time, recalcitrantly bound N may be removed from the organic matter via decomposition activity of the ectomycorrhizal community, particularly when free inorganic N is unavailable. The input of C and simultaneous removal of N leads to progressively increasing C:N ratios, which may be interpreted as a sign of ectomycorrhizal decomposer activity. This process is driven by a demand for organically bound N for the fungus and associated tree, and made possible by the external source of C that the roots constitute.

In order to release N from chemically complex organic matter, extracellular enzymes with a high redox potential are necessary (Baldrian, 2008). These types of oxidative enzymes have been extensively studied in wood decomposing fungi (Hatakka, 1994; Hofrichter, 2002, Baldrian, 2008), and agaricomycetous peroxidases have the highest efficiency in degrading recalcitrant organic compounds (Baldrian, 2006, Steffen *et al.*, 2002). The

activity of such enzymes is commonly measured in forest soils (Sinsabaugh, 2005, 2010) and has been interpreted as a proxy for decomposition activity of complex, phenol-rich organic matter. Bending & Read (1997) observed that peroxidase activity could be generated by ectomycorrhizal and ericaceous fungi in pure culture. They also found that species with long rhizomorphs showed more peroxidase activity than others. Chambers *et al.* (1999) published a short part of a DNA sequence of an ectomycorrhizal extracellular peroxidase and later a whole peroxidase sequence was detected in the genome of the ectomycorrhizal fungus *Laccaria bicolor* by Martin *et al.* (2008). During the course of this thesis we were able to recover peroxidase encoding gene sequences from genomic DNA of 11 ectomycorrhizal species from four of the major agaricomycetous clades (Paper I). Furthermore, three ClassII peroxidase encoding genes were recently found and annotated within the genome of *Hebeloma cylindrosporum* (Marmeisse *et al.*, 2004; Koua *et al.*, 2009; PeroxiBase). Thus, many ectomycorrhizal fungi possess the genetic potential to produce powerful oxidative, extracellular enzymes, and this may be a potentially helpful tool to obtain nutrients from complex organic matter. Currently, the potential of ectomycorrhizal fungi as decomposers is gradually gaining acceptance (Koide *et al.*, 2008; Talbot *et al.*, 2008; Burke *et al.*, 2011).

It is clear that the capacity to form ectomycorrhizal symbiosis has been evolved several times independently during the evolution of the Agaricomycetes (Hibbet *et al.*, 2000; James *et al.*, 2006). It is even possible that in some clades, ectomycorrhizal fungi may be ancestors to saprotrophic fungi and vice versa. The oxidative enzyme system of white-rot wood decomposers is well studied from the genes up to the expression level, whereas the presence and potential of these enzymes are still poorly understood for most ectomycorrhizal fungi. If fungi with a saprotrophic lifestyle have evolved from ectomycorrhizal ancestors and vice versa at several independent occasions, it seems plausible that the genetic potential as well as active expression of extracellular oxidative enzymes has been maintained in many species with an ectomycorrhizal lifestyle.

2.3 Fungal Extracellular Enzymes in Organic Matter Decomposition

Fungi use extra-cellularly secreted enzymes to decompose different kinds of organic matter. There are two major groups of enzymes, catalyzing either hydrolytic or oxidative reactions. Hydrolytic enzymes break residues linked by bonds such as glycosidic-, ester- and peptide bonds by addition of water. Fungal hydrolytic enzymes are key enzymes for the degradation of cellulose and other non-lignified carbohydrates in a forest ecosystem.

In early decomposition stages, soluble compounds and non-lignified carbohydrates are rather rapidly degraded. Cellulose and hemicellulose are decomposed primarily by hydrolytic enzymes, such as endocellulases, β -glucosidases, β -glucanases, cellobiohydrolases and different kinds of xylanases (Baldrian, 2008). Together with cellobiohydrolases, glucanases hydrolyse cellulose to the endproduct of cellobiose, which is further hydrolysed by β -glucosidases into glucose. Xylanases are endo-type enzymes that hydrolyse hemicellulose by cleaving β -1,4-glycosidic bonds randomly. Xylans are thereby degraded into smaller fragments. Cellobiodehydrogenases are oxidative enzymes that are involved in cellulose degradation and possess a high redox potential. With the help of these enzymes, cellulose and lignin-decomposition can be combined. Very recently, another key oxidative enzyme in cellulose degradation has been detected and further studied by Westereng *et al.* (2011). This enzyme is metal dependent and may aid fungal substrate penetration, which eases cellulose hydrolysis.

After cellulose, the second major plant component is lignin (Kögel-Knabner, 2002), which has a complex chemical structure consisting of phenolic residues, which are linked in C-C and ether bonds. The fungal enzymatic ligninolytic system is based on oxidative enzymes such as laccases, peroxidases, hydrogen peroxide producing enzymes, and peroxygenases. Laccases in general possess a low redox potential and use molecular oxygen for substrate oxidation. These enzymes are mainly present in white-rot wood decomposers and litter degrading basidiomycetes but there are also a few reports of laccases being present in ectomycorrhizal taxa (Courty *et al.*, 2005) as well as the brown rot fungus *Coniophora puteana* (Lee *et al.*, 2004). Compounds that are produced during laccase mediated lignin degradation may further act as redox mediators and thereby aid oxidation of compounds that need a higher redox potential than that of laccases themselves (Baldrian, 2006). Peroxidases provide a higher redox potential enabled by the reduction of hydrogen peroxide (H_2O_2). Thereby, they oxidize either aromatic alcohols (lignin peroxidases) or manganese from Mn^{2+} to Mn^{3+} . The oxidized manganese is diffusible and is therefore able to randomly react with a large

range of substrates of non-phenolic and phenolic compounds (Hofrichter, 2002). Other enzymes involved in lignin oxidation are hydrogen peroxide-producing enzymes (Baldrian, 2008) and peroxygenases that transfer hydrogen peroxide to substrate molecules to enable their decomposition (Hofrichter *et al.*, 2010).

Over the course of decomposition from fresh litter to humus a shift in enzyme activity in accordance with the chemical composition of the organic matter was observed by Snajdr *et al.* (2011). At the very beginning of litter decomposition, hydrolytic cellulases were most active and mass loss the highest. After 4 months hemicellulose xylanase activity peaked with a slight decrease in the mass loss rate, and finally after 12 months ligninolytic enzymes were most active, and most of the cellulose and hemicellulose had been degraded. At this stage the mass loss rate was the lowest. In another study Berg *et al.* (2006) suggested a dependence on presence and concentrations of manganese at late stage decomposition. These observations indicate that fungal ligninolytic enzyme activity is a feature of late stage decomposition, when easily accessible C is already removed by hydrolytic enzymes.

3 ClassII Peroxidases

ClassII peroxidases were first observed to be synthesized by the model white rot fungus *Phanerochaete chrysosporium* (Glenn *et al.*, 1983; Tien & Kirk, 1983) and were later described by Welinder (1992) as fungal extracellular enzymes, which are principally attributed to the Agaricomycetes. These peroxidases belong to a larger superfamily of non-animal heme-peroxidases. Heme-peroxidases all have a covalently bound heme group (porphyrine plus iron) and their activity is thereby based on the Fenton reaction, where the iron, bound to its porphyrine, is oxidized by hydrogen peroxide. In the resting state of a heme-peroxidase, the iron has the valence state Fe^{3+} and is oxidized to Fe^{4+} by transferring electrons, leading to the reduction of hydrogen peroxide (H_2O_2) to water. By this reaction, the enzyme gets into the excited state named Compound I (Figure 2), which lacks two electrons compared to the resting state.

Compound I is thereby able to oxidize a variety of substrates, which act as electron donors in order to reduce the enzyme back into its resting state. The reduction occurs in two steps by accepting one electron at a time from the reacting substrate, via formation of Compound II (Wirstam *et al.*, 1999; Hofrichter, 2002). Hydrogen peroxide is one of the strongest known oxidizers and through its catalysis highly reactive hydroxyl radicals ($\bullet OH$) are formed. These radicals strongly react with their environment. A variety of different substrates may take part in these highly unspecific reactions. Intracellular peroxidases are active in organelles called peroxisomes, which protect the cells from the toxic hydrogen peroxide and radicals. The intracellular reduction of hydrogen peroxide by peroxidases is a mechanism to protect cells from oxidative stress. The large variety of substrates that can be used as reductants might have favored the acquisition of novel functions, facilitating differentiation into extracellular heme-peroxidases concurrent with the evolution of fungi.

