

ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

SILVESTRIA 235



Responses of Ectomycorrhizal Fungi to Changes in Carbon and Nutrient Availability

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**Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2001**

Abstract

Fransson, P. M. A. 2001. *Responses of ectomycorrhizal fungi to changes in carbon and nutrient availability*. Doctor's dissertation.
ISSN 1401-6230, ISBN 91-576-6319-X

Ectomycorrhizal (ECM) fungi may receive 20% of the total C fixed by their host plants and are essential components of host nutrient acquisition. As a consequence of the vast physiological diversity that exists among ECM fungi, changes in community structure may potentially alter C and nutrient allocation and turnover within forest ecosystems. Effects of atmospheric CO₂ enrichment and balanced nutrient addition on the community structure of ECM fungi were investigated. Significant effects of elevated CO₂, as well as elevated nutrient levels were found. Daily nutrient additions for 10 years did not cause reductions in the density of ECM roots or the degree of root colonisation, in contrast to other studies. Some species became more common due to nutrient additions; *Cenococcum geophilum*, *Amphinema byssoides*, *Tylospora fibrillosa*, tomentelloid species, and others, *Piloderma byssinum* and *P. croceum*, became less common. High variability among samples made individual species responses difficult to distinguish. Data suggest that the same species may respond similarly to both elevated CO₂ and nutrient additions. In laboratory experiments, CO₂ enrichment increased the production of extraradical mycelium by *Hebeloma crustuliniforme*, increasing mycelial spread and root colonisation. Under field conditions such a response could enable species to increase in abundance. The natural abundance of the stable isotope ¹³C in fruitbodies can be used as a tool to distinguish between the two functional groups ECM and saprotrophic fungi. However, some caution is necessary in the interpretation since values overlap between the two functional groups. The ¹³C values can also be used to reveal the host-origin of carbon in mycorrhizal fungi in mixed forests. Generalist fungi, which can be associated with several different tree species, were found to receive most of their C from overstorey trees, as indicated by their high δ¹³C values. This implies that large trees which are able to fix more C potentially subsidise smaller trees via a common ECM mycelial network.

Key words: Basidiomycetes, boreal forest, morphotype, whole-tree chamber, fertilisation, ¹⁵N, *Picea abies*, *Pinus sylvestris*, *Paxillus involutus*

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Appendix A

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

- I. Högberg, P., Plamboeck, A. H., Taylor, A. F. S. & Fransson P. M. A. (1999). Natural abundance of ¹³C abundance reveals trophic status of fungi and host-origin of carbon in mycorrhizal fungi in mixed forests. *Proceeding of National Academy of Science* 96: 8534-8539.
- II. Fransson P. M. A., Taylor, A. F. S. & Finlay, R. D. (2001). Elevated atmospheric CO₂ alters root symbiont community structure in forest trees. *New Phytologist* 152: 431-442.
- III. Fransson P. M. A., Taylor, A. F. S. & Finlay, R. D. (2000). Effects of continuous optimal fertilisation upon a Norway spruce ectomycorrhizal community. *Tree Physiology* 20: 599-606.
- IV. Fransson P. M. A., Finlay, R. D. & Taylor, A. F. S. (0000). Mycelial production, spread and colonisation of *Hebeloma crustuliniforme* and *Paxillus involutus* under elevated CO₂. *New Phytologist*. Submitted.

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Introduction

Fungi account for a large part of the biodiversity in boreal forests, and about 1100 fungal species in Sweden form a symbiotic association called ectomycorrhiza, with the roots of major tree species (Dahlberg *et al.*, 2000). Ectomycorrhizal (ECM) fungi have a direct influence both on the sequestration and emission of carbon (C) from the soil, and on nutrient circulation, and are thus important components of ecosystem processes. They are likely to be affected by changes in atmospheric CO₂ level or by addition of fertiliser, which will affect the availability of C and nutrients for the fungi. The experiments described in this thesis were designed to investigate ECM community structure in relation to C and nutrient availability, and, to a lesser extent, how changes in species composition are related to ecophysiological differences among species.

The mycorrhizal symbiosis

General aspects of the mycorrhizal symbiosis

The mutualistic association between fungi and plant roots known as mycorrhiza is found in all terrestrial ecosystems, and was first described by the German forest pathologist Frank in 1887 as 'mykorrhiza', which literally means 'fungus-root' (see Read, 1987). The majority of land plant species have the mycorrhizal association, and in the boreal forest ecosystem the predominant form of mycorrhizal association between fungi and trees or woody shrubs is called ectomycorrhiza. There are other types of mycorrhizal associations, *e.g.* arbuscular mycorrhiza (AM), which is the most common underground symbiosis, formed between fungi and hosts such as grasses and herbaceous plants. The fungi forming ectomycorrhiza are predominantly basidiomycetes (Basidiomycota), and in some cases ascomycetes (Ascomycota). Many of them form large fruitbodies which are commonly found growing on the forest floor, but up to 70% of the mycorrhizal root tips may be colonised by species forming inconspicuous resupinate fruit bodies or not forming fruit bodies at all (paper III). In the symbiosis the fungus forms a sheath (mantle) around the terminal plant roots from which hyphae extend out into the soil for nutrient acquisition (Fig. 1). The fungus also extends inward from the mantle, 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 plant and fungus. Both fungus and plant rely upon the other symbiotic partner; the tree for nutrient uptake and the fungus for a supply of C. Almost 100% of forest tree short roots are colonised by ECM fungi (Taylor *et al.*, 2000; paper III), and the root uptake of water and nutrients is greatly enhanced by the mycelium which increases the uptake surface area. The high colonisation level also means that most of the nutrients and water taken up by the tree will first pass through the fungus. In addition to the nutritional benefits, the ECM association can increase the tolerance of the host plant to

environmental stresses, e.g. heavy metals, drought and pathogen attacks. (Smith & Read, 1997)

The ECM fungal mycelium – production, spread and colonisation

Mycelial growth form, spread and mycorrhizal morphology differ among ECM species (Agerer, 2001). The colonisation of root tips depends on inoculum potential. Garrett (1970) defined inoculum potential as ‘the energy of growth of a parasite available for infection in a host, at the surface of the host organ to be infected’, and also discussed the ‘invasive force’ of a parasite. The definition can be applied to ECM fungi with modifications as follows: inoculum potential of an ECM species depends on (1) the production of mycelium, (2) the spread of the mycelium, (3) the ability to establish on uncolonised root tips and (4) the ability to outcompete other fungi and establish on already colonised root tips. The inoculum potential of already established mycelia in the soil may be high (Ek *et al.*, 1996) and seedlings germinating in a forest have a high probability of becoming colonised by the mycelial network that exists in the soil (Francis & Read, 1994; Amaranthus & Perry, 1994).

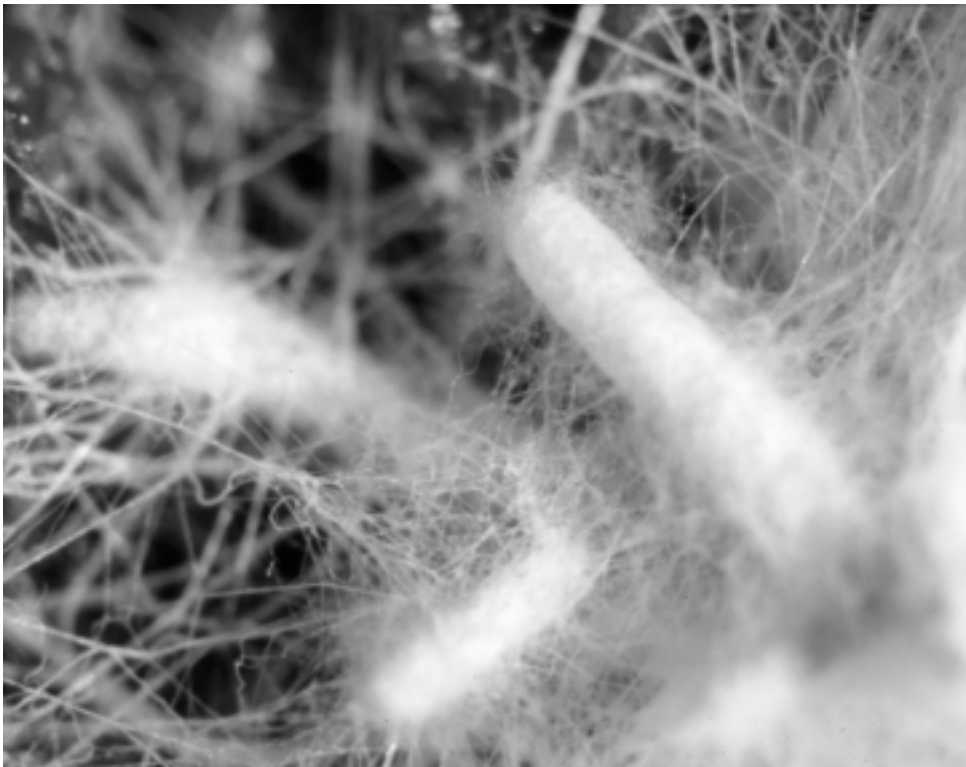


Figure 1. Mycorrhizal root tips formed by *Tricholoma albobrunneum* ('kastanjemusseron'), a fungus found growing in coniferous forests. Extensive extraradical mycelium grows out into the soil from the mycorrhizas. Photo: Petra Fransson.

