Functional Diversity in Nutrient Acquisition by Ectomycorrhizal Fungi

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2008

Acta Universitatis agriculturae Sueciae
2008:54

Cover: *Lactarius glaucescens* © Cajsa Nygren

ISSN 1652-6880
ISBN 978-91-85913-87-9

Tryck: SLU Service/Repro, Uppsala 2008

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Abstract

Nutrient uptake by boreal forest trees depends upon the symbiotic ectomycorrhizal (ECM) fungi that colonise more than 95% of the fine roots. In these ecosystems, nitrogen (N) is the most important growth-limiting nutrient for plants, followed by phosphorus (P). In the absence of anthropogenic influences, soil nutrients mainly occur in the form of organic compounds that are usually inaccessible to the plants. However, ECM fungi are able to utilise many of these organic resources via the production of a wide range of enzymes. What is known about the enzymatic capabilities of ECM fungi comes from experiments with a very small number of fast growing, easily culturable species. Very little is known about the functional capabilities of the most frequently occurring taxa that form ECM on fine roots.

In this thesis, the functional diversity of ECM fungi in nutrient acquisition was examined by assessing the ability of a wide range of ecologically important ECM fungi to use protein, nitrate and different P sources as nutrients. The ability to use protein by the excretion of extracellular proteases was widespread, which supports the theory that this trait is of considerable significance in the boreal forest. The ability to use mineral N was tested by assessing biomass production by a wide range of fungal isolates when grown on nitrate as the single N source. In addition, the genetic potential to reduce nitrate was examined by screening ECM fungal genomes. All isolates grew on nitrate and the nitrate reductase gene was found to occur widely among ECM forming taxa. All isolates grew on various P sources and differential patterns of nutrient use with respect to orthophosphate, organic P and apatite could be observed. Organic P sources were not necessary for phosphomonoesterase expression and, in addition, this enzyme expression was not correlated to growth rate.

The results from this study demonstrate that the enzymatic capabilities of ecologically important ECM fungi are continuously distributed between species rather than discrete. All the isolates had some ability to use all of the examined nutrient sources but the degree to which nutrients were utilised and converted to biomass varied considerably among taxa.

Keywords: ectomycorrhizal fungi, functional diversity, nutrient uptake, organic nutrients, proteases, nitrate assimilation, nitrate reductase, *nar1*, phosphorus uptake, phosphomonoesterase

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Always be wary of any helpful item that weighs less than its operating manual.
–Terry Pratchett

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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 Nygren, C.M.R., Edqvist, J., Elfstrand, M., Heller, G., Taylor, A.F.S. (2007). Detection of extracellular protease activity in different species and genera of ectomycorrhizal fungi. *Mycorrhiza* 17, 241-248.

II Nygren, C.M.R., Eberhardt, U., Karlsson, M., Lindahl, B., Parrent, J., Taylor, A.F.S. (2008). Growth on nitrate and occurrence of nitrate reductase genes in a phylogenetically diverse range of ectomycorrhizal fungi. *New Phytologist*. In press.

III Nygren, C.M.R., Rosling, A. Localisation of phosphomonoesterase activity in ectomycorrhizal fungi grown on different phosphorus sources (submitted manuscript).

IV Nygren, C.M.R., Karlsson, M., Lindahl, B., Taylor, A.F.S. *Hebeloma velutipes* does not express the nitrate reductase gene (*nar1*) when in symbiosis with *Pinus sylvestris* (manuscript).

Useful terms

Fungal terminology

Mycorrhiza	A symbiotic association between a fungus and plant roots.
Ectomycorrhiza	A mycorrhizal association where the fungus forms a sheath or mantle over the surfaces of terminal fine roots and grows in between the cortical cells of the root.
Sporocarp	The sexual structure of the fungi with spore-producing structures. Also known as fruit body.
Mycelium	Network of hyphae, the characteristic vegetative phase of many fungi.
Extramatrical mycelium	Hyphae extending from the mycorrhizas into the soil, essential for nutrient uptake and transport.
Hydrophilic	Literary mean "water loving". Hydrophilic substances are attracted to, and dissolve well in water.
Hydrophobic	Means "water fearing" and refers to substances that are repelled from water.
Rhizomorph	Aggregations of parallel-oriented hyphae that are often composed of wide vessel hyphae surrounded by narrower sheathing hyphae. Rhizomorphs are capable of transporting nutrients over long distances.

Enzymes involved in nutrient acquisition

Protease	An enzyme that breaks down protein. Also called proteinase or proteolytic enzyme.
Nitrate reductase	An enzyme that reduces nitrate molecules to nitrite.
nar	Nitrate reductase gene, coding for the nitrate reductase enzyme.
Nitrite reductase	An enzyme that reduces nitrite molecules to ammonium.
nir	Nitrite reductase gene, coding for the nitrite reductase enzyme.
Phosphomonoesterase	An enzyme that cleaves phosphate-ester bonds to release inorganic phosphorus from a range of substrates such as inositol phosphate, polyphosphates and phosphorylated sugars.

Molecular terms

Primer	A strand of nucleic acid that serves as a starting point for DNA replication.
Degenerate primer	A mixture of similar, but not identical primers used to amplify the same gene from different organisms.
PCR	Polymerase chain reaction, a technique used to amplify DNA fragments with the help of primers.
qPCR	Real-time quantitative polymerase chain reaction. Enables detection and quantification of a specific sequence in a DNA sample. Can be used to measure relative gene expression.
Cloning	The procedure of isolating a defined DNA fragment and obtaining multiple copies.

1 Background

1.1 Nutrient availability in the boreal forest

The boreal forest forms one of the largest terrestrial biomes on earth, covering most of Sweden, Finland, inland Norway, Russia, northern United States, inland Alaska and Canada (Figure 1). Altogether, this biome covers over 14 million square kilometers (Bonan, 1989). Boreal forests are relatively poor in plant species and only 3% of all vascular tree species on earth are found in these ecosystems.

Figure 1. The boreal forest is found throughout the high northern latitudes and is one of the largest terrestrial biome on earth. Picture adapted from http://en.wikipedia.org/wiki/taiga.

In Fennoscandia, the dominant tree species are Norway spruce (*Picea abies* [L.] Karst.), Scots pine (*Pinus sylvestris* L.) and birch (*Betula* spp.). Beneath the trees, the forest floor is dominated by ericaceous scrubs, lichens and

mosses. The soils tend to be young and nutrient-poor and fallen leaves, needles and moss can remain relatively intact on the forest floor for a long time in the comparatively cool and moist climate (Bonan, 1989). The highly lignified needles decompose slowly, creating a mat over the soil. Tannins and phenolic acids create very acidic conditions in the upper soil layers (Suominen *et al.,* 2003), which combined with the low temperature as well as low nutrient availability for decomposer organisms tend to limit decomposition processes. These climatic, chemical and biological factors result in accumulation of organic matter at the soil surface. Consequently, the nutrients are sequestered in compounds such as humified material, plant litter and live or dead microbial tissue (Tamm, 1991).

The most important limiting nutrient for plant growth in the boreal forest ecosystems is nitrogen (N) (Barbour *et al.,* 1987) and this nutrient is sequestered in a range of organic compounds ranging from simple amino acids and amino sugars to complex polypeptides and chitin (Leake and Read, 1997). The ability of forest trees to utilise organic N sources is limited to simple amino acids (Näsholm and Persson, 2001). However, most boreal forest trees form ectomycorrhizas (ECM) with a wide range of soil fungi, and it is believed that this symbiosis is essential for N uptake in these ecosystems (Smith and Read 1997).

In the absence of anthropogenic influences, N inputs from atmospheric deposition into boreal systems are low, ca. 1-3 kg N ha^{-1} $year^{-1}$ (Binkley *et al.*, 2000; Persson *et al.,* 2000; Brenner *et al.,* 2005) mainly in the form of nitrate (NO_3^-) originating from lightning discharges (Aneja *et al.*, 2001). During periods of perturbation such as snow melt, freeze-thawing and dry-wetting cycles, the soil mineral N levels can temporally increase, mainly in the form of ammonia (Schimel and Clein, 1996; DeLuca *et al.,* 1992).

Nitrogen-fixation is carried out by free-living bacteria and bacteria in symbiosis with certain legumes (Fabaceae) and other plants (e.g. *Alnus*). Rosen and Lindberg (1980) estimated the total N-fixation to ca 0.5 kg N ha^{-1} yr^{-1} in coniferous areas in central Sweden. However, DeLuca *et al.* (2002) found an N-fixing symbiosis between a cyanobacterium and the feather moss *Pleurozium schreberi* that alone fixes between 1.5 and 2.0 kg N ha^{-1} yr^{-1}. This indicates that the N fixation may be considerably higher than previously thought.

The second most limiting nutrient for plant growth in the boreal forest ecosystems is phosphorus (P) (Dalal, 1977). In the soil, there are three main sources of P; in solution as orthophosphate, ionically bound in primary and secondary minerals and bound in organic compounds (Jennings, 1995).

The major part of soil P (sometimes as much as 90%) is sequestered in the organic compounds phosphomonoesters and phosphodiesters (e.g. phospholipids and nucleic acids, Cosgrove 1967). Phosphomonoesters constitute a minor component of fresh litter, but accumulates in the soil to constitute 20 - 50 % of the total organic P in soil (Richardson, 1994; Schachtman *et al.,* 1998). The P uptake by forest trees has also been shown to be greatly enhanced in plants colonised by ECM fungi (Colpaert *et al.,* 1997; Conn and Dighton, 2000).