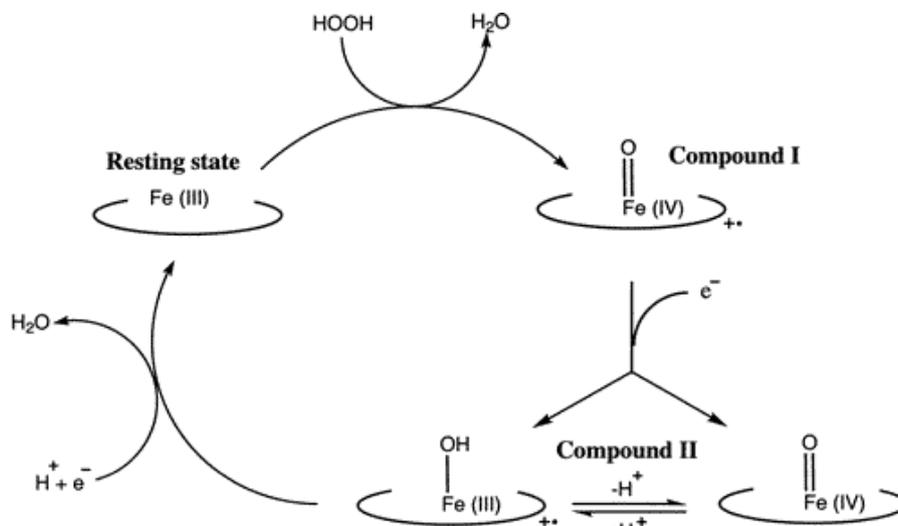


Figure 2. Reaction scheme for the basic catalytic steps of heme-peroxidases (Wirstam *et al.*, 1999).

The common substrates that react with ClassII peroxidases are either manganese (Mn) or aromatic alcohols. After oxidation, these substrates are converted into radicals and in this form further react unspecifically and randomly with other substrates within their proximity. ClassII peroxidases do not target specific compounds in organic matter but are able to degrade a wide range of substrates. Their high redox potential and random mode of action enables ClassII peroxidases to efficiently attack and degrade chemically complex and irregular compounds. This class of enzymes is a highly effective fungal tool in lignin decomposition during degradation of wood.

3.1 Subclasses of the ClassII Peroxidase Family

ClassII peroxidases are divided into three main subclasses, all of which have essentially the same three-dimensional protein structure (Banci *et al.*, 1997). Slight differences in protein sequence between these subclasses lead to differences in their electron transfer pathways. Based on these differences, protein sequence based phylogenetic analysis may be used to identify and classify peroxidase gene and protein sequences (Martinez, 2002).

Manganese peroxidases (MnP; EC 1.11.1.13) use manganese as an electron donor for reduction of the enzymes states Compound I and II. The residues in their protein structure, ExxxE (E35, E39) and D (D179) (Martinez, 2002; Morgenstern *et al.*, 2010) are responsible for manganese binding and electron transfer. The specific oxidation steps of Mn-peroxidases are: oxidation of Mn^{2+}

to Mn^{3+} , and chelating Mn^{3+} with organic acids, such as oxalic acid. The radical is thereby transferred from the excited enzyme state to the chelate, which can then react with organic compounds in the proximal environment. Manganese peroxidases are the most phylogenetically widespread subclass of Class II peroxidases (Hofrichter, 2002; Morgenstern *et al.*, 2010). Their activity has commonly been observed in decaying wood (Hofrichter, 2002, Baldrian, 2008) and litter as well as soil (Sinsabaugh, 2005, 2010) and they are known to be more effective than lignin-peroxidases in the degradation of humic compounds (Steffen *et al.*, 2002; Baldrian, 2008). Due to the high diffusibility of the chelated, oxidized manganese, MnPs are able to react with organic substrates in a more versatile manner than lignin-peroxidases. Phylogenetically, Mn-peroxidases are divided into two major groups, long Mn-peroxidases and short Mn-peroxidases. Long MnPs, with an average protein sequence length of 383 residues, form a monophyletic clade and have hitherto primarily been identified in species belonging to the Polyporales together with a few species from the Corticiales and Hymenochaetales orders. Short MnPs, with an average protein sequence length of 363 residues, are phylogenetically more similar to so called versatile and lignin-peroxidases than long MnPs (Morgenstern *et al.*, 2010).

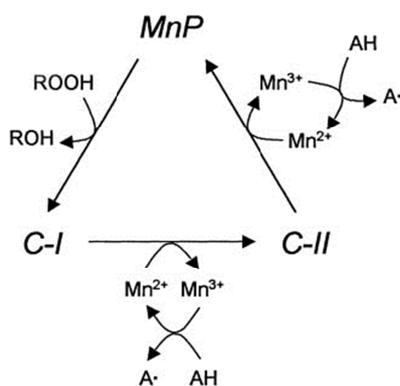


Figure 3. Reaction scheme of Mn-peroxidase (MnP) catalysis, adapted from Ruiz-Duenas *et al.*, 1999. The peroxide (ROOH) oxidizes the native Mn-peroxidase (MnP) into Compound I (C-I), now lacking two electrons. Compound I in return oxidizes Mn^{2+} into Mn^{3+} . In this reaction a free radical is formed on an aromatic compound ($\text{A}\cdot$), serving as electron donor (AH), and compound I is reduced into Compound II (C-II) by one electron. The reaction is repeated from Compound II to the resting state of the Mn-peroxidase by an additional electron donor.

Lignin peroxidases (LiP; EC 1.11.1.14) oxidize both non-phenolic and phenolic aromatic compounds including the fungal metabolite veratryl alcohol (VA). An exposed tryptophan Trp171 (W) and the lack of Mn-binding residues are the main characteristics that distinguish LiP protein sequences from MnPs. Lignin-peroxidases are commonly reported from white-rot fungi and are specialized in decomposing lignin, although they do not interact directly with the lignin molecules, as previously assumed, but rather catalyze lignin oxidation indirectly via aromatic alcohol mediators (Hatakka, 1994; Martinez, 2002).

Versatile peroxidases (VP; EC 1.11.1.16) combine catalytic properties of both LiPs and MnPs and possess both manganese binding sites and the exposed VA binding site (Trp164; Martinez, 2002; Pérez-Boada *et al.*, 2005). Both lignin peroxidases and versatile peroxidases possess alternative manganese-independent oxidation pathways, which are described as long-range-electron-transfer (LRET). They involve electron transfer between distinct amino-acid residues close to the heme pocket (Schoemaker *et al.*, 1994; Doyle *et al.*, 1998; Johjima *et al.*, 1999; Pérez-Boada *et al.*, 2005).

Another group called “Other ClassII peroxidases” (CII) encompasses the so-called “Ancestral Peroxidases” and “New Peroxidases”. Both lack the key residues for manganese and veratryl alcohol oxidation. Furthermore, ancestral CII-peroxidases have a shorter C-terminus, whereas the new CII-peroxidases are mainly present in Ascomycota. Due to the lack of key residues, CII-peroxidases are usually not included among the lignin-degrading group of LiPs, MnPs, and VPs, but are considered as hypothetical hybrid peroxidases with a lower redox- potential (Morgenstern *et al.*, 2008; Ruiz-Dueñas *et al.*, 2009).

4 Methods in Fungal Ecology

4.1 Community Analysis – from Sporocarp Records to High-Throughput Sequencing

Gathering knowledge about fungal diversity and community composition has long since depended on sporocarp records. Species identification was based on morphological traits, such as sporocarp structure, shape, and color, as well as microscopic features, such as hyphal structure, and spore size and shape. Sporocarp occurrence has also been used to indicate the ectomycorrhizal status of a forest ecosystem (Smith & Read, 2008), but was complemented by morphological examination and identification of the ectomycorrhizal root tips (e.g. Agerer, 1987-2002). Often, the below-ground picture of ectomycorrhizal communities is different from that obtained by above-ground sporocarp inventories (Gardes & Bruns, 1996).