Colonisation of roots can either be primary or secondary. In primary colonisation mycorrhizas are formed on uncolonised root tips. When the fungus is established, new roots usually expand from within the fungal mantle, and are in fact precolonised. In cases when there is a rapid proliferation of root tips, *e.g.* when a wetting front passes through the soil, the root tip may break out through the mantle and the abundance of non-mycorrhizal roots may be high (paper III). In secondary colonisation, there is direct competition between species or individuals, and a strong competitor may establish on and outcompete an already established fungus. ECM fungi can form composite mycorrhiza when one fungus is taking over root tips from another fungus (Wu *et al.*, 1999). Fungi with rapid growth rates in culture, *e.g.* *Paxillus involutus* and *Suillus bovinus*, colonise roots more rapidly than slow growing fungi (Shaw *et al.*, 1995). In the same study, Shaw *et al.* (1995) showed that some species stimulated both the growth and root colonisation of other fungi (*Lactarius rufus* stimulated *P. involutus* and *S. bovinus*), while others (*Laccaria laccata*) suppressed mycorrhizal formation of *P. involutus* and *S. bovinus*.

Carbon cycling in boreal forests

Carbon and a changing environment

Two major processes determine the net exchange of CO₂ between the atmosphere and the terrestrial biosphere - photosynthesis (the autotrophic fixation of CO₂) and respiration (Fig. 2). There may be a fine balance between these two processes (Valentini *et al.* 2000), and forests can act both as sources and sinks for CO₂ (Lindroth *et al.*, 1998). Valentini *et al.* (2000) showed that total soil respiration, most notably that of roots and soil microorganisms, can be the main determinant of the C balance of forests. Global atmospheric levels of CO₂ continue to increase due to burning of fossil fuels and land-use change (Schimel *et al.*, 1996), and we are likely to see a doubling of the CO₂ levels over the next 100 years (see Keeling *et al.*, 1995). This is a problem that has been discussed intensively in the context of global climate change ecology during the last 20 years (*e.g.* Craig & Holmen, 1995; Falkowski *et al.*, 2000). The possibility of sequestering CO₂ into forest biomass has been suggested as a more long-term solution to handle the C originating from antropogenic sources (Dixon *et al.*, 1994; Schimel *et al.*, 1996; Watson *et al.*, 2000), but the problem is of a complex nature and not easily solved (Falkowski *et al.*, 2000). The assumption that atmospheric CO₂ and increasing N deposition would drive C uptake in forests (*e.g.* Vitousek *et al.*, 1997) is not always valid - Nadelhoffer and colleagues presented work in 1999 which suggested that increasing N does not necessarily stimulate C uptake in temperate forests. One component of the intricate web of organisms and related processes that affect the C balance of forests are the mycorrhizal fungi, and their potentially large role in C cycling in boreal forests has been acknowledged (Allen, 1996). Most C-balance models do not generally consider separate groups of organisms, but rather deal with whole compartments of the forest. For

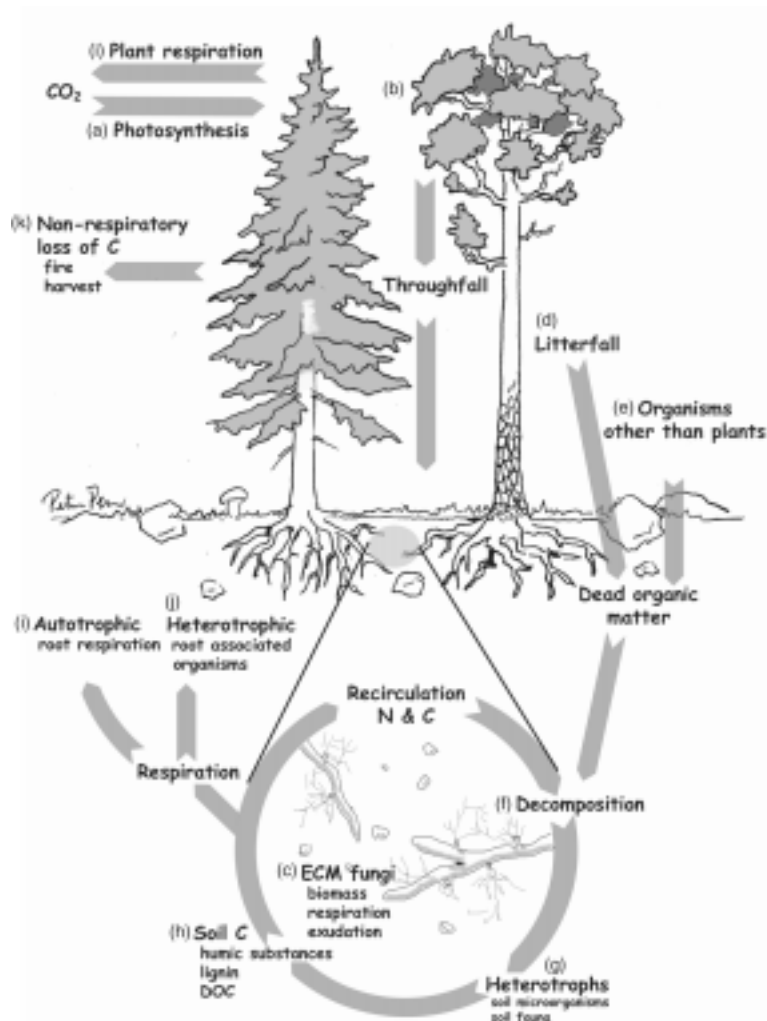


Figure 2. C-cycling in boreal forests. CO₂ from the atmosphere is fixed by plants via photosynthesis (a). Part of the current assimilates are then incorporated into plant biomass (b), released or allocated to associated microorganisms such as ECM fungi (c). The allocation through ECM fungi into the soil and root turnover are together with litter fall from the vegetation (d), the major input of C to the soil compartment. A proportion of organic matter deposited in soil, whether it originates from plants (d) or other forest organisms (e), will be decomposed by fungi, bacteria and soil animals (f) and incorporated, mainly into the microbial biomass. New compounds are synthesised by the decomposer community (g), and C may also remain and accumulate in the soil in more or less recalcitrant organic forms, such as humic substances and lignin (h). The pool of soil residues acts as a store of C and nutrients, and the soil compartment contains the largest pool of C in the boreal forest ecosystem. C finally returns to the atmosphere from autotrophic respiration by plants (i), heterotrophic respiration (j) mainly by microorganisms, and non-respiratory release via disturbances such as fire and via harvest of forest residues which may ultimately be combusted (k). Illustration: Peter Robertz.

example, the soil is merely a black box with an input and output of C. However, to understand the underlying mechanisms of C-cycling we need to study the organisms in each compartment.