1.2 Ectomycorrhizal fungi

Mycorrhizas are symbiotic associations formed between a group of specialised soil fungi and roots of higher plants and literary means "fungus-root". The first description of this association was made by the German forest pathologist Frank in 1885. Mycorrhizas are present in all terrestrial ecosystems and play a central role in nutrient uptake (Smith and Read, 1997). In addition, mycorrhizal plants are often more resistant to diseases caused by microbial soil borne pathogens (Duchesne *et al.,* 1989) and to the effects of draught (Parke *et al.,* 1983).

The most common mycorrhizal association on trees and woody shrubs of the boreal forest is formed by ECM fungi. The first fossil record of ECM fungi is about 50 million years-old (LePage *et al.,* 1997) but Brundrett (2002) suggests that ECM symbiosis have evolved together with gymnosperms that emerged 190 million years ago. Moyerson (2006) put forward the theory that ECM fungi were present in ancestors of the Dipterocarpaceae approximately 135 million years ago.

Read and Perez-Moreno (2003) suggests that the ECM symbiosis has developed as an adaptation for plants to colonise soils where nutrients are bound in organic compounds. Plant roots alone are incapable of extracting most organic nutrients; however the mycelium of the ECM fungus can access these nutrients through excretion of extracellular enzymes.

ECM fungi are predominantly from the two phyla Basidiomycota and Ascomycota and globally, as many as 7-10 000 fungal species and 8000 plant species may be involved in this symbiosis (Taylor and Alexander, 2005).

The ECM symbiosis provides the fungus with carbon (C), provided by the plant *via* photosynthesis. The C is translocated in the form of soluble sugars and organic acids from the leaves to the root tissue. In the common apoplast of the plant/fungus interface, the C is transferred from the root as sucrose (Nehls *et al.,* 2007). Plant-derived enzymes called acid invertases

then cleave the sucrose into glucose and fructose that the fungi are able to take up (Nehls *et al.,* 2007).

Through the large absorbing surface area of the fungal mycelium, the plant's access to nutrients from the soil increases significantly (Smith and Read, 1997). When colonising a root, the fungus forms a mantle around the fine root tips of the tree from which hyphae extends inwards, between the plant cortical cells, forming a network of specialised cells called the Hartig net. This is the interface for exchange of C and nutrients between the two symbionts. In general, it is common to find at least 95 % of all tree short roots in the boreal forest colonised by ECM fungi (e.g. Taylor *et al.,* 2000).

Individual trees can form ECM associations with a diverse community of fungal species. Even within small areas (< 1 ha), a large number (> 100) of ECM fungi can be found, where individual host plants support many different fungal species simultaneously (Smith and Read, 1997). Multiple ECM fungal species can also coexist within a few centimetres on a single length of root (Gibson and Deacon, 1988) and sometimes even share a single root tip (Taylor *et al.,* 2000).

ECM fungi produce a range of morphologies with respect to their mantle anatomy and the extramatrical mycelia radiating into the soil as well as rhizomorph production. Agerer (2001) defined different exploration types of ECM fungi based on the amount of emanating hyphae and the presence and differentiation of rhizomorphs. In many species with extensive extraradical mycelium, the ECM mantles are hydrophobic due to water-repelling substances, hydrophobins (medium to long distance exploration types sensu Agerer, 2001), and the only hydrophilic component is formed by the hyphal tips. Other species have little extraradical mycelium and cover the root tip in a hydrophilic mantle (contact exploration morphotypes, Agerer, 2001). However, in both morphological variations it is the hyphal tips that are primarily involved in nutrient uptake (Unestam and Sun, 1995).

1.3 Functional diversity and responses to environmental disturbances

The concept 'functional diversity' has many definitions but here the following definition from Diaz and Cabido (2002) is used: 'the number, type and distribution of functions performed by organisms within an ecosystem'. A definition of functional diversity does not necessarily have to concern the organisms' nutrient acquisition, but feeding is a very important ecological process, being an essential component in energy and nutrient cycling in the ecosystems (Bengtsson, 1998). Therefore, it is not surprising

that many researchers have focused upon nutrient uptake when investigating functional diversity. In addition, when examining the functional diversity in ECM fungi it seems reasonable to concentrate on functions that influence the success of the host plant as well as the fungus itself.

Most ECM fungi are remarkably sensitive to changes in the abiotic environment in which they are growing (Erland and Taylor, 2002) and the differential responses to these changes may, to some degree, reflect functional diversity of ECM fungi. In particular, increases in mineral N availability can cause dramatic losses in species richness and substantially alter ECM community structures (Wallenda and Kottke, 1998). This is not surprising since mineral N levels (mainly in the form of nitrate) can increase by as much as 50-200 times *via* aerial deposition and/or forest fertilisation.

Some ECM genera, e.g. *Cortinarius, Piloderma* and *Suillus*, appear to be particularly sensitive to enhanced levels of soil N (Wästerlund, 1982; Brandrud, 1995; Fransson *et al.,* 2000; Taylor *et al.,* 2000; Lilleskov *et al.,* 2002). However, a few species from the genera *Laccaria, Lactarius, Paxillus* and *Russula*, have been found to increase fruit body production with augmented levels of soil N (Shubin, 1988; Lilleskov et al., 2001; Avis et al., 2003). Kåren and Nylund (1997) found, in contrast, no losses in species richness after mineral N additions. But all these studies on ECM communities found that the species composition changed after increased mineral N availability whether the species richness was constant or decreased.

In addition to altered sporocarp and ECM production, the quantity of external mycelium by ECM fungi has been found to decrease significantly after N additions in both laboratory studies (Wallander and Nylund, 1992; Arnebrandt, 1994) and in the field (Nilsson and Wallander, 2003). Alexander and Fairley (1983) also found that the number of colonised root tips decreased in fertilised plots.

These responses to added mineral N may result from differential abilities to metabolise nitrate. Those species that proliferate after N additions may be able to utilise nitrate more efficiently than those taxa which are negatively influenced. But the interpretation of the functional significance to an ecosystem of these changes in community structure is currently constrained by the almost total lack of knowledge of the functional capabilities of frequently occurring ECM taxa. By examining the ability to utilise nitrate and other nutrients in a wide range of taxa it may be possible to determine the factors that explain the response of ECM fungi to different disturbances. To date, there are very few studies that have compared enzyme production among a wide range of ECM species. This is probably due to the fact that

many ECM fungi have been considered to be very hard or impossible to grow in pure culture.

There is an increasing awareness that soil organisms, in particular mycorrhizal fungi, can have a significant influence upon above ground organism dynamics and on ecosystem processes as a whole. Increased ECM fungal diversity has been shown to result in increased plant productivity under certain conditions (Jonsson *et al.,* 2001; Baxter and Dighton, 2001; pageb2005). However, the plant response to ECM diversity can be dependent on the nutrient sources available (Baxter and Dighton, 2005).

By increasing the knowledge of the enzymatic capabilities of ECM fungi, it may be possible to understand why increased below ground diversity is potentially beneficial to above ground diversity. In addition, this knowledge will shed light on the factors that negatively affect many species after disturbance.

1.4 Enzyme systems involved in nutrient uptake and utilisation by ECM fungi

As most nutrients are bound in organic macromolecules, ECM fungi need to degrade these compounds in order to make the nutrients available for themselves and for the host plant. The hyphal tips of ECM fungi produce a wide range of enzymes which enable the plant host to access nutrients that would otherwise be unavailable to the plant (Lindahl *et al.,* 2005). Proteins can be degraded by fungal extracellular proteases, peroxidases contributes to the break-down of humus and chitin is degraded by chitinases (Burns and Dick, 2002). Since ECM symbiosis has evolved from saprotrophic ancestors (Hibbett *et al.,* 2000), considerable catabolic activities may be expected.

In addition to enzymes, ECM fungi also produce siderophores, compounds that incorporate Fe^{3+} ions in soluble complexes that can then be taken up by active transport mechanisms (Haselwandter and Winkelmann, 2007). Some ECM fungi (e.g. *Suillus* spp. and *Paxillus involutus*) are also known to produce a wide range of low molecular mass organic acids that can act as weathering agents on minerals such as apatite, potassium feldspar and quartz (Machuca *et al.,* 2007; Landeweert *et al.,* 2001).

Despite the great ecological importance of ECM fungi, almost nothing is known about the enzymatic capabilities of the species most commonly found on root tips. The current knowledge originates from a limited array of isolates from a handful of easily culturable ECM fungal taxa such as *Amanita, Hebeloma, Laccaria, Paxillus* and *Suillus*. Within Sweden, four large genera, *Cortinarius, Inocybe, Lactarius* and *Russula,* account for one half of the

diversity of ECM fungi (ca. 660 spp.), yet we know close to nothing about their functional capabilities. These taxa, together with species with resupinate fruitbodies (e.g. *Piloderma, Tomentalla* see Kõljalg *et al.,* 2000), are major components of mature ECM communities and form ectomycorrhizas most frequently on fine roots. For that reason they will be referred to as 'ecologically important species' from here on.

The enzyme systems that are further investigated in this thesis are presented below.

1.4.1 Extracellular proteases

The best-documented ability of ECM fungi to utilise organic nutrient sources is that of growth on proteinaceous substrates (Read and Perez-Moreno, 2003). Abuzinidah and Read (1986) categorised ECM fungi as protein or non-protein fungi, stating that some species specialize in using protein while others had very limited abilities. This classification remains widely used even though it is clear that ECM fungi display considerably intraspecific variation (Cairney, 1999).