Later, molecular techniques have widened the possibilities to identify fungal species and describe communities in natural substrates. Molecular fungal species identification became common with the development of fungal specific primers, targeting the ribosomal RNA gene internal transcribed spacer (ITS) region as a molecular marker (White *et al.*, 1990). Amplified fungal ITS fragments from ectomycorrhizal root tips and plant pathogens were matched against identified reference material by restriction fragment length polymorphism (RFLP). In RFLP, amplicons are digested by restriction enzymes, leading to DNA fragments of different lengths. These are visualized on electrophoresis gels, with identification based on pattern recognition (Gardes & Bruns, 1993; Karen *et al.*, 1997). A more accurate fragment length polymorphism method is terminal restriction length polymorphism (TRFLP), where the ends of the amplified ITS fragments are fluorescence labeled. Here, species identification is based on the exact length of the terminal fragment, as determined in a capillary sequencer. This method allows for the analysis of complex species mixtures and, thereby, enables a first molecular glimpse of

entire fungal communities below ground (Dickie *et al.*, 2002). Combining TRFLP on environmental samples together with a clone library with known TRFLP patterns, representing the whole sample set up enables faster identification of species present in individual samples.

Application of Sanger-sequencing (Sanger *et al.*, 1977) enabled the establishment of sequence databases, where primarily fungal ITS sequences originating from identified species are stored. An ITS-sequence database with mainly ectomycorrhizal sequences is the UNITE Database (<http://unite.ut.ee>; Abarenkov *et al.*, 2010). By comparing ITS sequences of unknown origin against such databases (Chenna *et al.*, 2003), information on the taxonomic placement of the species may be obtained, based on sequence similarity. Direct sequencing of the ITS region after molecular cloning of the PCR product from environmental samples in connection with TRFLP can give a higher resolution of the soil born fungal community than TRFLP alone. Still, morphotyping approaches are combined with molecular methods such as TRFLP and Sanger-sequencing.

Instead of obtaining 96 sequence reads per run for traditional Sanger-sequencing, the newly developed techniques of next-generation-sequencing enable the generation of about a 1,000,000 of sequences per run. The technology of 454-pyrosequencing has been developed by Roche and is now commonly used for large scale analysis of microbial communities, both bacteria and fungi, as well for whole genome sequencing (Rothberg & Leamon, 2008).

Pyro-sequencing is based on the detection of light at the very moment when a nucleotide is incorporated by the DNA-polymerase. Simultaneous, highly resolved detection of light enables massively parallel sequencing of templates in mixture. The name pyro-sequencing derives from the pyrophosphate generated during nucleotide incorporation. This pyrophosphate is converted to adenosine triphosphate (ATP), which is then used by a luciferase to convert luciferin to oxyluciferin. During the latter conversion, light is emitted. To single stranded DNA fragments, adaptors are ligated, which enable binding to beads. DNA concentrations are balanced so that, on average, a single DNA fragment binds to one single bead. These strands are then PCR-amplified in an oil emulsion, with beads isolated in micelles. After amplification, each bead carries 10 million copies of the original DNA fragment, enabling subsequent sequencing. The emulsion is then broken and the beads carrying the DNA strands are placed into wells on a fibre-optic slide. Such a slide contains over a million wells, in which the pyro-sequencing reactions take place. During the sequencing process, the slide is exposed to each of the 4 nucleotides separately, which enables reading of the DNA sequence.

The output of several hundred thousand sequence reads per run requires high computational power, and during the past few years, pipelines for analyzing high-throughput sequencing data have emerged. Currently, a debate on how to handle such large amounts of sequence data and how to best use all this information to address ecological questions is underway (Hibbet, 2009).

The basic bioinformatic steps that are necessary after retrieval of 454-pyrosequencing data include processing the dataset to remove sequences that are too short or of poor quality. To entirely use of the large capacity of 454-sequencing and to enable handling of large sample numbers, amplicons are provided with sample-unique sequence tags of about 8bp, attached to either one or both PCR primers. Recognition of sequence tags during data analysis enables association of each derived sequence with the originating sample.

Sequences are then assembled into clusters according to their similarity. For clustering of fungal ITS sequences, single linkage clustering has proven to be particularly useful. During single linkage clustering, all sequences that have a similarity above a given threshold to any sequence already in a cluster will be added to this cluster. In order to assemble basidiomycetous ITS sequences on a level that roughly corresponds to species, a threshold of 98.5% similarity yields satisfactory results.

4.2 Gene Expression – Enzyme Assays and Transcription

Analysis of extracellular enzyme production is a useful tool to investigate and understand how organisms interact with their environment, and to better understand ecosystem processes. Enzyme assays are based on mimicking the enzymes' *in situ* activity in an *in vitro* environment. Substrates that are specific to enzymes of interest are added to enzyme extracts, in order to initiate catalysis together with dyes that interact with the reaction product. Activity is measured photometrically. The higher the enzyme activity is, the more dye molecules are produced or maintained in the reaction and the higher will be the optical density. Alternatively, methods may be based on substrates that yield fluorescent products upon cleavage.

Enzyme assays of environmental samples display the amounts of enzymes produced by the biota present at the time of sampling, providing insights into their momentary activity. However, it is difficult to directly link such observations of over-all enzyme activity to the individual taxa that produced the enzymes. Here, detection of mRNA transcription by reverse transcriptase-PCR (RT-PCR) may be a useful. If enzyme-specific PCR primers can be developed, which discriminate fungal taxa at genus or species level, ecological functioning of the genus or species may be investigated. Obtained cDNA

sequences may be compared with reference sequences, derived from genomic DNA from identified material.

4.3 Multivariate Statistics

In order to gain insight into how community data correlates with environmental parameters, multivariate statistics, such as canonical ordination, is required. Multivariate statistics allow analysis of several dependent variables at the same time. Prediction variables can consist of environmental data, such as pH, temperature, soil depth, or sampling time. In ecological community studies, the records of several species are used simultaneously as dependent variables (terBraak, 1986; Ramette, 2007).

One method to conduct multivariate ordination is by correspondence analysis (CA). Here, species data are not related to explaining variables. Instead, samples are related to each other based on community similarity and the method may thus be used in an exploratory manner, with relationships to environmental variables based on subjective evaluations. The basis of a CA is a unimodal model, where the eigenvalue (variance described by an axis) of the CA axis equals the correlation coefficient between species scores and sample scores. These scores derive from weighing the abundance of the species and the weighted average of samples where these species occur. Hereby, the ecological distance between samples (differences in species composition) is estimated. Species- and sample scores represent coordinates of ordination axes. The largest part of variation is distributed on the first ordination axis; the second axis describes the largest part of the residual variation; the third even less, and so on, with all axes being orthogonal (uncorrelated) to each other. A summary of the data is displayed in a two-dimensional ordination plot of two axes (default would be axis 1 and 2)

After conducting an exploratory ordination of the species data, a direct analysis may be conducted in order to test the significance of correlative relationships between environmental data and community composition. To directly analyze multivariate correlations with explaining parameters, canonical (or constrained) correspondence analysis (CCA) is used. In a CCA the correspondence analysis is implemented together with a linear regression analysis. The canonical axes are constrained by linear combinations of the environmental variables. Therefore, eigenvalues and total inertia will be smaller in a CCA, than in a CA, which represents the full multivariate variation. In order to test the significance of relationships between species composition and environmental variables, a Monte Carlo Permutation test can be implemented. The significance is calculated by permuting (repeatedly

shuffling) the dataset. Every single, randomly constructed data set is evaluated for correlative power, resulting in a mean F-value calculated from all permutations. The F-value, derived from the original data set (F_0), is ranked among all obtained F-values, in order to establish a probability estimate.