Forest responses to CO₂ enrichment

Increased levels of CO₂ in the atmosphere are known to increase plant photosynthesis and subsequent C supply into the soil (*e.g.* O'Neill, 1994; Rey & Jarvis, 1997; Ceulemans *et al.*, 1999). The increase in photosynthesis takes place within a period of hours to days. However, in short-term pot studies plants have been shown to experience considerable down-regulation of photosynthetic rates after the initial increase. The down-regulation may occur within days to weeks, and seems to be a result either of sink-limitations or environmental limitations (reviewed by Curtis & Wang, 1998; Ward & Strain, 1999). The issue of whether or not trees will experience a down-regulation or acclimation as a response to long-term exposure to elevated CO₂ has been debated. If down-regulation occurs in the field it may have large effects on the capacity of forests to sequester CO₂ and act as the predicted sink for atmospheric C. Down-regulation has usually not been seen in longer-term field experiments (Saxe *et al.*, 1996; Curtis & Wang, 1998; Norby *et al.*, 1999), possibly because the increasing nutrient demand of the trees is met through increased nutrient acquisition by the ECM fungi colonising the roots. However, down-regulation cannot be dismissed as a potentially important factor in the over-all C-cycling. Elevated CO₂ levels and the subsequent increased C supply into the soil are also known to have belowground effects on *e.g.* root production (Ceulemans *et al.*, 1999; Norby *et al.*, 1999), rhizodeposition and root exudation (Rouhier *et al.*, 1994; Berntson & Bazzaz, 1996; Hodge, 1996), mycorrhizal fungi (Hodge, 1996; Fitter *et al.*, 2000; Treseder & Allen, 2000) and other microbial organisms (Paterson *et al.*, 1997; Zak *et al.*, 2000).

The role of ECM fungi in the boreal forest ecosystem

Sequestration and emission of carbon

ECM fungi are of particular significance with respect to the CO₂ exchange between the forest and the atmosphere since they play a fundamental role in the belowground partitioning of host-derived photoassimilates. They influence both sequestration and emission of C from soil. Estimates show that up to 20% of the photosynthetically fixed carbon may be allocated to the ECM fungus by the host plant (Finlay & Söderström, 1992). Besides photosynthesis and the subsequent allocation of fixed C through the fungi, respiration is another main process for CO₂ exchange in which ECM fungi play an important role (Rygiewicz & Anderson, 1994; Colpaert *et al.*, 1996). Högberg *et al.* (2001) attributed a large and almost immediate reduction in soil respiration following girdling of a pine stand to a cessation in the transfer of current assimilates, *i.e.* recently fixed C, to ECM fungi. The respiration was reduced by 54%. The strong dependence of

ECM fungi on current assimilates from their plant hosts has also been demonstrated in the laboratory (Lamhamedi *et al.*, 1994).

Partitioning of host-derived photoassimilates in ECM fungi

The fungus is, as pointed out in the previous section, a strong sink for C (Cairney, Ashford & Allaway, 1989), even stronger than the plant root tissue itself (Colpaert *et al.*, 1996). The C transferred to the fungus has four major fates: 1) lost through respiration; 2) incorporated into biomass; 3) incorporated into enzymes involved in nutrient acquisition and 4) released in interactions with other microorganisms and fungivores. Respiration accounts for the largest fraction and up to 60% of the C allocated to the fungus can be respired (Rygielwicz & Andersen, 1994). The same authors also reported that hyphal respiration might represent approximately 20% of total belowground respiration and that belowground respiration by mycorrhizal plants was 35% higher compared to non-mycorrhizal plants. The extraradical hyphae are known to be the most active part of the plant-fungus association (Cairney & Burke, 1996), with a high turnover rate compared to mycorrhizal root tips. Biomass production consumes the second largest part of the allocated C, and many ECM species produce an extensive extraradical mycelium. Furthermore, ECM fungi produce a range of organic substances that are used in the mobilisation and uptake of nutrients, *e.g.* enzymes (Leake & Read, 1991; Cairney & Burke, 1994; Bending & Read, 1995b) and organic acids (Ahonen-Jonnarth *et al.*, 2000). Finally, there are differences among species in efficiency and utilisation of allocated C (Bidartondo *et al.*, 2001), but the extent of these differences is largely unknown since C-budgets for most ECM species are still missing.

Mycelial networks and transfer of carbon

Formation of mycelial links between mycorrhizal seedlings has been demonstrated on a number of occasions in the laboratory (Finlay & Read, 1986a; Finlay & Read, 1986b; Ek *et al.*, 1996; Fitter *et al.*, 1998). In the case of achlorophyllous plants, such as some orchids and monotropoid plants, transfer of C always occurs. These plants are completely dependent on the C supplied by their symbiotic partner (Bruns & Read, 2000; McKendrick *et al.*, 2000; Bidartondo & Bruns, 2001). In 1997, Simard *et al.* demonstrated both bi-directional transport and net transport of C between two ECM tree species (*Betula papyrifera* and *Pseudotsuga menziesii*) in the field connected by a common mycelium. They showed the occurrence of both hyphal and soil pathways for the transfer of C and source-sink regulation. This network may be important for seedling establishment, and as Read (1997) commented the possible subsidising of C by overstorey trees to understorey trees may now shift our ideas about competition between plants towards a more resource orientated view, where the contribution of fungal linkages to diversity is considered. The concept of inter-plant C transfer has nevertheless been questioned. Fitter *et al.* (1998) proposed a mycogenic view of the phenomenon of interplant C transfer in which transfer is not believed to have an impact on the plant fitness or C budget. The

movement of C from the fungus back to arbuscular mycorrhizal (AM) plant shoots may, according to Fitter *et al.* (1998), not take place. Robinson and Fitter (1999) pointed out that, for example, C transfer via soil and the quantity of C transferred need to be considered carefully. The work on mycelial networks will most probably continue in the future because of the potentially large ecological significance of these questions. Heterotrophic assimilation of C has also been suggested to be of ecological importance as a supplementary C source for young forest trees, since ECM plants utilise organic nutrient sources and up to 9% of the plant C can be derived from protein sources (Abuzinadah & Read, 1989). In addition to the potential transfer of C, transfer of nutrients via a common mycelium is also likely to be important (Arnebrant *et al.*, 1993; Ek *et al.*, 1996).

Nutrient cycling in boreal forests and nutrient use by ECM fungi

ECM fungi are important for nutrient cycling in forests, along with another large functional group of fungi - the saprotrophs. Boreal forests have developed under conditions in which nitrogen (N) is characteristically bound to a large extent in organic matter and not available to plants (Tamm, 1991). Nowadays, however, N deposition and fertilisation affect the forest environment. Decomposition processes in the forest may be slow, Persson *et al.* (2000) reported low mineral nutrient availability along an European transect, and there is commonly an extensive accumulation of organic matter (Swift, Heal & Anderson, 1979). Plants thus need to access the nutrients, *e.g.* N and phosphorous (P), contained within this organic matter to sustain growth. Most of the mycorrhizal root tips are usually found in the organic part of the forest floor (Smith & Read 1997). The fundamental role that ECM fungi play in the acquisition, uptake and transfer of nutrients from the soil to the trees is of great ecological importance. Interactions between ECM fungi and saprotrophs may also be of much greater significance than has previously been recognised (Lindahl *et al.*, 1999). Patterns of C and nutrient cycling are likely to be influenced by changes in both the activity and species composition of soil microbial communities (Paterson *et al.*, 1997; Olsson & Wallander, 1998; Conn & Dighton, 2000), and within ECM fungi C allocation and nutrient dynamics are tightly linked (Ekblad *et al.*, 1995; Wallander, 1995).

The utilisation of organic nutrients by ECM fungi, an idea first presented over 100 years ago by Frank (see Read, 1987), has been demonstrated on a number of occasions and is now widely accepted (Abuzinadah & Read, 1986; Abuzinadah *et al.*, 1986; Finlay *et al.*, 1992). ECM fungi may utilise amino acids, peptides and proteins with the help of various proteases and oxidases, and their enzymatic capabilities may vary considerably (Cairney, 1999). Recent evidence also suggests that mycorrhizal fungi may play a fundamental role in weathering of mineral particles and superficial rock (Jongmans *et al.*, 1997). Organic acids produced by the fungus release the base cations essential for plant growth (Wallander *et al.*, 1997; Wallander & Wickman, 1999).