Enzymes involved in protein break-down, collectively called proteases, act by hydrolyzing the peptide bonds that link amino acids together in polypeptide chains (Rao *et al.,* 1998). Proteases are divided into four major groups according to the character of their catalytic active site and conditions of action: serine proteases, cysteine proteases, aspartic proteases, and metalloproteases. A single fungal species may produce several different proteases depending on nutrient source and the pH of the environment (Gomi *et al.,* 1993; Sandhya *et al.,* 2005; Ogawa *et al.,* 1990).

To date, there are only two studies in which extracellular proteases produced by ECM fungi have been purified and characterised. Zhu *et al.* (1990) found a protease of unknown grouping in *Hebeloma crustiliniforme* with a molecular weight of 37.8kDa that was stable at a pH between 2.0 and 5.0. Nehls *et al.* (2001) found two proteases (45kDa and 90kDa) excreted by *Amanita muscaria* with pH optima at 3 and between 3 and 5.5 respectively. In addition, they determined the sequence for the 45kDa protease and found that it was very similar to aspartic proteases from other fungal species.

1.4.2 Nitrate reductases

Nitrate uptake is an energy-consuming process that requires active transport by NO_3^-/proton symporters, which results in an increase of the external pH as protons accompany the anion absorption (Galván and Fernándes, 2001; Javelle *et al.,* 2004), in a ratio of 2:1 (Eddy and Hopkins, 1985). After NO_3^- uptake, conversion to NH_4^+ requires eight reducing equivalents (Jennings,

1995). The high energy cost of nitrate assimilation is reflected in the tight control over the expression of the NO_3^- and nitrite (NO_2^-) reducing enzymes (Marzluf, 1997; Guescini *et al.,* 2003; Jargeat *et al.,* 2003). The genes coding for these enzymes are called *nar1* and *nir1*, respectively. For a simple summary on nitrate assimilation see Figure 2.

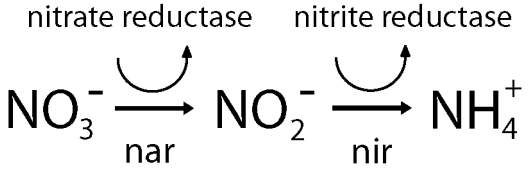

Figure 2. When nitrate is assimilated into the fungus it is first converted to nitrite by the nitrate reductase enzyme and then further to ammonium by the nitrite reductase enzyme. The genes coding for these enzymes are called *nar1* and *nir1*, respectively.

The use of NO_3^- as an N source has so far only been examined in a small number of ECM fungi and the results suggest that utilisation is very variable, both between and within species (France and Reid, 1984; Ho and Trappe, 1987; Anderson *et al.,* 1999). A few ECM species (e.g. some *Pisolithus* isolates) seem to prefer to grow on NO_3^- rather than on NH_4^+ (Scheromm *et al.,* 1990; Aouadj *et al.,* 2000), while others show limited (Sawyer *et al.,* 2003) or no growth (Norkrans, 1949) on NO_3^-.

To date, only two nitrate reductases from ECM fungi have been characterised, one from the basidiomycete *Hebeloma cylindrosporum* (Jargeat *et al.,* 2000) and one from the ascomycete *Tuber borchii* (Guescini *et al.,* 2003).

1.4.3 Phosphatases

Phosphomonoesterases are the enzymes primarily responsible for degradation of the organic P resources in soils (Burns and Dick, 2002). These enzymes cleave phosphate-ester bonds to release inorganic P from a range of substrates such as inositol phosphate, polyphosphates and phosphorylated sugars (Eleanor and Lewis 1973; Tibbett *et al.,* 1998).

The ability of ECM fungi to produce phosphomonoesterase has been investigated in fungal isolates that are easily grown in culture. However, the method commonly used, the *p*NPP-assay, has some draw-backs. Tibbett (2002) states that the large variations between studies in growth temperature, assay temperature, filtration (where the *p*Nppase can be lost) and washing steps can influence the results heavily.

In addition to phosphomonoesterases, some mycorrhizal fungi have also been shown to produce phosphodiesterases (Ho, 1987; Leake and Miles, 1996). Together with phosphomonoesterases these enzymes facilitate access to phosphorus in nucleic acids. However, phosphodiesters have a very low persistence in soil, rarely exceeding 1% of the total P (Paul and Clark, 1989).

This thesis focuses on the enzymatic capabilities of ecologically important ECM taxa and aims to relate the different physiological abilities to the ecology of the species. To examine the enzymatic abilities of ecologically important fungi will also provide basic physiological information that so far has been lacking. In addition, this knowledge may shed light on the factors that negatively affect many species after mineral N additions.

2 Objectives

The overall aim of this work was to characterise functional diversity in N and P acquisition by ECM fungi. The specific objectives were:

- To obtain pure cultures from ecologically important but intractable ECM species, i.e. species that form dominant components of mature forest ECM communities such as *Cortinarius, Lactarius, Russula, Tomentella* and *Tricholoma*. These cultures were subsequently used in **Paper I-III**.

- To examine how widespread the ability to use organic N is in ECM fungi by investigating the production of extracellular proteases and to further characterize the numbers and molecular weight of these proteases. We hypothesized, since these fungi have evolved in the boreal forest with very little mineral N, that the ability to use protein as an N source is widespread (**Paper I**).

- To investigate the ability of ECM fungi to metabolise nitrate by growing them on nitrate as sole N source and screening for the occurrence of genes coding for the nitrate reductase enzyme in a wide range of ECM fungal species. We hypothesised that those taxa which proliferate with elevated N levels (e.g. *Lactarius*) may be able to utilise nitrate more efficiently than those taxa which are negatively influenced (e.g. *Cortinarius, Piloderma* and *Suillus)*. (**Paper II**).

- To examine the capabilities of ECM fungi to utilise different P sources in the form of orthophosphate, organic P and apatite. To

investigate if the phosphomonoesterase activity differs during growth on the different P substrates and to assess the localization of this enzyme activity within the mycelium (**Paper III**).

- To assess the nitrate reductase expression by *Hebeloma velutipes* in symbiosis with *Pinus sylvestris*, and to compare this with the gene expression found in pure culture (**Paper IV**).

3 Methods

A brief summary of the methods used is given here. More detailed information can be found in the respective papers and the references cited therein.

3.1 Fungal material (Paper I-III)

A major problem in ectomycorrhizal research is that only a few tractable species have been isolated in pure culture. Consequently, we base our knowledge of functional capabilities of ECM fungi on just a few species that are easily grown in pure culture, e.g. species of *Hebeloma, Laccaria, Paxillus* and *Suillus*. However, these species are in general not major components of mature forest ECM communities (Horton and Bruns, 2001). The most species rich and perhaps the most ecologically important genera such as *Cortinarius, Lactarius, Russula, Tomentella* and *Tricholoma* are usually considered to be extremely difficult, if not impossible, to isolate into pure culture (Smith and Read, 1997). The first aim of this thesis was to obtain cultures of these untractable taxa.

Cultures were obtained mainly from fresh sporocarp material by breaking off a part of the cap and removing small pieces of fungal material close to the gills. The explants were plated into modified Melin–Norkrans (MMN) media (Marx 1969). For most species, ca. 50 explants were usually taken from each sporocarp.

To confirm the identities of the isolates, ribosomal DNA (the ITS region) was sequenced using the primer ITS1F and ITS4 (White *et al.,* 1990) and the obtained sequences were then compared with the sequence databases UNITE (Kõljalg et *al.,* 2005) and GenBank (Benson *et al.,* 2005).

3.2 Organic N utilisation (Paper I)

To assess the potential of the ECM fungal isolates to use organic N, two different methods were used, milk powder plates and zymograms. Milk powder plates are agar plates with a thin layer of insoluble milk protein on top. Hence, the casein in the milk renders the upper layer of agar opaque. Diffusion of extracellular proteases from the mycelial front creates a clear zone around the fungal colony as the proteins are broken down. This method is suitable for detecting even very low levels of protease production by slow growing ECM fungi. In total, 118 isolates were grown on milk plates.

The other method used was the zymogram technique, which involves growing fungi in liquid cultures and then separating the secreted enzymes with electrophoresis in a polyacrylamide gel containing gelatine. After electrophoresis, removal of SDS (sodium dodecyl sulphate) from the gel by washing in a 2.5% Triton X-100 solution allows the enzymes to renature and to degrade the protein substrate, gelatine. Staining of the gel with Coomassie blue allows the bands of proteolytic activity to appear as clear bands of lysis against a blue background. Thirty-five isolates were grown in liquid culture and tested for extracellular protease activity.

3.3 Nitrate utilisation (Paper II)

To examine the potential of ECM fungi to use mineral N in the form of nitrate, a wide range of species (106 isolates representing 68 species) were grown in liquid culture with nitrate as sole N source. The biomass, the levels of nitrite and ammonia released into the media and the pH changes were used as indicators of nitrate use. The nitrate and ammonia levels were measured with a flow injection analyser. Determination of NO_2^- and NH_4^+ used the methods of Henriksen and Selmer-Olsen (1970) and Svensson and Anfält (1982), respectively.

In addition, the genetic potential to produce the nitrate reductase enzyme was investigated by screening 43 strains (representing 31 species and 10 genera) of ECM fungi for the occurrence of the nitrate reductase gene. Degenerate primers were designed by comparing the only known ECM basidiomycete nitrate reductase gene from *Hebeloma cylindrosporum*, with fully sequenced genomes from other basidiomycetes. The primers were located in conserved regions and amplified a ca 700bp fragment of the *nar1* gene (Figure 3).