After performing a CCA, the usual causal interpretation would be that a certain environmental factor explains the species distribution and community composition with a certain level of significance. When relating enzyme activities to fungal community composition, however, the causal relationship is expected to be opposite. The environmental data (enzyme activities) might be interpreted as the dependent variable and the species data as the explanatory variable. The fungal taxa produce enzymes and, thus, their pattern of distribution will explain the observed enzyme activity. Another way to analyze such reversed causal relationships is to use the sample scores, derived from a CA as an index of community composition, and to include this parameter as an explanatory variable in a statistical model.

5 Objectives

The main hypothesis of this thesis is that ectomycorrhizal fungi produce ClassII peroxidases in order to forage boreal forest humus for N. Ectomycorrhizal fungi obtain C from their host trees and thus have a secure source of energy. However, they need to mobilize nutrients from the soil in order to cover the demand of their hosts as well as themselves. In boreal forest ecosystems N is recalcitrantly bound to organic matter while free inorganic N is scarce. In order to access the vast organic N pool, it is here hypothesized that ectomycorrhizal fungi utilize ClassII peroxidases to degrade complex macromolecules into assimilable compounds. As a side effect of this enzymatic oxidation, stored C may also be released as CO₂ or leached in dissolved forms.

The motivation for the project was the theoretical hypothesis built on the current and future increase in atmospheric CO₂ concentrations. Elevated CO₂ may lead to higher photosynthesis rates with subsequent increased allocation of C to roots and ectomycorrhizal symbionts. Increased C availability under strong N limitation is likely to intensify the demand and foraging for soil N. Due to the increased N demand of the symbiotic system, production of ectomycorrhizal ClassII peroxidases may be stimulated, with concomitant losses of soil C. The implications of ectomycorrhizal, co-metabolic C release may be considered in the context of the boreal forest ecosystem as an important global C sink.

Anthropogenic N input is hypothesized to have the opposite effect. Increased availability of accessible N to ectomycorrhizal fungi and their host plants would decrease the need to mobilize organic N. This may then lead to a decreased ClassII peroxidase production and thereby decreased turnover of organic matter. The short-term effect of N-amendment on ectomycorrhizal peroxidase production was a central question investigated within this thesis.

The specific objectives of this thesis were:

- I - To investigate the genetic potential of ectomycorrhizal fungi to produce ClassII peroxidases and gain a deeper understanding of the phylogeny and evolution of these enzymes. Upon database screening, degenerate PCR primers were developed to target as wide a range of ClassII peroxidase sequences as possible. Analysis of the obtained sequences in a phylogenetic context enabled conclusions to be drawn about their possible classification, activity, and evolution. **Paper I**
- II - To elucidate the activity of ectomycorrhizal ClassII peroxidases and their response to N-amendment in the field, as well as detecting *in situ* mRNA transcription of ClassII peroxidases by ectomycorrhizal species in forest soil. A field study was initiated at two different forests sites. Control and N-treated plots were sampled, in order to test for Mn-peroxidase activity and *in situ* mRNA transcription. Enzyme activity and fungal community composition was investigated for potential spatial correlation. A down-regulation of Mn-peroxidase activity was expected in response to N-amendment with respect to both enzymatic activity and gene transcription. **Paper II**
- III - To compare the potential of root-associated fungi and saprotrophic litter colonizing fungi to colonize and decompose needle litter and humus. Litter or humus containing mesh bags were reciprocally placed in the soil profile of a boreal forest. After harvest, the bags were analyzed for fungal exploration and exploitation, by analysis of mass loss, respiration and fungal community composition. **Paper III**

6 Presence of ClassII Peroxidase Genes in Ectomycorrhizal Fungi – The genetic Potential (Paper I)

Ectomycorrhizal fungi are widely phylogenetically distributed over the agaricomycetous clades. In order to target the full variation of ClassII peroxidase genes within the Agaricomycetes, we developed highly degenerate primers. The forward primer was directed towards the calcium (Ca^{2+}) binding site (GGADGS) close to the N-terminus and the reverse primer targeted a sequence coding for the calcium binding site and a heme-binding residue (PFDST) towards the C-terminus. The primer design was based on amino-acid and nucleotide sequences from all ClassII peroxidase subclasses, retrieved from the peroxidase specific database PeroxiBase (<http://peroxibase.toulouse.inra.fr/>) and the National Center for Biotechnology Information (NCBI). Genomic DNA from 70 different ectomycorrhizal species and two white-rot wood decomposing species was used as template in carefully optimized PCR reactions. Obtained PCR products were isolated by cloning into *E. coli* cells and subsequently sequenced by the Sanger method. Manually edited sequences were confirmed as coding for ClassII peroxidases, using the nucleotide blast and blastx algorithms against databases at NCBI (Altschul *et al.*, 1997). Converted protein sequences were aligned together with reference sequences from previously sequenced agaricomycetous ClassII peroxidase genes, and investigated for the presence of functional ClassII peroxidase specific sites.

Altogether, 21 novel Mn-peroxidase coding sequences were obtained from 13 different agaricomycetous species. Eleven of these species were ectomycorrhizal, representing 15 of the recovered genes. They were spread across three of the major basidiomycetous clades of Euagaricales, Russulales, and Gomphales (Phylogeny according to Hibbet *et al.*, 2000). Among them were 9 different sequences from 5 *Cortinarius* species. There were single

sequences each in *Russula xerampelina*, *Russula sardonia*, *Lactarius fulvissimus*, and *Lactarius rufus*. Furthermore we detected a ClassII peroxidase coding gene in the two phylogenetically distant ectomycorrhizal species *Hygrophorus agathosmus* and *Gomphus clavatus*. From the two wood decayers *Hypholoma fasciculare* and *Phellinum ferrugineofuscum*, we were able to amplify three different sequences, each.

All novel genes contained the typical residue for manganese binding (aspartic acid, Asp175, D), while the exposed tryptophan (Trp171 or Trp164, W), necessary for veratryl alcohol oxidation, was absent. Therefore, we concluded that these genes code for Mn-peroxidases. The russulacean sequences and the sequence from *Cortinarius infractus* were truncated, most likely due to the presence of an alternate binding site for the reverse primer.

In a phylogeny, the cortinarioid peroxidases formed their own weakly supported group together with the sequences from the wood decayer *Hypholoma fasciculare*. The sequence similarity between ectomycorrhizal genes and those of a well-established white-rot fungus supports a possible function of *Cortinarius* Mn-peroxidases in degradation of recalcitrant organic matter, rich in phenolic compounds. The russulacean peroxidase genes formed their own highly supported clade in the neighbor joining analysis and did not cluster together with other russuloid Mn-peroxidases from *Heterobasidion*, *Amylostereum*, *Bondarzewia*, and *Echinodontium*. Interestingly, the Mn-peroxidase gene sequence obtained from the basal ectomycorrhizal species *Gomphus clavatus* clusters with bootstrap support together with the so called “long” Mn-peroxidases (Morgenstern *et al.*, 2008), previously restricted to typical white rot fungi with good capacity to degrade lignin. This finding was confirmed in a study by Morgenstern *et al.* (2010), who re-analyzed the peroxidase-encoding genes recovered from this study together with other newly discovered Mn-peroxidase genes from the wine pathogen *Fomitopsis mediterranea*.

Two of the *Cortinarius* species, as well as both of the investigated wood decomposers, contained several different genes that, according to their sequence similarity, indicate late gene duplications. Such expansion of gene families contributes to the fitness of an organism and may imply that the genes are under a positive selection pressure (Lynch & Conery, 2000; Wapinski *et al.*, 2007) supporting an active expression of the genes.

In addition to the already described short ClassII peroxidase sequence in *Tylospora fibrillosa* (Chambers *et al.*, 1999) and the Mn-peroxidase encoding gene in *Laccaria bicolor* (Martin *et al.*, 2008), we succeeded to demonstrate the presence of Mn-peroxidase genes from a phylogenetic wide range of ectomycorrhizal fungi. From the majority of investigated species we were,

however, unable to amplify ClassII peroxidase genes. Failed amplification does not necessarily imply that the genes do not exist. Still, no ClassII peroxidase gene sequence could be found in the newly published genome of *Amanita bisporigera* (<http://genomics.msu.edu/Amanita/blast/blast.html>), confirming that peroxidase genes are not a general feature for all ectomycorrhizal species. In contrast, three duplicate genes of ClassII peroxidase encoding genes were recently found in the genome of the ectomycorrhizal species of *Hebeloma cylindrosporum* (Marmeisse *et al.*, 2004; PeroxiBase), although we were unable to recover ClassII peroxidase-encoding genes from this genus.