Community structure of ECM fungi

Community studies

The spatial heterogeneity of the boreal forest soil is normally high. ECM fungal communities in boreal forests are diverse (Dahlberg *et al.*, 2000). In Sweden many forests display a high abundance of fruit bodies during autumn, and a total of 4000 macromycetous species have been found (Ryman & Holmåsén, 1992), many of which are ectomycorrhizal. Community studies have traditionally been based on fruit bodies (*e.g.* Rühling & Tyler, 1991; Brandrud, 1995) which are easy to assess, instead of belowground parts. However, large discrepancies have been shown between what is found in an aboveground and belowground sampling (Gardes and Bruns, 1996). In recent years most work has been based on mycorrhizal roots (*e.g.* Erland *et al.*, 1999, Peter *et al.*, 2001), the work being done using both morphological and molecular methods. The mycorrhizal roots, the functional unit of the symbiosis, should show possible responses by the fungus to treatments more accurately and even more rapidly than changes in the fruit bodies. Responses of the extraradical mycelium, which has a high turnover rate and activity level (Cairney & Burke, 1996), to environmental perturbations are also highly interesting to monitor. We are now able to work with soil extracts of DNA and even distinguish different taxa with new methods such as DGGE (denaturing gradient gel electrophoresis). It is, however, still not possible to quantify the amount of fungal mycelium of individual mycorrhizal species. Only a few of the species found in the field have been used in laboratory studies. Our current knowledge of ECM fungal physiology is largely based on the easily cultivable species, *e.g.* *Hebeloma crustuliniforme*, *Laccaria bicolor*, *Paxillus involutus*, *Pisolithus tinctorius*, *Suillus spp.*, *Rhizopogon roseolus* etc. The relative contribution of these species to soil processes in the field may be questioned since they are generally of low abundance in mycorrhizal root communities (Fig. 2). We still do not know to what extent the function of different ECM species can be considered to be the same, but recent comparative studies have shown a great diversity in physiological function both within and between species (Cairney, 1999).

ECM fungal diversity and function

The large diversity of organisms is suggested to be important for the resilience and productivity of ecosystems (*e.g.* Chapin *et al.*, 2000), and Amaranthus (1998) has proposed the same to be true for ECM fungi in boreal forests. Nevertheless, Bengtsson (1998) pointed out that it is probably more important to understand the linkages between key species or functional groups and ecosystem function, rather than focusing on species diversity per se. van der Heijden *et al.* (1998) showed in micro- and macrocosm experiments simulating grasslands that belowground diversity of AM fungi is a major factor contributing to the maintenance of plant biodiversity. They demonstrated increased aboveground plant biomass with increasing mycorrhizal diversity, and their results showed that ‘microbial

interactions could drive ecosystem functions such as plant biodiversity, variability and productivity'. The influence of ECM fungal diversity on plant performance was investigated in *Betula populifolia* seedlings inoculated with one, two and four ECM fungal species in a study by Baxter and Dighton (2001). They found changes in plant growth (mainly shoot biomass) and nutrient responses that could be explained by changes in ECM diversity. Shoot biomass decreased and mycorrhizal root biomass increased with increasing ECM diversity. This may however have been explained by increased root colonisation (Leake, 2001). Shoot N concentration, whole plant P content and P concentration also increased with increasing diversity (Baxter and Dighton, 2001), however, plant biomass did not differ with individual ECM species or composition, in contrast to the results of van der Heijden *et al.* (1998). Finally, it is important to keep in mind that these two experiments are simplifications of natural systems.

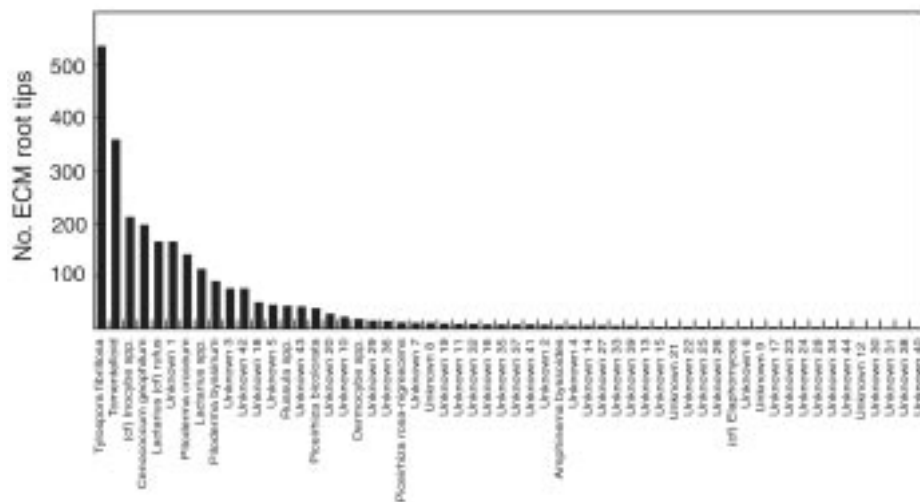


Figure 3. Species abundance curve showing the number of root tips colonised by different ECM fungal taxa. Commonly a few species dominate the community, and a high number of species form the tail of the distribution curve. The corticoid fungi *Piloderma croceum*, *P. bysseinum* and *Tylospora fibrillosa* and the ascomycete *Cenococcum geophilum* can be very abundant. Up to 70% of the total number of root tips may be colonised by these species. Data from study III.

Stable isotopes

General background and methodology

C and N exist in different forms in nature. About 98.8% of all C is made up by the isotope ^{12}C , and 1.1% by the heavier isotope ^{13}C . For N, the figures are 99.6% for the ^{14}N isotope and 0.4% for ^{15}N , and all of these isotopes are stable. Small differences in the isotopic ratios of $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ result from fractionation in physical, chemical and biological processes (Högberg, 1997). In chemical reactions more energy is required to assimilate a heavier isotope than a lighter one, and the diffusion rate is slower for a heavier isotope. Photosynthesis is an example of a biological process in which a discrimination against the ^{13}C isotope takes place. This results in differences in ^{13}C abundance between trees growing in the overstorey and trees growing in the understorey in forests (Brooks *et al.*, 1997). The overstorey trees will have higher illumination since they are not shaded, and therefore they have a higher photosynthetic rate. They will also be more water stressed compared to the shaded understorey, resulting in that stomata being partially closed. Because of these factors overstorey trees discriminate less against the heavier isotope ^{13}C . The natural abundance of stable isotopes can be analysed in living and dead material with isotope ratio mass spectrometry (see *e.g.* Lajtha & Michener, 1994). The abundance values are given in the unit δ , which represents the deviation in parts per thousand from a standard. In the case of C negative values are obtained in relation to the international Pee Dee Belemnite standard, a marine limestone fossil. For N, the ratio in air is taken as zero, and values range from negative to positive.

Stable isotopes in mycological research

A rapid expansion in the use of natural abundances of stable isotopes in ecological research has been seen during the past decade. Stable isotope values can be used to find patterns and mechanisms at the organism level as well as to trace food webs and examine ecosystem cycling of elements (Lajtha & Michener, 1994). Within the field of mycology it is mainly the natural abundances of ^{13}C and ^{15}N isotopes that have been used. Stable isotopes are also used in tracer studies (Hodge *et al.*, 2001). Since analysis of soil mycelia is not feasible, most studies on natural abundances have been based on ^{15}N and ^{13}C content of fruitbodies. Some general patterns are becoming apparent from the mycological studies. Ectomycorrhizal fungi are generally more depleted in ^{13}C and more enriched in ^{15}N than saprotrophic fungi (Hobbie *et al.*, 1999; Högberg *et al.*, 1999; Kohzu *et al.*, 1999, paper I). Among the saprotrophs, litter fungi are more enriched in ^{15}N than wood decomposers, with both saprotrophic groups enriched relative to their substrates (Gebauer & Taylor, 1999; Hobbie *et al.*, 1999; Kohzu *et al.*, 1999). Another general pattern is emerging in which ECM fungi are considerably enriched in ^{15}N relative to their host plants (Gebauer & Dietrich, 1993; Högberg *et al.*, 1996; Taylor *et al.*, 1997; Michelsen *et al.*, 1998; Hobbie *et al.*, 1999). Given that almost 100% of the root tips are colonised by ECM fungi

(Taylor *et al.*, 2000, paper III), it would be expected that most of the N taken up by the tree would have to pass through the fungi. The ^{15}N signatures of the fungi and the host plant should therefore be similar. The reason(s) for the anomaly between the signatures remains unclear, but fractionation against the heavier ^{15}N isotope during the transfer of N from the fungus to the plant has been suggested as a possible cause (Högberg *et al.*, 1996; Taylor *et al.*, 1997; Hobbie *et al.*, 1999). Another possibility, suggested by Gebauer and Dietrich (1993), is the transfer of ^{15}N -depleted mineral N (NH_4^+ and NO_3^-) directly from soil via the fungus to the plant without any metabolic processing. The fungi would preferentially utilise ^{15}N -enriched organic compounds as N sources. This process would be more important in systems where mineral N is a significant proportion of the available soil N.