To acquire sequences for the amplified nar fragments, the obtained PCR products were cloned by ligation into a vector which was used to transform

chemically competent *Escherichia coli* cells. The cloned products were sequenced and a neighbour joining tree was constructed and bootstrap values of the individual branches were calculated.

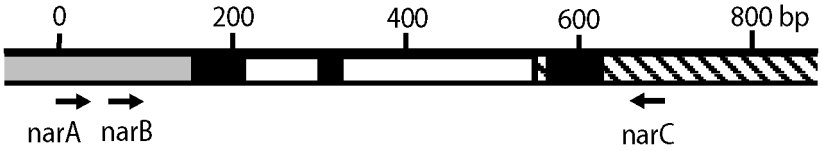

Figure 3. A fragment of the nitrate reductase gene in ectomycorrhizal fungi amplified with degenerate primers. The sequenced region was ca. 700bp long. The primers narA and narB are located in the region coding for the molybdopterin binding domain (marked with grey) and the reverse primer narC is located in the region coding for cytochrome b5-like Heme/Steroid binding domain (marked with stripes). The black areas mark the approximate location of the three introns found in the characterized *nar1* gene of *Hebeloma cylindrosporum*.

Where the primers failed to amplify any nitrate reductase products, a Southern blot hybridisation was performed. This technique uses a probe to check for the presence of a DNA sequence. Whole genome DNA is cut into smaller fragments with restriction enzymes after which the fragments are separated on an agarose gel using electrophoresis. All the negatively charged DNA fragments in the gel are then transferred and fixed to a sheet of positively charged nitrocellulose membrane. The membrane is exposed to a hybridisation probe, a single DNA fragment (the PCR product from the nitrate reductase gene in this case) whose presence in the target DNA is to be determined. The probe is labelled with radioactive ^{32}P in order to be visualised on an X-ray film. The location of the DNA fragments corresponding to the probe is visualised as black bands on a white background.

3.4 Phosphomonoesterase activity and growth on different P sources (Paper III)

The ability of ECM fungi to use different P sources was assessed by growing 19 isolates (representing 16 species) on agar plates where P was supplied as inositolphosphate (an organic P source), in mineral form as orthophosphate or as apatite (the most common P-containing mineral in Earth's crust).

The method used to measure phosphomonoesterase activities was carried out on intact systems to avoid damaging the cells and measured extracellular and surface-bound enzyme activity. By overlaying the surfaces of the living fungal colonies with a thin gel containing the fluorogenic substrate 3,6,8 –

Tris - (dimethylaminosulfanyl) – 1 - pyrenol phosphate pyridine salt, the phosphomonoesterase activity could be determined. When the substrate is cleaved by the enzymes in the colony it becomes fluorescent and can then be measured with a fluorescence spectrophotometer. The advantage of adding the substrate in agar is that the location of the enzymatic activity in the mycelia can be determined. To assess if the phosphomonoesterase expression varies depending on P source, the activity was measured over the organic P plates as well as the orthophosphate and apatite plates.

3.5 Nitrate reductase expression by *Hebeloma velutipes* when in symbiosis with *Pinus sylvestris* (Paper IV)

The nitrate reductase gene in the fungus *Hebeloma cylindrosporum* was found to be constitutively expressed in pure culture (Jargeat *et al.,* 2000). However, determining gene expression in mycelia is problematical and there is increasing evidence that enzymatic activities measured in pure culture only represent a potential activity and may not reflect ecological activities (Perez-Moreno and Read, 2000).

To examine the spatial expression of the nitrate reductase gene in symbiosis with a host tree, soil microcosms were set up with *Hebeloma velutipes* (localized in the same species complex as *H. cylindrosporum*, see Aanen *et al.,* 2000) in symbiosis with *Pinus sylvestris*. Wells with acid washed sand were inserted into the microcosms and one well in each microcosm was treated with ammonia (that is known to down-regulate the nitrate reductase gene in pure culture, Jargeat *et al.,* 2000).

Mycelia were harvested and the expression of the nitrate reductase gene and the control gene α-tubulin (*tub1*) was measured. This was done by extracting total RNA from mycelia in the tested areas in the microcosms. In order to be able to measure gene expression, the total RNA was then amplified linearly by T7 transcription. Finally, the gene expression was analysed using quantitative real time PCR.

4 Results and Discussion

ECM fungi are dominant and important components of soil microbial communities in the boreal forest, where they carry out a central role in nutrient cycling processes. It is likely that many of these fungi fulfil broadly similar ecological functions and that some degree of functional redundancy exists in ECM fungal communities (Allen *et al.,* 1995). However, given their taxonomic diversity, communities of ECM fungi are still likely to possess a vast amount of functional heterogeneity or diversity.

This thesis addresses the question of how much functional diversity is expressed, with respect to nutrient acquisition, by the ECM fungi of the boreal forest. Here, we show that ECM fungi are able to use protein, nitrate and organic P as nutrient sources, but that the ability to use different nutrients is quantitative rather than qualitative. Practically all fungi have the ability to utilise the tested substrates as nutrient sources, but the ability may be highly variable between species.

4.1 Growing ecologically important ectomycorrhizal fungi

Despite repeated attempts to obtain isolates from fresh sporocarp material, only a small percentage of species from some genera produced cultures. In particular, species within *Cortinarius*, *Russula* and *Inocybe* were the most intractable with no isolates obtained from *Inocybe*. In most cases where cultures were obtained, the mycelium was usually derived from a single explant, even when ca. 50 were plated out. Surprisingly, once growth was initiated, the *Cortinarius* isolates had a relatively rapid growth rate (5 mm wk^{-1}). *Lactarius* species, even though some of them were amongst the slowest growing, were the most amenable to pure culture isolation. Once in culture, *Lactarius, Hydnum, Piloderma, Russula* and *Tricholoma* showed measurable but very slow growth on agar, ca. 1–15 mm $month^{-1}$ (Figure 4). However,

several *Lactarius* isolates had growth rates comparable to *Amanita* or *Suillus* species with growth rated around 20-30 mm month^{-1} (e.g. *Lactarius lacunarum, L. sanguineus, L. chryssoreus*). The extremely slow growth rates of some isolates dictate that any analysis of enzymatic capabilities should not be dependent on a large biomass production.

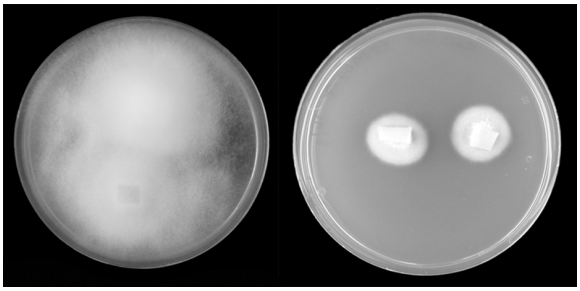

Figure 4. The growth rates of ectomycorrhizal fungi in pure culture can vary greatly. The fungus to the left is the fast growing species *Amanita spissa* after one month of growth and the right-hand picture shows *Russula integra* after six month of growth.

In addition to the freshly obtained ECM cultures, cultures from Ursula Eberhardt (Fungal Biodiversity Centre, Utrecht, The Netherlands) and Andy Taylor (Macaulay Institute, Aberdeen, UK) were compiled into an extensive culture collection that was used in the following experiments.

4.2 Organic N utilisation (Paper I)

The majority (30/33) of ECM species produced detectable protease activity. The detection was, however, method dependent. Milk powder plates proved to be the most effective method, suitable for screening slow-growing isolates for extracellular protease production. Zymograms could only detect proteases in four species.

The break down of the milk protein was visible as a cleared zone around the colony in the milk plates and this cleared zone varied considerably between species (Figure 5a). Some mycelia degraded the milk powder proteins over extensive areas while others had narrow cleared zones in the immediate vicinity of the mycelial front. Even a very thin mycelium was sufficient to produce a transparent or clear zone in the milk agar. Some isolates did not produce a clear zone around the mycelia and this may be due to wall-bound enzymes or to low diffusion rates. However, these isolates had created a cleared zone beneath the mycelium. Retaining the enzymes in close proximity to the hyphae could be expected to greatly enhance the uptake of any assimilable breakdown products produced by these enzymes.

In addition to the 33 species tested in paper I, the whole culture collection was grown on milk plates. Seventy species (108 isolates) out of 77

(118 isolates) produced extracellular proteases, indicated by cleared zones around or under the mycelia. The ability to degrade proteins were found in the taxa *Amanita, Amphinema, Boletus, Cenococcum, Cortinarius, Gyrodon, Hebeloma, Hydnum, Laccaria, Lactarius, Leccinum, Meliniomyces, Paxillus, Piloderma, Pisolithus, Rhizoscyphus, Russula, Sarcodon, Suillus, Tomentella, Tricholoma* and *Xerocomus*. All tested taxa contained at least one isolate that produced extracellular proteases.

The zymogram approach only detected extracellular proteases in *Amanita muscaria, Russula chloroides, Lactarius deterrimus* and *L. quieticolor* (Figure 5b). The molecular weight of these proteases was around 45, 90 or 150kDa. Intraspecific variation was obtained in *A. muscaria,* one isolate secreted two proteases with a size of ca. 45 and 90kDa and another produced a single protease at 90kDa.