To design general primers that successfully and easily amplify all members of gene families across wide phylogenetic groups of organisms is a major challenge. It is much easier to develop primers targeting sub-groups of sequences within families in restricted groups of organisms. With rapid advances in high throughput sequencing, it becomes more and more affordable to obtain sequence information of specific genes by shot gun sequencing of whole genomes. Producing a DNA sequence library enables bioinformatic screening for specific sequences of interest.

The genus *Cortinarius* contained the highest number of amplified Mn-peroxidase encoding genes, which suggests that this genus might be particularly important in soil organic matter turnover. Furthermore, this genus usually dominates ectomycorrhizal communities in low-productive boreal forest ecosystem (Lilleskov *et al.*, 2001; Lindahl *et al.*, 2010, Paper II). Active transcription of Mn-peroxidase genes in such nutrient limited ecosystems could constitute a clear advantage by enabling nutrient retrieval from recalcitrant organic matter. Thereby, in ecosystems with low nutrient availability, *Cortinarius* species might outcompete other ectomycorrhizal species that are more dependent on the uptake of nutrients in mineral form. Since ClassII peroxidases also often are expressed during fungal combative interactions (Hiscox *et al.*, 2010), expression of Mn-peroxidase genes would, potentially, imply an advantage for ectomycorrhizal fungi during interference competition for space and substrate, something, which may contribute to the strong spatial patchiness displayed by *Cortinarius* species (paper II). The presence of genetic potential alone does not imply active gene expression *in situ*. The majority of the here recovered Mn-peroxidase genes belong to ectomycorrhizal species that are hard to cultivate, and most likely, these enzymes are only expressed in symbiosis with a host plant and not in culture. The establishment of symbiosis with *Cortinarius* species in laboratory systems has yet not been possible. Therefore, detection of ectomycorrhizal Mn-peroxidase gene expression directly in the field remains the best option to verify an active role of these genes.

7 ClassII Peroxidases and Ectomycorrhizal Fungi in Forest Soils – From Genes to Forest (Paper II)

To investigate, whether ectomycorrhizal Mn-peroxidase genes are actively expressed in forest soil, we established a field study in two different forest sites. One site was a nutrient limited boreal Scotts pine forest near Jädraås in middle Sweden, and the other a subarctic birch forest close to Abisko in Lapland, Northern Sweden (Figure 4).



Figure 4. Location of the two forest sites in Sweden

We hypothesized that ectomycorrhizal fungi produce Mn-peroxidases in order to retrieve N from complex organic sources. At each site we implemented a highly localized N-amendment ($10\text{g}/\text{m}^2$ of NH_4SO_4) of 30 20×20 cm plots with 30 unamended control plots. We expected an active expression of Mn-peroxidase genes in control sample plots and a down regulation of expression in N-treated ones. The main aim of this study was to investigate the direct, short-term effect of N amendment on Mn-peroxidase production by specific fungal species. Sampling of humus was therefore conducted as soon as two days after the N-treatment. To test the success of N amendment, $\text{NH}_4\text{-N}$ concentration was measured in freshly prepared samples. Enzyme activities of Mn-peroxidases, laccases, and cellulases were measured by colorimetric enzyme assays. Laccase and cellulase activities were very low or hardly detectable for all samples. The average Mn-peroxidase activity was more than twice as high in control plots as in the N-treated plots. A two-way ANOVA of Mn-peroxidase activity data showed that both sample origin and N-treatment had significant effects.

However, in each forest, there were only a few samples that showed distinctively high activities. This patchy spatial distribution of enzyme activity suggests that there were certain key players present in these particular samples that were responsible for the observed high activity. Fungal community analysis by 454-pyrosequencing of all samples was therefore conducted, in order to reveal these key players. A CCA was performed in order to establish correlations between the species distribution among samples and the observed Mn-peroxidase activity. This analysis revealed a significant co-localization of high Mn-peroxidase activity with several *Cortinarius* species. Other positively correlated species were *Piloderma olivaceum*, *Suillus variegatus* and *Tricholoma fucatum*. A Mann-Whitney U test confirmed a positive correlation between the abundance of basidiomycete DNA and Mn-peroxidase activity. Among basidiomycetes, the presence of *Cortinarius* species were more correlated with high Mn-activity than other taxa. Due to the high abundance of *Cortinarius* species in samples with high enzyme activity, *Cortinarius* specific Mn-peroxidase PCR primers were designed based on sequence information from paper I. A clone library of cortinarioid Mn-peroxidase sequences was produced, based on material taken from genomic DNA, prepared from sporocarps representing the nine most positively correlated *Cortinarius* species. Messenger RNA was prepared from humus samples with high peroxidase activity, which contained these *Cortinarius* species in a high abundance as well as from N-treated plots containing the same *Cortinarius* species but with low peroxidase activity. RT-PCR products were cloned and sequenced, and obtained sequences from mRNA in soil samples were aligned together with the sequences from identified sporocarps. A neighbor-joining tree with bootstrap analysis was calculated from the alignment.

We were able to detect several different sequences of Mn-peroxidase coding mRNA in a single control sample, as well as a single sequence in a sample from an N-treated plot. The phylogenetic analysis of *Cortinarius* Mn-peroxidases helped to identify the soil derived sequences. The control sample contained three transcripts coding for Mn-peroxidases from *C. semisanguineus*, one sequence that could be assigned to *C. acutus*, and another uncertainly placed sequence. The transcript from the N-treated sample was ascribed *C. obtusus*. In this study, transcribed mRNA coding for Mn-peroxidases could for the first time be directly linked to ectomycorrhizal fungi *in situ* within the forest soil. Some transcripts could even be confidently assigned to individual species. Previously, Kellner *et al.*, (2010) were able to detect Mn-peroxidase transcription in forest soil, but of unknown fungal origin.

In conjunction to the scattered high peroxidase activity, agaricomycetous species were only detected in a few sampling plots each. In contrast, most

ascomycetous taxa were more evenly distributed over the plots. A reason for this patchy distribution may be that many basidiomycetes form larger mycelia than most of the detected ascomycetes. The mycelium of ectomycorrhizal individuals may often stretch over large distances (Douhan *et al.*, 2011), often forming chord like structures, aggregated into more or less dense hyphal mats. Also Genney *et al.*, (2006) found a patchy distribution of ectomycorrhizal fungi across the organic horizon of a *Pinus sylvestris* forest and linked this observation to different exploration types. The patchy distribution of ectomycorrhizal mycelia may explain the observed activity hotspots, where Mn-peroxidases were expressed locally.

All the ectomycorrhizal species that co-localized with high peroxidase activity belong to rhizomorph forming groups. Rhizomorphs enable ectomycorrhizal fungi to establish large networks for transport of nutrients over longer distances. The positively correlated species belong either to the long-distance (*Suillus variegatus*, *Tricholoma fucatum*.) or to the medium-distance fringe exploration types (all nine *Cortinarius* species, *Piloderma* sp.; Agerer, 2001). Bending and Read, (1997) reported peroxidase activity especially for long rhizomorph forming ectomycorrhizal fungi. Furthermore, based on stable-isotope signatures, Hobbie and Agerer, (2009) proposed preferred N uptake from organic matter by long-distance and medium-distance exploration type forming ectomycorrhizal taxa. Rhizomorph formation may enable ectomycorrhizal species to forage relatively distant nutrient patches and re-allocate the mobilized N back to the roots - a strategy that would be advantageous when exploiting solid organic substrates with little mobility. All species belonging to the russulacean family were negatively correlated with Mn-peroxidase activity. The russulacean sequences obtained in Paper I were however short and we do not have any information about the presence of manganese binding sites. Species like *Russula* and *Lactarius* do not form distinct rhizomorphs (Agerer, 2001). Since root tips from these species are often found closely attached to leaf litter and other debris, they could obtain nutrients opportunistically in dissolved forms, released by decomposer activity of free-living saprotrophs.