Objectives

The work in this thesis had three main objectives:

- i) To determine the host-origin of carbon in ECM fungi with different host-specificity in mixed forests and whether natural abundances of stable isotopes can be used to reveal trophic status of fungi (paper I).
- ii) To investigate how elevated atmospheric CO_2 levels and balanced fertilisation affect the community structure of ECM fungi colonising spruce roots (paper II-III).
- iii) To investigate how elevated CO_2 affects the production, spread of and root colonisation by mycelia of individual ECM fungal species (paper IV).

Results and discussion

Fungal ecology and the use of natural abundances of stable isotopes

A major problem in fungal ecology has been to establish the trophic status of species in the field. We lack a way to distinguish the two large functional groups, mycorrhizal and saprotrophic fungi, from each other, and our conclusions are often based on anecdotal field evidence (paper I). We used stable isotope techniques to investigate ecological questions concerning this, and also to investigate host-specificity related questions. The ECM association has been said to show relatively low specificity (see references in Duddridge, 1987), and a large number of potential partners exist for both fungus and host. From the host plant perspective the specificity might indeed be low, with a large number of different fungi reported from the same tree species (Trappe & Fogel, 1977; Molina *et al.*, 1992), but there are a number of ECM fungi that seem to form the symbiosis with only a single tree genera.

Natural abundance of ^{13}C and ^{15}N in fungal fruit bodies

We analysed a range of presumed saprotrophic and ECM fungal fruit bodies collected in two mixed forests, Åheden in northern Sweden and Stadsskogen in central Sweden. Both forests had Scots pine growing as the overstorey, and with Norway spruce approaching the position of co-dominant tree species. The understorey consisted of broad-leaved tree species. The analysis was done using isotope ratio mass spectrometry, to compare $\delta^{13}\text{C}$ (paper I) and $\delta^{15}\text{N}$ abundances (Taylor *et al.*, In preparation) in the two groups of fungi. We also investigated whether $\delta^{13}\text{C}$ abundance could reveal the host-origin of C in ECM fungi with different host-specificity (paper I). This was done by comparing different tree species with a range of ECM fungi associated with them, and these fungi were either generalists or host-specific, according to literature relevant for the area (Hansen *et al.*, 1992). Host-specific fungi associated with the overstorey trees could be expected to have higher ^{13}C values compared to fungal species associated with the understorey trees.

A total of 135 fungal species (118 ECM and 17 saprotrophic), representing 25 ECM and 15 saprotrophic genera, was analysed in the study (paper I). We found significant differences in ^{13}C abundance between the different groups of ECM fungi and saprotrophic fungi at both sites investigated, with ECM fungi being more depleted in $\delta^{13}\text{C}$ than saprotrophic fungi (Fig. 4). The investigation revealed interesting information about individual species, *e.g.* *Chalciporous piperatus*, a presumed mycorrhizal species, had high $\delta^{13}\text{C}$ values and may in fact be saprotrophic (Fig. 5). As expected, the overstorey tree species (Scots pine and Norway spruce) had the highest $\delta^{13}\text{C}$ values, and the understorey broad-leaved species had values that were up to 2-3‰ lower values. The host-specific ECM

fungi, *i.e.* fungi growing only together with one host tree species (for example pine-specific or spruce-specific), were found to be consistently higher in value compared to their hosts (Fig. 4). This pattern was consistent, and the difference in enrichment between fungi and host was between 1.2-2.9‰, comparable to the trophic shift between decomposing basidiomycetes and wood reported by Gleixner *et al.* (1993). Finally, the generalist fungi, which can be associated with several different tree species, were found to receive most C from overstorey trees, as indicated by their high $\delta^{13}\text{C}$ values. This implies that large trees, which are able to fix more C, potentially subsidise smaller trees via a common ECM mycelial network.

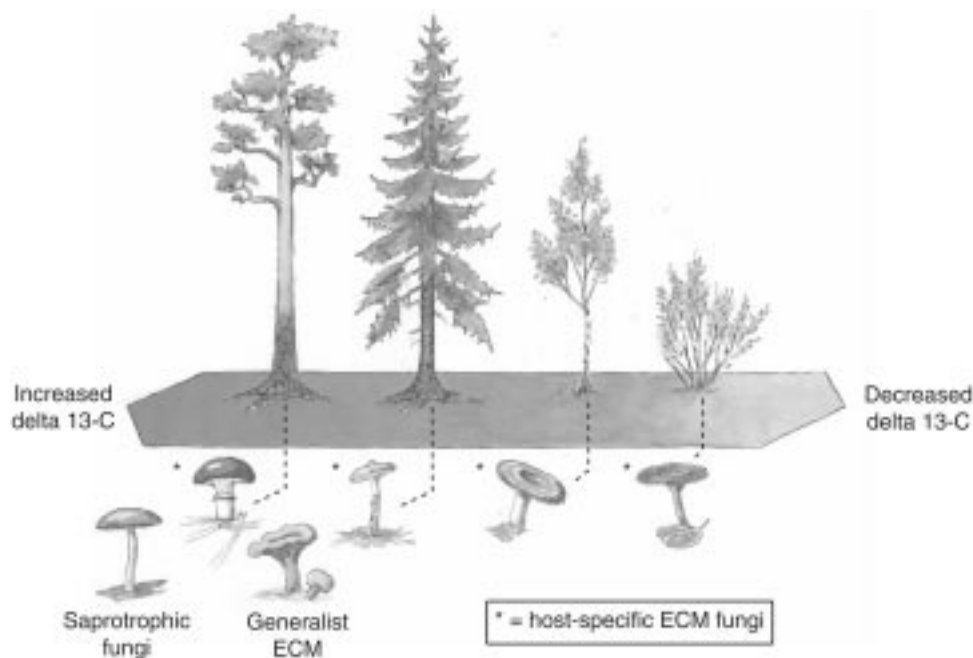


Figure 4. Natural abundances of the stable isotope ^{13}C in host trees, ECM and saprotrophic fungi in a mixed forest. Overstorey trees, in this case Scots pine and Norway spruce, have the highest ^{13}C values among the trees. Saprotrophic fungi are enriched compared to ECM fungi. Host-specific ECM fungi associated with the overstorey trees are enriched compared to host-specific fungi associated with understorey trees. The lines connecting the host-specific fungi with their hosts represent the isotopic shift that takes place during the transfer of C between tree and fungus. Finally, generalist fungi receive most of their C from the overstorey although they may be associated with understorey trees as well as dominating trees. Illustration: Peter Roberntz.

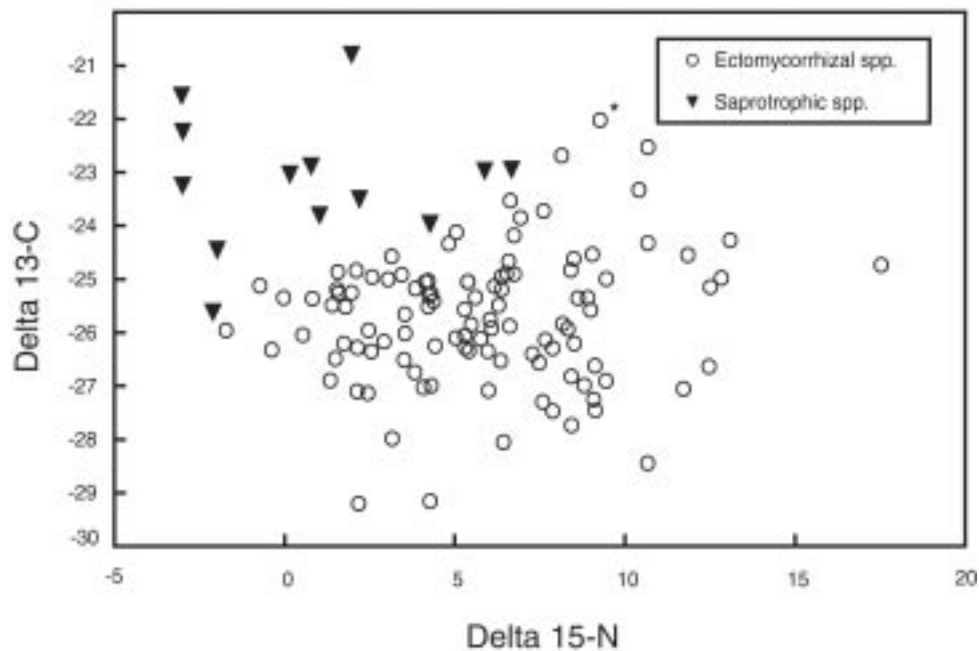


Figure 5. Natural abundances of the stable isotopes ^{13}C and ^{15}N can be used to distinguish between ECM and saprotrophic fungi, although caution should be taken in the interpretation since there is some overlap between the two groups. Saprotrophic fungi are enriched in ^{13}C compared to ECM fungi, and depleted in ^{15}N . * = *C. piperatus*, a suggested mycorrhizal species which had high ^{13}C values. Data from paper I and Taylor *et al.* (In preparation).