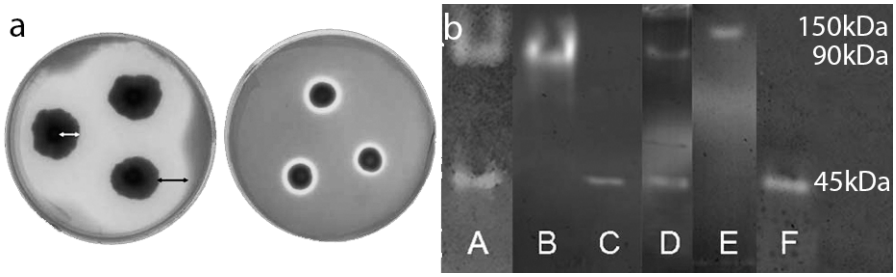

Figure 5. a: Growth of two ectomycorrhizal fungi (left: *Lactarius zonarius*; right: *Hydnum rufescens*) after 1 month on media containing insoluble milk powder where cleared zones indicate the activity of secreted proteases. The white arrow indicates mycelial growth and the black arrow the cleared zone. b: Zymograms showing extracellular protease activity in culture filtrate derived from the growth of ectomycorrhizal fungi on liquid medium containing casein hydrolysate as an N source. Lane A: *A. muscaria*, two proteases with a molecular weight of around 45 and 90 kDa. Lane B: Another isolate of *A. muscaria* UP500, one 90-kDa protease. Lane C: *L. quieticolor* one protease, 45 kDa. Lane D: Same isolate of *L. quieticolor* at another time point, two proteases 45 and 90 kDa. Lane E: *L. deterrimus*, around 150 kDa and Lane F: *R. chloroides*, 45 kDa. The bands are from different gels except for B and C.

The lack of detection of extracellular proteases in most species with the zymograms may have several explanations. The SDS in the loading buffer may have caused irreversible damage to the protein structure, which prevented renaturation. Support for this comes from the observation that the ericoid endophyte *Rhizoscyphus ericae* (previously *Hymenoscyphus ericae*) isolate did not produce any bands in the zymograms, but is known to have very high extracellular protease activity (Leake and Read, 1990). However, the proteases appeared to be remarkably stable; it was possible to transfer a

plug of agar from the cleared zone of one plate onto a new milk powder plate and obtain a new cleared zone.

It is also possible that aggregation of the protease molecules, either with themselves or other proteins, into larger units restricted entry into the zymogram gel. As the molecular weights of fungal acidic proteases are generally in the range of 30 to 45 kDa (Rao *et al.,* 1998), we hypothesise that the 90-kDa and 150-kDa proteases are dimers and trimers of the 45-kDa protease.

To date, protease production has only been conclusively demonstrated in a small fraction of the huge diversity of ECM fungi. The data presented in Paper I significantly increase the number of ECM species known to produce proteases. More importantly, most of these species are representatives of ecologically important taxa for which there was little, if any, previous data due to problems related to their culturability. The ability to produce extracellular proteases is widespread in ECM fungi. This is not surprising since the majority of N is sequestered in organic compounds in the boreal forest.

4.3 Nitrate utilisation (Paper II)

4.3.1 Growth on nitrate

Biomass production varied considerably between the 106 isolates, but all isolates had some ability to grow on nitrate (Figure 6). Daily growth rates spanned one order of magnitude from a minimum of 0.2 (*L. mitissimus*) to a maximum of 2.4 mg day^{-1} (*Meliniomyces bicolor*), with a mean of 0.80 ± 0.06 mg day^{-1}. In general, species of *Amanita, Lactarius* and *Russula* had comparable and the lowest rates of mycelial growth, while *M. bicolor*, *Rhizoscyphus ericae, Rhizopogon roseolus, Paxillus involutus, Xerocomus communis, Laccaria bicolor* had the highest rates. *Suillus* grew well on NO_3^- and, along with *Piloderma* and *Tricholoma*, produced significantly greater biomass than *Lactarius* and *Amanita* (Figure 6).

Since nitrate uptake into cells is accompanied by the influx of two protons (Galván and Fernándes, 2001) the pH of the culture medium is expected to increase after growth on nitrate. The majority of the species increased the pH of the culture medium with the greatest increases associated with *Cenococcum geophilum, M. bicolor, Tricholoma album, R. ericae* and *Hebeloma* sp. There was a significant correlation between biomass production and pH of the medium. However, a number of isolates, in particular *Tricholoma* spp. and suilloid fungi, either maintained or lowered

the original pH of the medium. Many ECM fungi, including *Suillus*, are known to produce a wide range of low molecular mass organic acids that may act as weathering agents (Machuca *et al.,* 2007; Landeweert *et al.,* 2001). Lapeyrie *et al.,* (1987) observed elevated oxalic acid excretion into the growth when growing *Paxillus involutus* on nitrate. One possible explanation for the anomalous pH values is that these fungi are releasing large quantities of low molecular mass organic acids into the culture medium and thus counteracting the rise in pH associated with nitrate uptake.

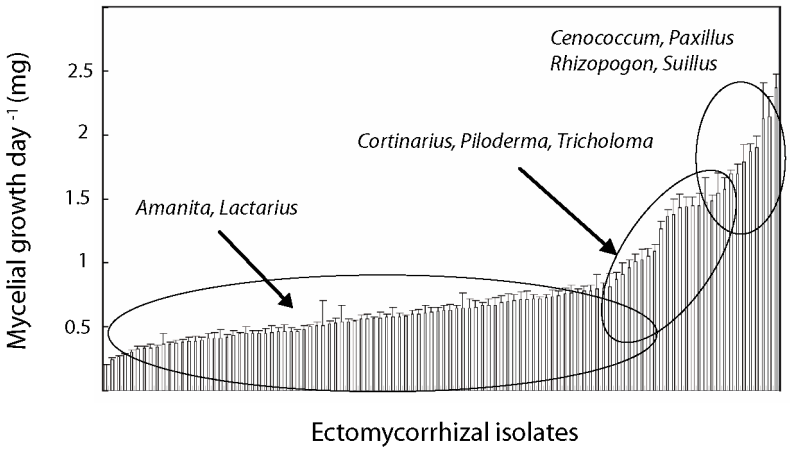

Figure 6. Mycelial growth on nitrate as sole N source of 106 isolates of ectomycorrhizal fungi.

In general, the levels of NH_4^+ and NO_2^- in the cultures filtrates were low but many isolates from the genus *Lactarius* produced high NH_4^+ concentrations relative to the majority. Boeckstaens *et al.* (2007) recently examined loss of ammonium from *Saccharmomyces cerevisiae* cells when growing on different N sources. They suggested that cells may control the internal ammonium concentrations by releasing it through non-selective cation channels. But Boeckstaens *et al.* also suggested that cells were unable to prevent ammonia (NH_3) diffusing through the plasma membrane and that released NH_3 was reabsorbed as ammonium by the non-selective cation transporters involved in its release. It is possible that uncontrolled loss of NH_4^+ or NH_3 by *Lactarius* species in our study may have restricted biomass production. However, it is questionable if ammonia would be released from the mycelium of *Lactarius* spp. in a natural system where the host root would act as a strong sink for ammonia (Chalot *et al.,* 2006).

Most NO_2^- values in the culture filtrates were close to the lower detection limit of the analysis method. But some isolates, especially *C. geophilum, L. acerrimus, Suillus granulatus*, *Paxillus involutus* and *Pisolithus arhizus*, produced much higher amounts of NO_2^-. This could suggest differential rates of the nitrate reductase and nitrite reductase activities.

4.3.2 Occurrence of nitrate reductase encoding genes

We obtained 34 nar amplicon sequences from the 43 tested strains with the degenerate primers. The nitrate reductase gene was found to occur widely in ECM boletales and in all four of the clades within the agaricales that contain ECM forming taxa (Matheny *et al.,* 2006). Only a single sequence of the nar gene was amplified from each fungal species.

No sequences corresponding to nar genes were recovered from taxa within *Amanita* and the Russulaceae with any combination of PCR programmes or primers. There are several plausible explanations for this. The presence of introns in the primer site could have prevented amplification. However, no gene fragments were amplified from cDNA constructed from mRNA from any isolates from these groups, suggesting that primer failure was not due to introns. In additional attempts to obtain gene sequences from these taxa, ten other primer pairs were designed from different conserved parts of the nar gene (data not shown), but these also all failed to detect the nar gene even though several could amplify the gene from the genus *Hebeloma*.

The Southern hybridisation using the *Hebeloma mesophaeum* nar PCR product as a probe, yielded bands in all *Amanita, Lactarius* and *Russula* species, indicating the presence of a nar gene in these taxa. This would suggest that amplification failure is due to mutations in the primer binding sites. The primers were designed from highly conserved regions with narA and narB located in the region coding for the reducing active site and narC located in the region coding for cytochrome B5 where heme-Fe is bound (Campbell and Kingshorn, 1990). The conserved nature of these primer binding sites in all other investigated taxa suggests that there has been a loss of selective constraints in the nar gene in taxa within the Russulaceae and *Amanita* and that this has resulted in accumulations of mutations in the primer binding sites.