Even though high Mn-peroxidase activity was only measured in a few control samples, the average activity in untreated samples was more than twice as high compared to N-treated ones in both forests. Even when *Cortinarius* species were highly abundant in N-treated plots, there was still no- or low Mn-peroxidase activity. This short-term N-effect was observed on an organism level *in situ* and suggests that organisms adapted to nutrient limited conditions are phenotypically sensitive to changes in N availability. Direct short-term N-effects on ClassII peroxidase activity have previously been observed in

cultures of the white rot wood decomposer *Phanerchaete chrysosporium* (Kirk & Farrel, 1987). Effects of N deposition on enzyme activities in forest ecosystems have hitherto only been investigated within long-term fertilization experiments, where decreases in peroxidase activity have been observed in soils (Saiya-Cork *et al.*, 2002; DeForest *et al.*, 2004; Waldrop *et al.*, 2004; Zak *et al.*, 2011; Averill & Finzi, 2012). When N availability increases as a result of anthropogenic influence, long-term effects on ectomycorrhizal fungi are more likely to be indirect, as the host tree respond to improved N access by restricting C allocation to the ectomycorrhizal symbionts (Högberg *et al.*, 2003, 2010). In this changed situation, ectomycorrhizal species specialized in organic matter oxidation may become obsolete as N foragers and thereby lose their competitive advantage towards other ectomycorrhizal taxa, which may be superior in house holding with host derived C, leading to changed community composition (Lilleskov *et al.*, 2001, 2002).

Loss of C as a result of ectomycorrhizal Mn-peroxidase activity is likely to occur primarily as a co-metabolic process. Previous studies have indicated that lignin degradation by peroxidases only takes place in the presence of other, more accessible C sources (Kirk & Farrel, 1987). Having access to host derived sugars, ectomycorrhizal fungi seem in a good position to carry out such co-metabolic degradation. Our results and previous studies suggest that ectomycorrhizal fungi turn over organic matter primarily in order to mobilize N. Still, C may be lost during degradation of organic N-containing substrate, either as dissolved organic C (DOC), due to dissolution as molecular sizes decreases, or as CO₂, resulting from complete extracellular oxidation (Hofrichter *et al.*, 1999). Another scenario is that oxidation by ectomycorrhizal fungi facilitate further C transformation by prokaryotes, which may be able to metabolize small, structurally diverse degradation products to CO₂. Via such “indirect” decomposition of organic matter, ectomycorrhizal fungi are likely to have a major influence on C dynamics in low-productive boreal forests. Elevated atmospheric CO₂ levels have been shown to positively influence mycorrhizal fungal biomass and respiration (Treseder, 2004; Fransson *et al.*, 2007) especially for rhizomorph-forming species. Due to higher photosynthesis rates, more C is allocated to the roots of the host tree and mycorrhizal symbionts, stimulating biomass production as well as production of oxidative enzymes (Carney *et al.*, 2007). In order to meet the increased demand for N induced by increased C availability, ectomycorrhizal fungi would have to increase their Mn-peroxidase expression. Increased activity of organic matter decomposing enzymes in response to elevated atmospheric CO₂ may lead to losses of C from the forest floor, which in a long term perspective could abolish the C storing function of boreal forest ecosystems (Drake *et al.*, 2011).

8 Comparing Decomposition Potential between Root-associated Biotrophs and Free-living Saprotrophs (Paper III)

In the light of the recently demonstrated capacity of ectomycorrhizal fungi to produce potent oxidative enzymes, we aimed at investigating the relative efficiency of root-associated fungal communities and free-living saprotrophs in decomposition of litter and humus. In a field experiment, we reciprocally transplanted litter and humus substrates between the litter and humus layer in a boreal forest in middle Sweden (Sala; Figure 5). Our initial assumption was that the vertical structuring of the fungal communities, with free-living saprotrophs in the upper litter layer and root-associated fungi in the humus layer (Lindahl *et al.*, 2007), would persist, in spite the addition of small amounts of foreign substrate qualities. This manipulation would enable to investigate the potential of natural communities to explore and exploit substrate qualities from outside their normal realized niches. Thereby, we could compare the decomposition potential of different communities when colonizing equivalent substrates.



Figure 5. Location of the Sala forest site

Litter at two different decomposition stages; freshly fallen litter, named litter1 (L1) and partly decomposed litter, named litter 2 (L2), as well as humus were collected, filled into nylon mesh bags, and sterilized by gamma-irradiation. The litter bags were inserted in the organic top-soil at three different depths: on the surface (L1), below the uppermost litter (L2) and in the humus layer (H), in a factorial design

(Figure 6). The experiment was initiated at the beginning of the growing season and was replicated in eight blocks. Weight loss and respiration were used as proxies for decomposition activities and measured in recently recovered bags after 4 and 16 months of incubation, covering two growing seasons in total. The fungal community composition in the litterbags was analyzed by Titanium-454-pyrosequencing. Effects on the fungal community composition were evaluated indirectly by correspondence analysis (CA), and tested for significance by canonical correspondence analysis (CCA) followed by MonteCarlo permutations. Effects of placement, substrate, and time on mass loss and respiration were evaluated in a general linear model (GLM) with replicate block as a co-variable. The Axis 1 sample scores from the CA, describing variation in fungal community composition, were also included as an additional explaining variable in the GLM, in order to test the impact of the fungal community on mass loss and respiration.

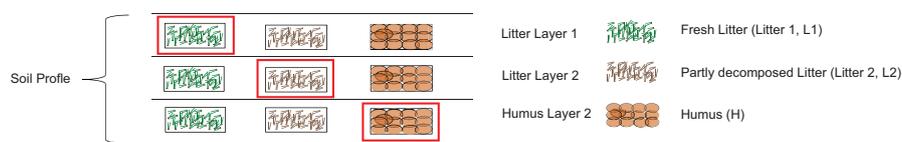


Figure 6. The factorial design of a field experiment, where natural organic substrates of different qualities were incubated in different layers of forest soil. The red frames indicate the control treatments of substrates, which were placed in their native layer.

Overall, all substrates were colonized by the fungal community native to the respective horizon the substrates were incubated in. Free-living saprotrophs, such as *Mycena* spp., consistently dominated in all materials incubated in the litter layers, and the different substrates buried in the humus layer were primarily colonized by a humus community, such as *Piloderma* spp., and other root-associated fungi, such as *Oidiiodendron* spp.. The vertical distribution of the soil fungal community mirrored a typical spatially separated fungal community of free-living saprotrophs and root-associated fungi in a boreal forest ecosystem (Lindahl et al., 2007). In addition to this spatial community segregation, the substrate type also had a smaller, but highly significant effect on the community composition, with humus deviating from the litter substrates in all horizons.

Mass loss is a proxy for decomposition over time, whereas respiration displays the microbial activity in the very moment of measurement. In this study, the results from both mass loss and respiration followed the same pattern

and are thus further referred to as combined “decomposer activity” conducted by the colonizing community.

After four months of incubation, fresh and partly decomposed litter was significantly more decomposed when incubated in the two litter layers, compared to when it had been buried in the humus. In GLMs of factors affecting decomposition, the fungal community composition (CA-axis 1) had equal or higher explanatory power than vertical placement of the litter bags, implying that the negative effect of incubation in the humus was primarily due to biotic effects, rather than abiotic factors, such as moisture and temperature. The community of free-living litter saprotrophs was thus more effective in decomposing fresh and partly decomposed litter than the humus-inhabiting, root-associated community. These results confirm earlier findings by Colpaert and vanTichelen (1996), who observed higher decomposer rates of litter colonized by the saprotroph *Lepista nuda* compared to litter inoculated with ectomycorrhizal fungi, growing in association with host seedlings in a microcosm experiment. Root-associated fungi have access to a C supply from plants and may therefore be less inclined to degrade external sources of C. Active foraging for nutrients, such as N and phosphorus in organic material (Read & Pérez-Moreno, 2003) may take place selectively, not leading to large-scale losses of organic matter. Although some ectomycorrhizal fungi have the potential to produce oxidative degrading enzymes, their capacity to produce many hydrolytic enzymes, necessary for large-scale litter degradation, has been questioned (Martin *et al.*, 2008).