In addition, ECM fungi were found to be more enriched in ^{15}N compared to saprotrophic fungi (Taylor *et al.*, In preparation), and when the information about $\delta^{15}\text{N}$ abundance is included in the comparison between ECM and saprotrophic fungi, the pattern becomes even clearer (Fig. 5). Mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values differed significantly between fungal groups, but there was a potential overlap at the species level. Intraspecific variation was low compared to interspecific variation. Potential influences of site, host and season are factors that have not been assessed in earlier studies. We found significant differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in relation to collection site, host association and inter-yearly variation, but these questions need to be addressed more closely before general patterns can be found (Taylor *et al.*, In preparation).

Usefulness of stable isotopes

The large ecological groups of fungi, mycorrhizal and saprotrophic fungi, are generally considered to share the same substrates within ecosystems. Within a functional group species have been expected to have similar isotope signatures. This is, however, not the case at all. The natural abundance of ^{13}C can be used as

a tool to distinguish between the two functional groups ECM and saprotrophic fungi. $\delta^{13}\text{C}$ values can thus be used to reveal trophic status of fungi, but also to reveal the host-origin of carbon in mycorrhizal fungi in mixed forests. To investigate the trophic status of fungi based on fruit body material a combination of $\delta^{13}\text{C}$ values and $\delta^{15}\text{N}$ values should preferably be used. They should, nevertheless, still be used with caution since overlapping values between the two functional groups have been reported (e.g. Kohzu *et al.*, 1999; Taylor *et al.*, In preparation). The species needs to be highlighted as the taxonomic level at which most information can be obtained from ^{13}C and ^{15}N abundance data from fungi (Taylor *et al.*, In preparation). Although data on the natural occurrence of stable isotopes in fungal material may be difficult to interpret, the information available so far provides us with an excellent opportunity to continue exploring fungal physiology in laboratory experiments. Large differences in $\delta^{15}\text{N}$ values have been found among ECM species. Two examples of the great variation in $\delta^{15}\text{N}$ values from the study by Taylor *et al.* (In preparation) are *Lactarius* species which range from -0.2 – +9.5, and *Suillus* species which range from 2.4 (*S. variegatus*) to 15.0 (*S. bovinus*). These results provide us with knowledge about which species to continue investigating with respect to fractionation processes in the transfer of N between fungus and host.

Effects of elevated atmospheric CO₂ and balanced fertilisation on ECM community structure

Changes in the community structure of ECM fungi colonising the roots of 36-yr-old Norway spruce trees was investigated following three years of treatment with twice the atmospheric concentration of CO₂ (paper II). The effects of a balanced fertilisation treatment alone and irrigation treatment upon ECM community structure were also investigated separately (paper III). Increases in the availability of atmospheric CO₂ and soil N may have profound effects on the forest ecosystem and soil microorganisms (O'Neill, 1994; Ceuleman *et al.*, 1999; Cairney & Meharg, 1999; Treseder & Allen, 2000). In paper II we report, for the first time, effects of elevated CO₂ on the community structure of ECM fungi growing in association with large forest trees.

Changes in community structure

The study site, Flakaliden, in northern Sweden was planted with Norway spruce after clear-felling in 1963. A balanced nutrient solution (macro- and micro-) was supplied daily throughout the growing season. Since the start of the experiment in 1987 the fertilisation treatment has included a total of 1125 kg N ha⁻¹, added as both ammonium and nitrate. In 1996, whole-tree chambers were installed on two plots around individual trees growing either with or without fertiliser additions (paper II). One half of the enclosed trees on each plot received ambient levels of CO₂ (350 ppm), the other half received elevated levels of CO₂ (700 ppm). In 1997, before the CO₂-treatment started, and in 2000, soil cores (a total of 90 each time) were taken from the organic soil layer beneath each of the enclosed trees

and reference trees. Samples were also taken from the fertiliser treatment (paper III). These were compared with control plots receiving no treatment and an irrigation treatment receiving water without added nutrients. A total of 270 soil cores were sampled from three replicate plots of each treatment. Live root tips were extracted, examined and classified into mycorrhizal morphotypes, using macroscopic and microscopic features (Agerer, 1986-1998). The term morphotype was used to designate a recognisable group of mycorrhizal root tips. In paper III the vertical distribution of ECM morphotypes between soil layers was also investigated. A sub-sample of the soil cores, which included both organic and mineral layers, was analysed using a model based on the binomial distribution.

A clear shift in ECM community structure was found in response to elevated CO₂ both with and without fertiliser treatment (Fig. 6; paper II). The pattern was less pronounced for the fertiliser treatment, possibly because fifteen years of fertilisation may already have shifted the community in the fertilised plot so that the CO₂ treatment had little effect once started. Significant effects of the fertilisation treatment were also evident in both studies (paper II, III). Surprisingly, the magnitude of the three year CO₂ treatment effect on the ECM community was similar to that seen after 15 years of nutrient additions (Fig. 6). Irrigated plots receiving only water did not differ significantly from the untreated control plots (Fig. 7). We do not know if the change in ECM community structure induced by elevated CO₂ is transient, but evidence from long term studies on the effects of N-fertilisation upon ECM communities suggests that once initiated, changes in community structure may be more long lasting (Garbaye *et al.*, 1979). The results show that increasing atmospheric CO₂ levels and fertilisation can affect root symbionts of forest trees and potentially alter C and nutrient allocation and turnover within forest ecosystems.

In both community studies a large number of morphotypes were found, about 50 different morphotypes were found in the study in paper II, and about 60 in the study in paper III. Both studies also revealed a large heterogeneity among individual samples, but despite this large variability within the data set, clear patterns could be revealed with the multivariate statistical analysis (Fig. 6, 7). When it comes to individual ECM species or morphotypes, however, possible responses to the different treatments may have been masked by the high variability, especially in study III. There was no measurable effect of either elevated CO₂, fertiliser or irrigation treatment on morphotype richness or the total number of root tips. Root colonisation was generally high, in paper III over 93% of the roots were colonised (100% colonisation level in six of the nine investigated plots).

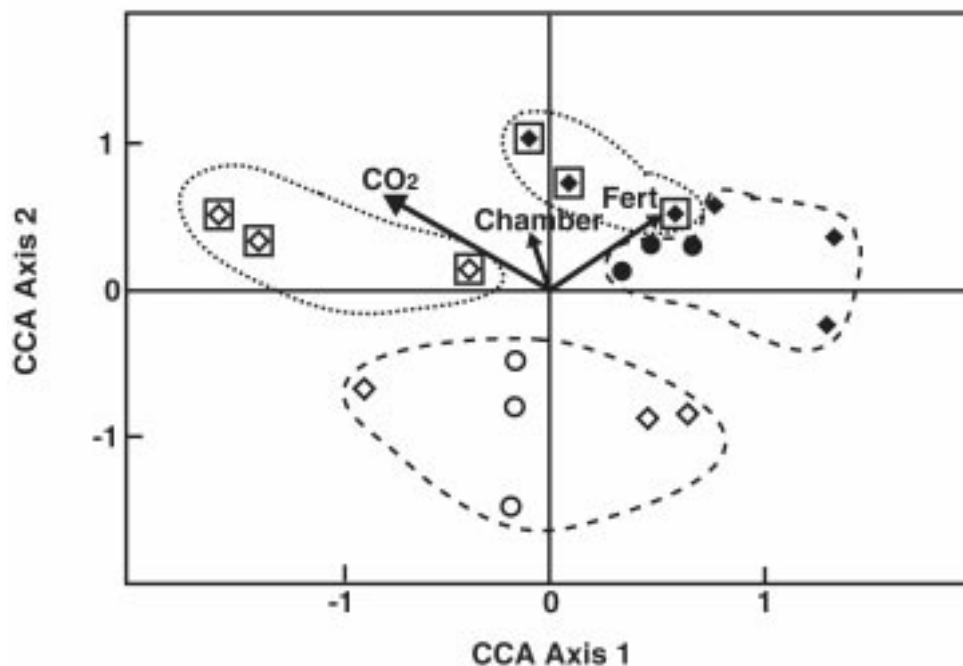


Figure 6. Effects of elevated atmospheric CO₂ on the community structure of ECM fungi colonising the roots of *ca.* 40-year-old Norway spruce trees were investigated. Individual trees in whole-tree chambers received elevated (700 ppm) or ambient (350 ppm) levels of CO₂. Canonical correspondence analysis (CCA) revealed significant effects of the CO₂ treatment. The same pattern, but less pronounced, was found in the fertilised plot. This was possibly explained by the same species reacting to both treatments. Open symbols – unfertilised trees, closed symbols – fertilised trees. Ambient CO₂ trees with (◇) and without (○) chamber and elevated CO₂ trees with chamber (◩)