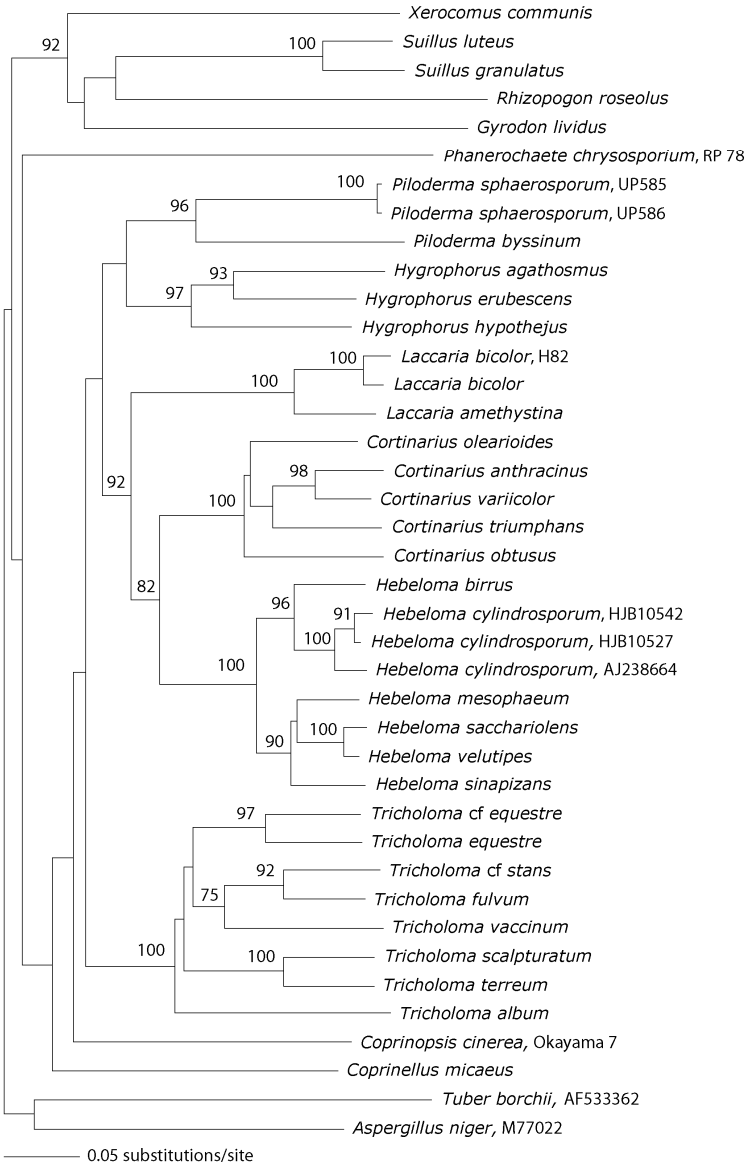

Figure 7. A rooted neighbour joining tree of amplified coding regions (ca 550 bp) of the nitrate reductase gene from a range of ectomycorrhizal fungi. The numbers on the branches refer to a bootstrap analysis carried out with 1000 replicates. The sequences were obtained by cloning and sequencing PCR products obtained with degenerate primers. Sequences for *Phanerochaete chrysosporium*, *Laccaria bicolor* and *Coprinopsis cinerea* were obtained from whole genome sequences. The sequence from *Tuber borchii*, *Aspergillus niger* (chosen as an outgroup) and one of the *Hebeloma cylindrosporum* sequences were acquired from GenBank.

The Neighbour-joining analysis of the obtained nar gene fragments successfully grouped species of the same genera together and all genera represented by multiple species received high bootstrap support (Figure 7). In addition, there was sufficient variation within the gene fragment to clearly distinguish the taxa at the species level, with the exception of *Hebeloma sacchariolens* and *H. velutipes*. Higher taxonomic groupings were also upheld within the tree, with the bolete taxa forming a well supported group separated from the other ECM taxa.

4.3.3 A theory linking differential responses to nitrate in the field to the morphology of the mycorrhizas

Chronic (atmospheric deposition) or drastic (forest fertilisation) additions of N into forest ecosystem usually result in significant changes in ECM communities (Avis *et al.,* 2003; Lilleskov *et al.,* 2001, 2002; Peter *et al.,* 2001). We hypothesized that the taxa which proliferate with elevated N levels (e.g. *Lactarius*) may be those that are able to utilise nitrate more efficiently than those taxa which are negatively influenced (e.g. *Cortinarius, Piloderma* and *Suillus)*. There was however, no support for this idea from the growth on nitrate, with *Lactarius* isolates compromising the main bulk of the species with the slowest biomass increments.

An explanation for the proliferation of some ECM taxa under elevated N may relate to the nature of the mycorrhizas which they form and the pathways of mineral N uptake and metabolism. The uptake of NH_4^+ occurs *via* passive transport along an electrical potential difference across the plasma membrane through specific membrane bound proteins (see Boeckestans *et al.,* 2007), while NO_3^- uptake is energy-consuming involving a nitrate transporter protein (Javelle *et al.,* 2004; Slot *et al.,* 2007). The high energetic cost of nitrate uptake and subsequent reduction means that any mechanism that enabled the nitrate to enter directly into the host tissue without any metabolic processing by the fungus would be advantageous to ECM fungi.

Most *Lactarius* and *Russula* taxa form hydrophilic, smooth mantles with few emanating hyphae (Contact exploration types, sensu Agerer, 2001). The hydrophilic nature of these structures may allow nitrate to diffuse through the mantles and pass directly into the host plant. Movement of nitrate through the apoplast could deliver N to the fungal/plant interface in the Hartig net without a need for the fungus to process the mineral N and thus avoid the C drain that this would entail. This enhanced N supply could result in down regulation of monosaccharide uptake back into the root cortical cells (Nehls *et al.,* 2007) and the fungus receiving additional C, leading to increased growth and potentially greater fruit body production.

By contrast, *Cortinarius*, *Piloderma, Suillus* and *Tricholoma* species produce mycorrhizas with hydrophobic mantles and extensive mycelial systems in which nutrient uptake takes place some distance from the mycorrhizal root tips (Agerer, 2001). In order to avoid potential toxicity effects of nitrate,

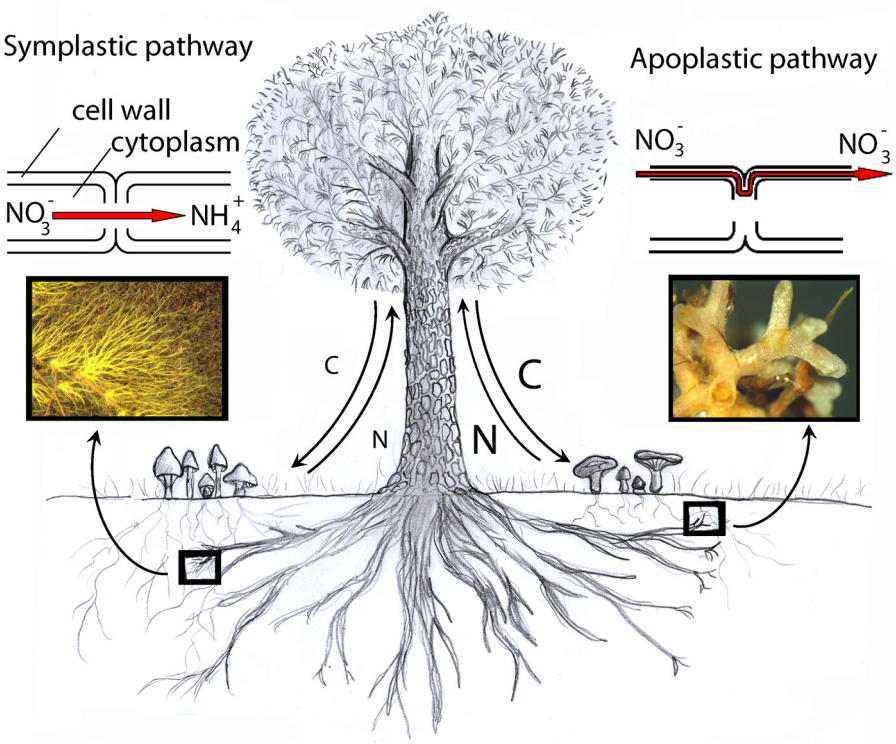

Figure 8. A hypothesis that could explain the differential responses to N fertilisation of different fungi. When nitrate is taken up by morphotypes with hydrophilic, copious mycelia (left, e.g. *Cortinarius*) it must first be converted through an energy consuming process to ammonium via nitrite. Morphotypes with hydrophilic mantles and little extraradical mycelium (right, e.g. *Lactarius*) might be able to pass to transport nitrate directly *via* an apoplastic pathway thus avoiding the cost of metabolism. More N would be transported *via* fungi with smooth, hydrophilic mantles that would receive more C in return. This could result in the increased sporocarp production by these latter species. Photos of ectomycorrhizas by Andy Taylor.

nitrite and ammonia (Stöhr, 1999), these taxa must first metabolize these compounds before translocating them to the host. This would create a significant C drain, thereby leading to a reduction in mycelial growth and reduced fruiting. For a summary of this theory, see figure 8.

One notable exception among ECM fungi to this potential link between mycorrhizal morphology and the negative effects of N additions is *Paxillus involutus*, which often responds to N addition by producing large numbers of fruit bodies (Shubin, 1988). *Paxillus involutus* mycorrhizas develop an extensive soil mycelium, but the species grows well on nitrate. Intriguingly, Ek *et al.* (1994) found that *P. involutus* was able to transfer N as nitrate through the mycelium to the host plant, suggesting a mechanism for avoiding potential toxic effects of nitrate.

In conclusion, even though ECM species are generally considered to be adapted to ecosystems where mineral N, especially nitrate, is present in trace quantities, many of them appear to readily metabolize nitrate as an N source. The widespread abilities to use both organic and mineral N in ECM fungi supports the view that in these nutrient poor conditions, the fungi have the ability to acquire N from a wide range of potential sources.