In contrast, humus as a substrate was only marginally affected by decomposition, regardless of where in the profile the samples had been incubated or which fungal community that colonized them. Thereby, we may conclude that the low rates of humus degradation are not primarily dependent on the fungal community composition, but due to substrate quality. Humus has a lower C-quality than litter and is supposed to be more chemically complex with a higher concentration of phenolic substances and therefore rather recalcitrant to decomposition (Kögel-Knabner, 2002). Our results suggest that free-living saprotrophic fungi are not much more efficient in decomposing humus than root-associated fungi, and that the dependence of decomposition on the fungal community composition declines with increasing age and decreasing quality of the substrate. However, these results have to be considered with the limited time frame of this experiment in mind. Longer incubation times of several years may be necessary, in order to detect community dependent differences in humus decomposition rates.

Although the general trend was that all samples incubated in the humus layer were colonized by a typical humus community, after 16 months of

incubation, litter bags incubated in the humus layer were also colonized by the litter degrading species *Mycena galopus*. The retarded decomposition in the humus layer, observed at the first harvest, was also less obvious at the second harvest, and from its high relative abundance, we assume that presence and activity of *Mycena galopus* increased litter decomposition in the humus later in the experiment. Earlier studies have shown that *Mycena* species are efficient explorers and exploiters of newly introduced substrates (Boberg, 2009; Frey *et al.*, 2000, 2003) re-allocating N from more decomposed substrate within their mycelium to the fresh substrate. Due to its superior capacity as a litter degrader (Colpaert & van Tichelen, 1996) and, presumably, powered by resources reallocated from surface litter, the *Mycena* was able to seek out the hidden litter resources in the humus, establishing itself and partially replacing the root-associated community. Correspondingly, we found that two ectomycorrhizal species, *Piloderma sphaerosporum* and *Tylospora fibrillosa*, were able to colonize humus, even when it was incubated at the surface, putatively exploiting this material for nutrients (Bending & Read, 1995). Old, well decomposed, and chemically complex organic material has a higher N concentration, though it is chemically bound in complex organic forms (Berg & McLaugherty, 2003; Schmidt-Rohr *et al.*, 2004). These results indicate, that ectomycorrhizal fungi seem to be well adapted to, or even specialized in, colonizing humus and utilize it as a nutrient source. Some ectomycorrhizal fungi possess the enzymatic capacity for complex organic matter decomposition (Paper II) and have a secured C source through their interaction with host plants. Thereby, ectomycorrhizal fungi may have a competitive advantage towards saprotrophic litter degraders in colonizing humus, as the latter group depends on C obtained by decomposition and therefore requires substrates of better quality.

This study showed that the vertical separation of free-living saprotrophs and root-associated biotrophs in boreal forest soils depends on mutual exclusion by antagonistic interference as well as specificity towards different substrate qualities. Free-living saprotrophs were more efficient degraders of litter components, but were nevertheless prevented access to litter substrates placed in the humus, at least during the early phases of the study. This result corroborates previous observations in laboratory microcosms that root-associated fungi may exclude saprotrophs from soil substrates, by antagonistic mycelial interactions (Lindahl *et al.*, 2002).

Manipulations in the field are essential for understanding ecosystem processes, and the litter bag approach has been promoted as a less intrusive alternative. However, disturbances always lead to alterations in processes, as well as in behavior and abundance of R- and K- strategic organisms. Within

the first growing season, we found abundant colonization of opportunistic saprotrophic fungi, such as *Penicillium* and *Trichoderma* species, in all treatments and replicates. These R-strategists putatively benefited from low molecular weight compounds released in our substrate bags as a side effect of sterilization by gamma-irradiation prior incubation in the forest. Normally, in the absence of disturbance, the abundance of these fungi in boreal forest soils is rather low (Lindahl *et al.*, 2010), and the here observed high abundance decreased over the second growing season, during which the opportunists were replaced by other saprotrophs and root-associated fungi.

9 Conclusions

This thesis highlights that a phylogenetically wide range of ectomycorrhizal fungi possesses the genetic potential to produce oxidative organic matter degrading Mn-peroxidases (Paper I). The potential of Mn-peroxidase production is therefore not limited to wood decomposing fungi and other saprotrophs. These results widen the knowledge of ectomycorrhizal genetic potential, and open up for considerations about the functional ecological role of ectomycorrhizal fungi as potential decomposers of organic matter.

This is the first time that ectomycorrhizal Mn-peroxidase activity has been demonstrated in forest soils and the observed transcript was linked to the genus of *Cortinarius* (Paper II). Manganese-peroxidase transcripts in forest soils have so far only been detected by Kellner *et al.* (2010) who could not link them to any species. On a larger scale, Mn-peroxidase activity was spatially correlated with the presence of several *Cortinarius* species, but after N-addition the activity declined. This further strengthens our conclusion that ectomycorrhizal fungi, especially members of the genus *Cortinarius* and other rhizomorph forming species produce peroxidases in order to mobilize N from complex organic compounds. Rhizomorph-forming taxa dominate ectomycorrhizal communities of low-productive boreal forest ecosystems (Lindahl *et al.*, 2007) where N availability is low as a result of being chemically bound in complex organic compounds in the humus. The ability to exploit complex organic pools of N is likely to be a major competitive advantage for rhizomorph-forming ectomycorrhizal fungi in such ecosystems, and production of these enzymes may represent a costly adaptation to low mineral N availability. As a result of increased N-deposition, these ectomycorrhizal taxa may be outcompeted by non-rhizomorph-forming ectomycorrhizal fungi (Lilleskov *et al.*, 2001 & 2002; Figure 7).

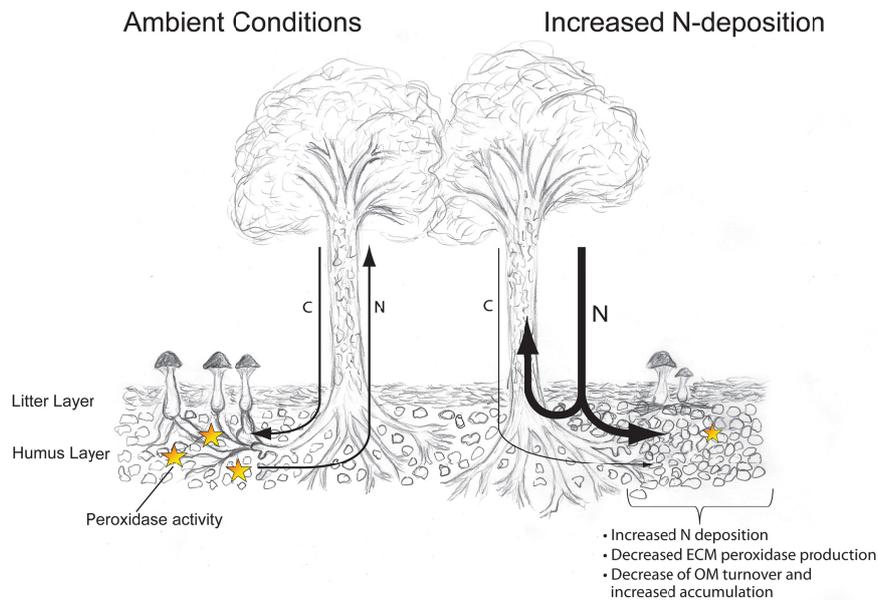


Figure 7. A hypothetical scenario of responses of the mycorrhizal plant-fungus-soil system to increased mineral nitrogen (N) deposition. The tree gets direct access to mineral nitrogen, and therefore down-regulates C allocation to ectomycorrhizal fungi. In response, ectomycorrhizal Mn-peroxidase activity in the soil (represented by stars) declines, leading to decreased losses of soil C.