Only one ECM species, *C. geophilum*, was significantly more abundant on the fertilised plots than on the control plots (paper III). Several morphotypes nevertheless, showed a tendency to become more common with fertiliser treatment. *A. byssoides*, *T. fibrillosa* and tomentelloid types showed an increased abundance in the fertilised plots (paper II, III). In contrast, *P. byssinum* and *P. croceum* showed a tendency to become less common with fertilisation, and the changes in abundance of *Piloderma* species could not be related to the CO₂ treatment in any particular way. An unknown basidiomycete appeared to be favoured by the CO₂ treatment. Nevertheless, comparison between the two sampling years for the CO₂-study indicated that the fungal community colonising trees receiving only CO₂ treatment became more similar to those of the fertiliser treatment. Two morphotypes were associated with a specific soil layer: *C. geophilum* mycorrhizal root tips were more common in the organic layer than the mineral layer ($P = 0.04$), and for *T. fibrillosa* the opposite relationship was

demonstrated ($P = 0.02$). All *Lactarius spp.* root tips were found in the mineral layer, although the data did not fit the model used to test the vertical distribution. *Piloderma* did not show any significant association with a particular soil fraction.

What do community studies tell us?

Descriptive studies provide a basis for continued exploration of functional aspects of the mycorrhizal symbiosis. On the other hand, laboratory experiments provide information that can be extrapolated to the field situation, and be used to suggest an explanation for observed changes in community structure. Changes in C and N availability, through CO₂-enrichment or fertilisation, have been shown to affect ECM community structure in the field. A few studies have observed changes, due to elevated CO₂, in the community structure of ECM fungi colonising seedlings or saplings (Godbold & Berntson, 1997; Godbold *et al.*, 1997, Rey & Jarvis, 1997), but never for forest trees. Other studies have failed to detect any significant changes in ECM community structure due to elevated CO₂ levels (Markkola *et al.*, 1996; Rygielwicz *et al.*, 2000). The former studies indicate that ECM morphotypes forming extensive extraradical mycelia and rhizomorphs may become more common at elevated CO₂ levels. We report, for the first time, significant differences in ECM community structure for large trees grown in ambient or elevated CO₂ levels (paper II). We cannot however say from our study if there is an increase in species producing large amounts of extraradical mycelium, or if there is an increase in the actual production of mycelium in the soil.

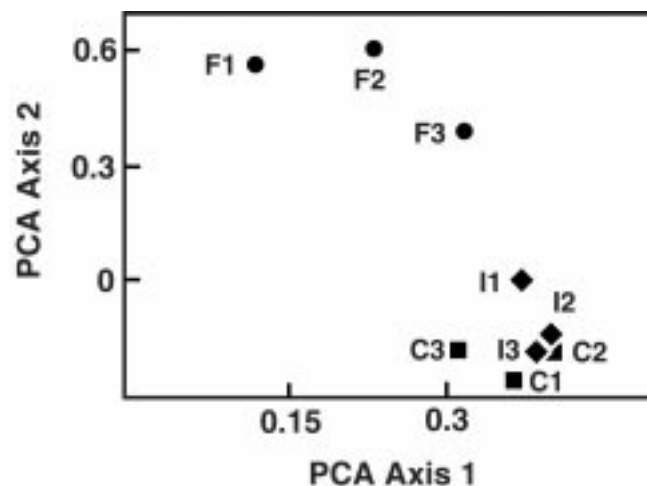


Figure 7. Effects of a balanced fertilisation treatment on the ECM root community structure were investigated. ECM fungi colonising Norway spruce trees had received daily additions of a fertiliser, during the vegetation season, for 15 years. Principal components analysis (PCA) revealed a clear shift in community structure due to the fertiliser treatment. Irrigation treatment alone had no effect on community structure. Trees receiving fertilisation treatment (●), irrigation treatment (◆) or no treatment at all (■).

Many earlier studies have shown effects of fertilisation on ECM community structure, using methods based on fruit bodies (*e.g.* Rühling & Tyler, 1991; Wiklund *et al.*, 1994) and mycorrhizal roots (*e.g.* Kårén & Nylund, 1996; Jonsson *et al.*, 2000; Peters *et al.*, 2001). The different studies have used different types of fertiliser (*e.g.* NO_3^- -N, NH_4^+ -N and N-free) and methods of application (single or multiple applications, usually in large doses). Reductions in the percentage of colonised tips are generally seen after fertilising with single, high N additions (see Wallenda & Kottke, 1998). In our study (paper III) with continuous application of fertiliser, a clear shift in community structure was seen however no effect on colonisation level was found. In accordance with earlier studies a high diversity of ECM fungi was found (paper II, III), and this appears to be a common feature of boreal forests (Dahlberg *et al.*, 1997; Väre *et al.*, 1996).

Despite the fact that a large number of papers have been published on the effects of various treatments on ECM community structure, there are a few crucial questions that have generally not been addressed. Sampling of ECM communities is problematic, and attention has been drawn to sampling strategies recently by Peter *et al.* (2001) and by Taylor (In press). Temporal and spatial aspects of ECM community structure, based on mycorrhizal root tips, are still not thoroughly investigated. One study by Taylor and Alexander (1989) points towards large differences in community structure during a vegetation season. It is crucial that sampling takes account of temporal shifts in community structure. An investigation in our laboratory (Rosling *et al.*, Personal communication) has addressed one aspect of spatial variation, namely that of vertical distribution. Normally samples are taken from the top part of the soil profile, but the distribution of taxa may vary considerably down the profile. Most studies have ignored data about mycorrhizal species in the lower mineral horizons.

Carbon availability affects mycelial production and spread

Fungal mycelia respond to changes in both C and nutrient availability, and enriched CO_2 environments have been shown to increase the production of ECM fungal mycelia when associated with a host (Ineichen *et al.*, 1995; Rouhier & Read, 1998) as well as change ECM community structure in the field (paper II, Godbold & Berntson, 1997; Godbold *et al.*, 1997, Rey & Jarvis, 1997). The present study (paper IV) aimed at investigating one possible mechanism behind the observed changes in community structure, namely how an increased C availability affects the inoculum potential of ECM fungi. The production and spread of mycelia and colonisation of pine roots are two components of inoculum potential, which were studied. Changes in the production of mycelium and the potential to colonise roots may affect competition among species, which in turn may lead to changes in ECM community structure.

Mycelial responses to CO₂ enrichment

Two different types of microcosm systems were used in this study: pots for the production experiment and shallow boxes for the spread experiment. Pine seedlings, inoculated either with *H. crustuliniforme* or *P. involutus*, were grown at two levels of atmospheric CO₂ – ambient (350 ppm) or elevated (700 ppm) levels. The production experiment lasted for six weeks. To study mycelial spread, one inoculated seedling was grown in a line together with three uncolonised bait seedlings, for approximately four months. All microcosms were watered on a regular basis with nutrient solution. At the end of the experiments, seedlings were harvested, plant biomass and numbers of mycorrhizal root tips were recorded. Fungal biomass was analysed in roots and the growth substrate using the chitin method described by Vignon *et al.* (1986).