4.4 Use of different P sources and phosphomonoesterase activities (Paper III)

4.4.1 Growth on different P sources

The mycelial radial growth varied considerably between the 19 isolates and between the treatments, but all isolates had some ability to grow on orthophosphate, organic P and apatite. In general, the average mycelial area was largest in the orthophosphate treatment, followed by the organic treatment. The lowest average growth was found on apatite.

Three isolates of *Amanita muscaria*, *A. spissa*, *Cortinarius glaucopus, Laccaria bicolor* and *Meliniomyces bicolor* (Figure 9a) grew significantly better on the orthophosphate plates compared to the plates with organic P. The opposite was observed in the isolates of *Lactarius chrysorrheus*, *Suillus bovinus* and *S. variegatus* (Figure 9b), which grew more on organic P. In addition, the mycelia of *L. chrysorrheus* produced much thicker mycelia on the organic substrate. However, in general, the isolates appeared to produce mycelia of similar density in all treatments.

ECM fungal community composition has been observed to vary with depth in a vertically stratified soil profile (Rosling *et al.,* 2003). In such profiles the forms of P vary from complex organic forms in the litter layer, to simpler organic forms and mineral forms further down in the profile (De Brouwere *et al.,* 2003). It is plausible that different P utilisation strategies among ECM fungi are a contributing determinant for the composition of mycorrhizal communities through vertically differentiated soil profiles. The

isolates in this study are mostly isolated from fruit bodies and information on where in the soil profile they proliferate is not available. However, *S. variegatus* is frequently found in the upper soil layers (Genney *et al.,* 2006) and in our study this species grew better on organic P and orthophosphate compared to apatite.

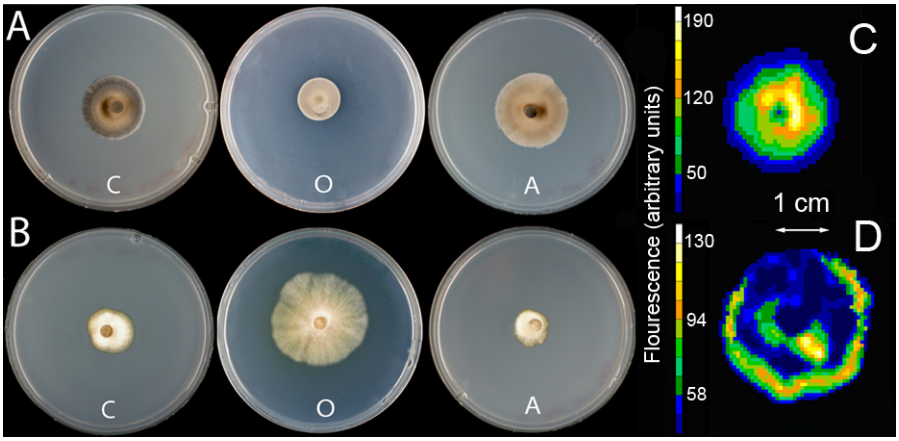

Figure 9. A: *Meliniomyces bicolor* and B: *Suillus variegatus* grown on agar plates for four weeks. Phosphorus was supplied as orthophosphate (c), in organic form as phytic acid (o) or in mineral form as apatite (a). Phosphomonoesterase activity over living mycelia of C: *Amanita muscaria* and D: *Suillus bovinus* after growth on phytic acid in arbitrary units after incubation with an overlay gel containing fluorogenic substrate. Note that the scales for the arbitrary units differ between picture C and D.

In general, pH changes appeared to be species dependent, but independent of the substrate. The isolates of *A. muscaria*, *C. glaucopus*, and *S. luteus* increased the pH to levels between 5 and 6 while *L. chrysorrheus, M. bicolor, Rhizoscyphus ericae* and *Serpula lacrymans* decreased pH to below 4. The remaining isolates maintained the pH at 4-5. When growing on ammonia, the pH of the culture media decrease as protons are released (Jennings, 1995). In this study, the *A. muscaria* isolates and *C. glaucopus* instead increased the pH. However, the pH can increase when phosphate ions are transported through high affinity proton symporters (Kothe *et al.,* 2002). In addition to P transport by proton symporters, ECM fungi may also possess sodium symporters that would render the pH of the media unchanged (Kothe *et al.,* 2002).

Substrate acidification may also be an active mechanism to increase mineral dissolution under P limiting growth conditions (Rosling *et al.,* 2007). Among the isolates that did not produce any enzymatic activity, *L.*

chrysorrheus and *R. ericae* lowered pH to below 4. This may reflect a strategy to obtain P by stimulating weathering rather than phosphomonoesterase activity.

4.4.2 Phosphomonoesterase activity

The agar overlay method used in this study provided simultaneous whole colony analysis of the surface phosphomonoesterase activity. The enzymatic assay was well suited to screen a large number of isolates and had, in addition, the advantage of showing the spatial distribution of the activity over the mycelial surface. Overall, there was no significant correlation between mycelial size and enzymatic activity. The highest activity was found in the *A. muscaria* isolate when growing on orthophosphate. No phosphomonoesterase activity could be detected in any treatment for the species *C. geophilum, L. chrysorrheus, Piloderma* cf. *fallax* and *R. ericae*. Only a few isolates expressed significantly different enzyme activity between different media.

The majority of the species expressed the highest enzyme activity in the interior parts of the mycelia (Figure 9c). Lindahl and Finlay (2006) found the same pattern when growing wood-rotting fungi on spruce wood and screening for chitinolytic enzymes. They suggested that the fungi utilise endochitinases to degrade older parts of the mycelia in order to recycle the nitrogen and translocate it to newer parts of the mycelia. The same may be true for P use, the break-down of phospholipids in the cell walls of old hyphae may be the cause of the high enzyme activity. Another possibility is that the interior activity simply reflects a higher concentration of cells in the more dense mycelia of the interior parts of the colony. In contrast, two fungi, *S. bovinus* and *Laccaria bicolor,* had the highest activity in the actively growing parts at the edge of the colony (Figure 9d). This may reflect more expansive phosphorus uptake strategies. Both species are pioneer species and may be adapted to forage for nutrients more competitively than later colonisers.

The phosphomonoesterase activities did not require organic P for activation. This is consistent with Antibus *et al.* (1992) who found no general differences in enzyme production after growth on inorganic and organic P sources. In fact, one isolate of *A. muscaria* and *A. spissa* actually expressed significantly higher enzyme activity in the orthophosphate treatment compared to the organic treatment. In addition, the isolates of *M. bicolor* and *T. scalpturatum* only produced measurable amounts of enzyme when grown on the apatite. This could reflect increased enzymatic activity under P limiting growth conditions (Aleksieva and Micheva–Viteva, 2000). It is likely that phosphomonoesterases are always active, to some extent, at

the mycelial surface to activate proteins on the outer surface of the plasma membrane for physiological activity (Arnold 1987).

There was little intraspecific variation among the four *A. muscaria* isolates in this study. Only one *A. muscaria* isolate differed with respect to enzyme activity and had significantly higher enzyme activity on orthophosphate compared to the organic treatment. All the *Amanita* cultures have been isolated from the same forest in Uppsala. It is possible that the variation within these species had been higher if the isolates originated from more diverse environments.

The pH optima for most of phosphomonoesterases of the species included in the study is not known. It is plausible that the pH value of the media was unsuitable for induction of phosphomonoesterase activity in some cases. Previous studies using *C. geophilum* have demonstrated maximum phosphatase activity with no available P and at a pH of around 4 as well as phosphorus concentrations around 3.5 mM and a pH around 5 (Alvarez *et al.,* 2004). In *R. ericae,* the maximum activity of phosphomonoesterase was found between pH 5.5 and 6 (Leake and Miles 1996). The individual pH optima of the phosphomonoesterase activity must be known before the maximal enzymatic capacity for different species can be assessed. No extracellular phosphomonoesterases activity could be detected outside the mycelial front in any of the isolates in this study, indicating that the enzymes were wall-bound.

The agar overlay method proved to be a suitable method for screening fungal cultures for phosphomonoesterase activities. We show that the production of phosphomonoesterases is widespread in ECM fungi and to accurately assess the maximal enzymatic activity, the individual pH optima has to be considered.

Phosphomonoesterase activity does not need induction by organic P and most species in this study expressed higher enzyme activities in the central parts of the mycelia. This may reflect break-down and recycling of phospholipids from old hyphae or simply higher mycelial density.

4.5 Nitrate reductase expression in *Hebeloma velutipes* when in symbiosis with *Pinus sylvestris* (Paper IV)

The *P. sylvestris* root systems were fully colonised after seven weeks and after being transferred to flat microcosms, the compression of the peat ensured that the mycelial network grew mostly on the surface of this substrate. After four months, the mycelia had grown over the wells in the microcosm (Figure 1).

No expression of *nar1* could be detected in the undisturbed systems or in wells where ammonium sulphate had been applied. By contrast, *tub1* expression was detected in *H. velutipes* in both situations indicating that RNA extraction and analysis were performed correctly. Attempts to detect very low levels of *nar1* expression by linear amplification of RNA, up to 600 times, before reverse transcription also failed to detect *nar1* expression.

These results are in contrast to the constitutive expression of *nar1* by *H. cylindrosporum* in axenic cultures (Jargeat *et al.,* 2000). This difference may be related to regulatory differences between *H. velutipes* and *H. cylindrosporum*, but is more likely a consequence of differences in the experimental systems used. Hence, our study suggests that results on transcriptional regulation from pure culture systems may not always reflect the situation in more natural systems with a functioning mycorrhizal symbiosis.