Knowing that some ectomycorrhizal taxa may produce potent degrading enzymes, their decomposer potential has to be taken into consideration when investigating C turnover, especially in nutrient limited forest ecosystems. Being directly powered by recently fixed photo-assimilates, co-metabolic degradation of humus by mycorrhizal fungi would be regulated in a very different way from saprotrophic decomposition, with plant host-fungus interactions playing a central role. The N demand of the ectomycorrhizal fungus-host system is likely to have a strong regulatory influence on ectomycorrhizal turnover of organic matter in the humus layer. In a scenario of increased photosynthetic C fixation and C allocation belowground, due to elevated atmospheric CO₂ levels, the N demand of ectomycorrhizal fungi and their hosts may increase, potentially leading to higher production of ectomycorrhizal ClassII peroxidases. Thereby, more C would be lost from the forest floor, either as dissolved organic C or as CO₂ (Figure 8). In contrast, increased N-deposition decreases the N demand and inferentially decreases ectomycorrhizal organic matter decomposition (Figure 7).

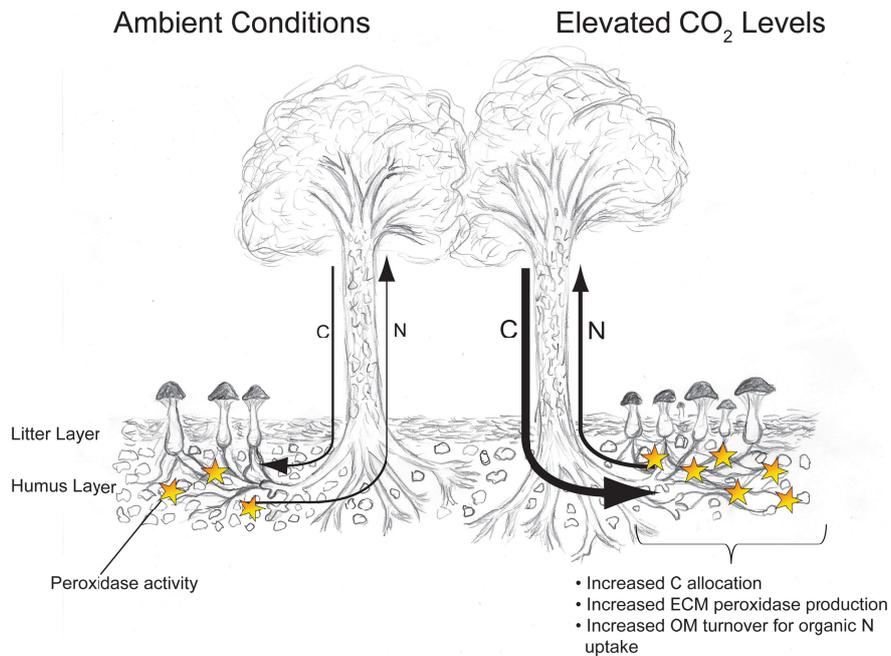


Figure 8. A hypothetical scenario of ectomycorrhizal responses to elevated CO₂ levels. With increased photosynthetic rate, more carbon (C) is allocated to the ectomycorrhizal mycelium, leading to increased Mn-peroxidase activity induced by an increased nitrogen (N) demand of the system.

Paper III demonstrated that the community composition followed primarily the vertical placement, indicating that competition drives the vertical separation of free-living saprotrophs and root-associated fungi, rather than the substrate type. However, root-associated fungi were less efficient in litter decomposition than free-living saprotrophs. Together with the finding that a few agaricomycetous species were able to colonize their native substrates outside their original horizon, which indicates that the substrate specificity influences the outcome of competition for space and resources. This competition may slow down decomposition (Gadgil & Gadgil, 1971, 1975; Koide & Wu, 2003) and therefore, the presence and activity of root-associated fungi may indirectly control C dynamics in boreal forest ecosystems. Due to overlapping fundamental niches and differences in the decomposition capabilities of both functional groups, the competitive balance between them is sensitive to environmental changes, such as nutrient and water availability and climatic changes. These changes may thus alter decomposition rates and thereby the C release in boreal forest ecosystems.

This thesis shows that ectomycorrhizal fungi in general may have two functions, besides their symbiosis, that influence the boreal forest ecosystem C cycle:

- 1) Decomposition of organic matter for N-acquisition.
- 2) Indirectly influencing saprotrophic litter decomposition by antagonistic interactions during competition for space and resources.

Constrained saprotrophic litter decomposition may counteract larger CO₂ releases derived from ectomycorrhizal decomposition described above (Figure 7 & 8), since saprotrophic litter fungi are more efficient decomposers than mycorrhizal fungi (Paper III; Colpaert & vanTichelen, 1996). Depending on the nutrient availability of a boreal forest ecosystem, the ectomycorrhizal community composition may differ (Lilleskov *et al.*, 2001, 2002), and a different community may influence the competition, altering decomposition, in different ways. Trees in nutrient limited forest ecosystems seem to be dependent on the presence of ectomycorrhizal fungi with high decomposer activity. Ectomycorrhizal fungi are more abundant under elevated CO₂ conditions and nutrient limitation (Treseder, 2004; Figure 8). Here, ectomycorrhizal fungi may thus have an increased competitive force towards the saprotrophic litter community, which decreases litter decomposition. In this manner, ectomycorrhizal may mitigate C releases from saprotrophic litter decomposition and thereby contribute to maintaining the C-sink of boreal forest ecosystems.

In general, environmental changes that disfavor root-associated fungi, including ectomycorrhizal fungi, such as clear cutting of forest stands, may in a long-term perspective lead to increased C losses from boreal forest soils. Understanding the ecological function of ectomycorrhizal fungi is therefore essential to be able to comprehend forest C dynamics and to sustain the boreal forest ecosystem as a global C sink with an appropriate forest management.

10 Future Prospects

We did not succeed to establish a direct regulatory response of ectomycorrhizal Mn-peroxidase gene transcription to increased N-availability, due to problems with establishing transcription levels in most samples. Optimization of mRNA preparation and PCR conditions would potentially help to enable a quantitative analysis of gene expression in the field, enabling confirmation of a direct down regulation of Mn-peroxidase gene transcription in response to N-addition. Ectomycorrhizal *Cortinarius* species are notoriously hard to cultivate, and the successful establishment of an ectomycorrhizal laboratory microcosm is a challenge. Still, microcosm studies with established symbiosis between a *Cortinarius* species and host seedlings would enable controlled investigations of several other parameters that may regulate Mn-peroxidase expression, such as up regulation under elevated CO₂ conditions, down regulation in response to shading, or induction in response to combat for nutrients in the presence of antagonistic fungal species (similar to Lindahl *et al.*, 1999, 2001). Sequencing the entire genome of a *Cortinarius* species would help to further elucidate the amount of duplicate Mn-peroxidase genes and their full-length sequences. This would enable a more certain phylogenetic placement of the genes within the Mn-peroxidase phylogeny.

New high-throughput sequencing methods have led to the initiation of numerous whole-genome sequencing projects, including a large variety of ectomycorrhizal fungi (JGI genome-projects: <http://genome.jgi.doe.gov/genome-projects/>). Annotated genomes will enable detection of new functional genes and comparative genomics may elucidate putative ecological niches of sequenced species. Full-length gene sequences will be directly available, and new PCR primers for detecting mRNA transcription can more easily be designed. The stored genome sequences can also be used as a reference library for blast searches. Genome-sequencing projects may act as a foundation for projects where the metatranscripts or the

metagenome from environmental samples are sequenced. Currently, such metatranscriptomic studies face a lack of reference sequence material to help interpretation of possible functions and identification of the organism hosting recovered genes. The more genomes are sequenced, the more reference data is available, helping to directly link observed genes in metagenomic studies to origin and function.

Sequenced genomes from other ectomycorrhizal species will also provide a better understanding of the distribution of Mn-peroxidase genes across a wider range of species. Presence and abundance of these genes could for instance be correlated with data of ectomycorrhizal species composition and distribution in a forest ecosystem, in order to predict putative activity hot spots, where increased Mn-peroxidase activity, and thus, increased organic matter turnover might occur. Furthermore, large-scale Mn-peroxidase transcriptomic analyses would be enabled, integrating their regulation upon changed environmental factors, such as the forest ecosystem N status, responses to increased atmospheric CO₂ conditions, or climatic changes. Results from such large-scale studies would help to substantiate climate C models, which predict global climate developments and are therefore important for political decision makers.

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