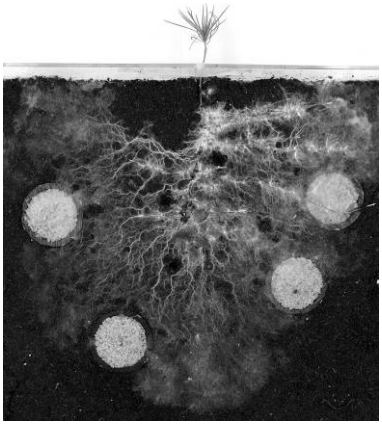

Figure 10. Ectomycorrhizal seedlings of *Pinus sylvestris* colonised by *Hebeloma velutipes* growing in flat microcosms. The mycelia grew over the quartz sand wells which were treated with $(NH_4)_2SO_4$ or Na_2SO_4. The mycelia were then harvested and the nitrate reductase and α-tubulin expression were measured.

This difference may be due to reduced enzymatic activities as a consequence of interactions with the soil substrate (Lindahl *et al.,* 2005). In addition, in a natural system, the plant host can act as a sink to prevent accumulation of nutrients that would otherwise repress enzymatic activity. However, in our study the presence of a host tree did not increase enzyme activity. It is possible that when grown in pure culture, the fungus can express nitrate reductase freely as it has access to the high glucose levels in the growth media. In contrast, a fungus in symbiosis may be carbon limited and therefore avoids the costly nitrate reductase production in the absence of substrate.

Although the axenic culture approach have some limitations, it can still be very informative; the enzymatic activities may be considered potential activities and the method has established that many ECM fungi have the

potential to utilise organic (e.g. Leake and Read, 1997; Read and Perez-Moreno, 2003: Paper I) and mineral (Smith and Read, 1997; Paper II) nutrient sources. In addition, it can also demonstrate that the external environment (e.g. nutrient availability, pH and temperature) may strongly influence production and activities of different enzymes.

5 General discussion and conclusions

In the boreal forest ecosystem where nutrients are bound tightly in organic compounds it is vital for ECM fungi to be able to access N and P from all available sources. We were able to show that taxa from the Cortinariaceae, Russulaceae and Tricholomataceae can be grown in pure culture, but that their isolation requires more persistence than other fast-growing, more tractable genera. By using alternative methods we could measure enzymatic activities in these slow-growing taxa. This thesis expands the knowledge on the enzymatic capabilities of those ECM fungi that constitute the dominant components of ECM communities in mature forests.

ECM fungi from a wide range of taxa were found to be able to metabolise organic N in the form of protein and organic P in the form of inositol phosphate. The ability to metabolize mineral N in the form of nitrate was also found to be widely spread among ECM fungi.

We show that, somewhat in contrast to Abuzinadah and Read's (1986) concept of protein and non-protein fungi, there is a wide range of proteolytic capabilities in between these two extremes. Virtually all ECM fungi can use protein as an N source, an expected feature in the boreal forest where most nutrients are sequestered in organic compounds (Paper I). Surprisingly, even though many ECM species are negatively influenced by N fertilisation (Wallenda and Kottke, 1998), all tested ECM fungi produced biomass when grown on nitrate as sole N source (Paper II). This is a strong indication that all available N must be taken up, even if the nutrients are usually only present in trace amount. In paper III, the fungi in all treatments were supplied with small concentrations of easily available orthophosphate. All isolates grew well and differential patterns of nutrient use with respect to orthophosphate, organic P and apatite could be observed. Paper IV supports the theory that enzymatic activities in pure culture can only be considered to be potential activities and do not necessarily reflect the ecological

functioning. However, enzymatic activities in axenic culture provide a fundamental, stable base to design more complex experiments from.

To date, most evidence shows that within plant species assemblages there tend to be a fairly continuous distribution of traits (e.g. Reich *et al.,* 2003; Diaz *et al.,* 2004). This thesis indicates that the ability of ECM fungi to use different nutrients is also continuously distributed between species. It is likely that nutrient uptake by ECM fungi would be better described quantitatively rather than qualitatively. This quantitative uptake may be measured in terms of biomass, enzyme expression, nutrients passed to the host plant or the uptake rate of the nutrient, depending on the question asked.

The number of functional groups or the functional group richness is often used as an approximation of functional diversity in an ecosystem (Wright *et al.,* 2006). These kinds of groupings assume that the traits of importance are discrete rather than continuously distributed between species. Therefore, it can prove to be problematic to design experiment that depends upon grouping ECM fungi, or any other organism group with discrete traits, in clusters depending on functional traits.

There is an increasing awareness that ECM fungi can have a significant influence upon above ground organism dynamics and on ecosystem processes (Jonsson *et al.,* 2001). In addition, other groups such as bacteria, protozoa and microarthropods that show specialization with regard to ECM species (Smith and Read, 1997) could be severely affected by the loss of a single ECM species or a group of similar species. Given the high degree of ECM diversity and the potential threats to this diversity, a greater understanding of the various functional roles of ECM fungi is needed. With increasing knowledge, it may be possible to determine potential ecosystem scale impacts of changes in community structure.

6 Future prospects

Interpretation of the functional significance of changes in community structure due to disturbances such as increases in N deposition (Wallenda & Kottke, 1998), soil acidification (Erland & Taylor, 2002) and additions of lime and ash (Taylor & Finlay, 2003) is still constrained by a lack of knowledge of the functional capabilities of most ECM taxa. In addition, the ecological relevance of screening for enzymes in artificial environments in the absence of host plants can be questioned (see Read and Perez-Moreno, 2003). The enzymatic measurements from these systems only reflect the potential activities and do not necessarily reflect the enzymatic activities in the soil. A solution to this problem would be to extract mRNA from soil samples and investigate, with specific primers, to what degree the ecologically important species contribute to uptake and break-down of certain nutrients.

The publication of the whole genome for *Laccaria bicolor* (Martin *et al.,* 2008), the first ECM fungus to be completely sequenced, enables better primer design for regions coding for different enzymes. For example, degenerate primers for the different groups of extracellular proteases can be designed and a wide range of ECM fungi could be screened for the presence of these genes. The expression of these proteases may be examined in pure culture by extracting mRNA and measuring the expression with real-time quantitative PCR. Even though most ECM fungi seem to be able to use protein, more functional diversity may be detected when comparing utilisation of different protein and peptide sources. In addition, specific primers could be designed and the contribution of ecologically important species to protein degradation in soil could be assessed as above.

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Acknowledgements

This thesis was made possible by the support of FORMAS, The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning.

Foremost I would like to thank my main supervisor Andy Taylor for giving me the opportunity to work with ectomycorrhizal fungi. Your never-ending enthusiasm for fungi and my project was a constant motivation. You encouraged me to work independently and explore different methods and techniques. I am very grateful for all your help, positive feedback and constructive criticism. We have also had many interesting discussions and lots of laughter and fun. Having you as a supervisor has never been boring!

Thanks also to Ursula Eberhardt, Magnus Karlsson and Björn Lindahl for support with the molecular work. Ursula always helped me navigate around all the sneaky zebra fish of GenBank and Magnus' experience in qPCR was very helpful. Björn is always up for interesting discussions and gave supportive advice on fungal genetics.

I am very grateful to two amazing persons at the Plant Biology and Forest Genetics Department: Ingrid Eriksson for saving me in desperate times and helping out with Southern blots; and Johan Edqvist for help with the zymograms and for many excellent sushi lunches and discussions.

Jeri Parrent and Malin Elfstrand are gratefully acknowledged for being very helpful with various aspects of molecular work. Katarina Ihrmark and Maria Johansson were always ready to answer questions and give advice in the lab. Thanks to Anna Rosling for the enjoyable collaboration on paper III. It was great fun working with you! Many thanks to Karin Backström for administrative help and for lessons on how to play nyckelharpa.

Thanks to all former and present colleagues at the department for being friendly and supportive. Special thanks go to Jonas and Ylva for help with

innumerable things and for being great friends. Thank you, Johan, for lovely forest excursions with epic amounts of *Macrolepiota procera*! Kalle, Johanna, Mikael and Greg made the conference in Australia a hilarious experience. Thanks to the crazy Rhizosphere-gang that roamed the streets of Montpellier in search of wine and new super-hero names led by Professor Pastis. I would especially like to thank Petra (or… Isotopia!). Thank you Fredrik for never-ending discussions on 'Lost' and Les, Hironari, Ina, Kerstin and Nicklas for lots of fun at and off work!

Thanks also to all the wonderful people I have had the opportunity to meet on conferences and courses. I hope to see you again in the future!

I am also very grateful to Ellen Larsson and Stig Jacobsson at the University of Gothenburg for introducing me to the wonderful world of mycology during my MSc studies.

My old and new friends distracted me from work and gave me moral support when progress was slow. Special thanks to Åsa and Csilla for long discussions on PhD studies and to Tove, Terese, Peter, Joanna, Ebba, Ingela, Anna and Maria for being great friends! Thanks to Thomas, Linda and Joakim for chaotic trips abroad and lovely dinners. Thanks to everyone I got to know through Sunday evenings full of drama and fun at ÖG. The choir Dissonantia enabled me to have lovely Monday evenings with lots of fika and songs.

Extra special thanks go to my wonderful family for always supporting me through all the strange things I have decided to do with my life. My grandparents Erik and Ulla are constantly ready to go and have serious talks with evil reviewers. My parents Inger and Gösta always believe in me when I need it the most, and Agnes is thanked for generally being the most wonderful person and sister in the world!

Finally, I would like to thank Martin for always being able to make me laugh and for your never-ending love and support. Thanks for being in my life and for wanting to share it with me.