In the present study significant differences were found in the amount of fungal mycelium produced by *H. crustuliniforme* under elevated CO₂ levels compared to ambient CO₂ levels. This is in accordance with earlier findings of increased mycelial growth of *P. involutus* and *P. tinctorius* (growing with pine seedlings) and *S. bovinus* (growing with pine and birch seedlings) under elevated CO₂ levels (Ineichen *et al.*, 1995; Rouhier & Read, 1998; Rouhier & Read, 1999). The amount of fungal biomass was about three times higher for the elevated treatment in our study. There were, however, no clear differences in plant biomass or number of ECM root tips between the treatments. In contrast to our results, Rouhier and Read (1998) reported increased numbers of ECM root tips for pine seedlings colonised by *P. involutus* and *S. bovinus* after four months of exposure to elevated CO₂. The plants, however, grew for a longer time in their experiment. Ineichen *et al.* (1995) found three times more pine-*P. tinctorius* root clusters after growing the inoculated seedlings in elevated CO₂ for three months. In the present study, plants colonised by *P. involutus* did not grow as expected, and at harvest the mycorrhizal roots appeared moribund. The extraradical mycelium has been shown to respond more strongly to changes in N-availability compared to the fungal tissue on roots (Wallander & Nylund, 1992), and in the latter experiment the mycelium recovered from the negative growth response once the N load was removed. Mycelial responses to changes in C and nutrient availability will most likely not only be stronger, but also more rapid compared to the mycorrhizal root response. The present results showed a biomass response by the fungal mycelium to elevated CO₂ which could not be detected in the ECM roots or the rest of the plant.

The mycelial spread experiment with inoculated seedlings showed a high degree of variability, and no significant differences were found for plant or fungal biomass. The general trend for *H. crustuliniforme* was however that plants grown in CO₂-enrichment had higher mean values of fungal biomass and numbers of ECM root tips. The growth of *Paxillus involutus* was more variable. For both fungal species, a higher number of root tips had been colonised further away from

the inoculated plant under elevated CO₂ levels, *i.e.* the mycelial spread further and colonisation was greater. For *H. crustuliniforme* plants, no ECM root tips were found on the last of three bait seedlings in the ambient treatment, but a low amount of fungal mycelium was detected on the roots. This mycelium had managed to reach the last growth section in the experimental box system, but had not yet managed to colonise any root tips. There were also generally no significant differences in plant biomass between treatments for this experiment, for either fungal species.

From the above results it is apparent that the amount of fungal mycelium produced under CO₂-enrichment may increase for some ECM fungal species under certain growth conditions. Both *H. crustuliniforme* and *P. involutus* produce large extraradical mycelia, and may possibly benefit from an increased C-availability in that they increase the mycelial component. In the present study the amount of nutrients given during the experiment was moderate, in order to avoid high nutrient availability and hence resemble the nutrient limitation under field conditions. With increased production of extraradical mycelium under elevated CO₂ levels the mycelium can spread further, as indicated in this study. The extension of mycelium between root systems may be faster, or it may be a mass effect.

With increasing mycelial production fungi have a larger inoculum potential and may colonise root tips more rapidly. Competition for resources, both for root tips to colonise and for nutrient sources in the soil, may thus be affected under conditions of increased C-availability. Species forming larger mycelia may explore the soil for nutrients more efficiently compared to other species not increasing the amount of mycelium. These types of responses in colonisation and nutrient acquisition may lead to changes in community composition. The outcome of interactions between ECM fungi will most likely depend on their competitive ability, which in turn is influenced by environmental factors and physiological traits of the fungi. Competition between ECM fungi has not been extensively studied. Papers published on the subject have mainly been on cultivable species, such as *Tuber spp.*, and other invading ECM species that are unwanted in plantations (Zambonelli *et al.*, 2000; Mamoun & Olivier, 1993). The specificity of fungi has been suggested to be important for the outcome of competition between fungi (Wu *et al.*, 1999; Zambonelli *et al.*, 2000). Wu *et al.* (1999) studied competition between three ECM fungal species (*P. tinctorius*, *S. luteus* and an unknown type) colonising the roots of *Pinus densiflora*. For example, *P. tinctorius* was gradually replaced on the root system by the unknown type (Wu *et al.*, 1999). McAfee and Fortin (1987) published a field study investigating effects of acid treatment on competitive interactions of ECM fungi, using *Pinus banksiana* seedlings which were outplanted after inoculation with either *L. bicolor* or *P. tinctorius*. In their experiment indigenous colonisation was suppressed by pre-inoculation. Mechanisms underlying the observed changes in

ECM community structure due to changes in C- and nutrient-availability, such as competition, need to be further examined.

General discussion

The role of ECM fungi in forests exposed to an enriched CO₂ environment

Atmospheric CO₂-enrichment may increase C fixation by plants and subsequent C supply into the soil. This affects both symbiotic organisms directly associated with the plant roots, and other soil microorganisms. Direct effects of CO₂ enrichment on soil microorganisms and processes are unlikely however due to the high levels of CO₂ already found in the soil environment (Paterson *et al.*, 1997). Possible responses to elevated CO₂ by ECM fungi are likely to be mediated through the plant. The strong dependence of ECM fungi on current assimilates (Söderström & Read, 1987; Lamhamedi *et al.*, 1994; Högberg *et al.*, 2001) indicates that the fungus may experience the effects of photosynthetic changes shortly after they are induced in the plant. We do not know how long it takes for the community composition to change in response to increased C-supply. In paper II the changes seen in community composition after three years of treatment were as large as those seen after 15 years of balanced fertiliser additions. The shift in, or replacement of, ECM species is a slower change compared to the first and immediate physiological responses to increased CO₂ levels. Plant responses on the physiological level are measurable at an early stage, while changes in community structure on the actual root tips can only be detected after some time. There should, of course, be a range of physiological responses in the fungi that precede the shift in species, both on a shorter and a longer time scale. Studies of how ECM fungi respond to elevated CO₂ levels, however, usually involve monitoring both plant and fungal biomass responses after an experimental time period that ranges from weeks to months (*e.g.* O'Neill *et al.*, 1987; Lewis & Strain, 1996; Rouhier & Read, 1998). The lack of measurements of short-term physiological responses in the ECM fungi is obvious, and needs to be addressed in the future.

Increased C allocation belowground as a result of CO₂ enrichment has been related to the root system development (*e.g.* Norby *et al.*, 1987; Ceuleman *et al.*, 1999). Root growth and death constitute a large part of the organic matter that enters the soil (Fitter *et al.*, 1997), and increased root production and turnover under CO₂ enrichment has been reported (Berntson & Bazzaz, 1996; Fitter *et al.*, 1997; Tingey *et al.*, 1997). Although root turnover is difficult to investigate and estimate (Eissenstat *et al.*, 2000), it is an important issue for mycorrhizal research, since the root tips constitute an essential resource for these organisms. Nevertheless, the turnover of mycelium is likely to be faster than that of roots. An increase in extraradical mycelium produced by ECM fungal communities under elevated CO₂, a response that has been suggested from field studies (Rey & Jarvis, 1997; Godbold & Berntson, 1997; Godbold *et al.*, 1997), could lead to a

faster turnover of C within the plant-fungus system (Fitter *et al.*, 2000). Furthermore, there have been reports of an increased amount of C lost through respiration in response to elevated CO₂ concentrations (Rouhier *et al.*, 1996; Gorisson & Kuyper, 2000), which could also mean a faster turnover of C in the system rather than an absolute increase in the amount of C retained belowground.

Nutrient availability is a crucial factor coupled to the role of ECM fungi in long-term responses of boreal forests to elevated levels of CO₂ for several reasons: (1) N, but also P, limits tree growth in most forest ecosystems, and ECM fungi play a central role in the mobilisation and acquisition of these elements (2) within ECM fungi there is a tight coupling between C and N nutrition (France & Reid, 1983; Turnau *et al.*, 2001), (3) ECM community changes due to elevated CO₂ have been reported (paper II), and these changes may affect C and N cycling in forest ecosystems. Elevated CO₂ and increased C input into the soil may have contrasting effects on microbial processes and nutrient availability within ecosystems. Microbially mediated decomposition has been shown to decrease in grasslands after 5 years of continuous exposure to elevated CO₂ (Hu *et al.*, 2001). This could eventually lead to lower nutrient availability. Diaz *et al.* (1993) proposed that elevated CO₂ may cause an increase in substrate release into the rhizosphere of non-mycorrhizal plants, leading to increased mineral nutrient sequestration by the expanded microflora and a consequent nutritional limitation of plant growth. In contrast to these suggested negative feedbacks, Zak *et al.* (1993) hypothesised that greater belowground C inputs in response to elevated CO₂ should elicit an increase in soil microbial biomass and increase rates of organic matter turnover and nitrogen availability. The long-standing hypothesis that litter quality would decrease in an environment with increasing CO₂, as a consequence of increasing C/N ratio, has not been supported by long-term field studies (Norby *et al.*, 1999). The consensus now is that there is insufficient evidence to support this idea (Norby & Cotrufo, 1998), and thus some of the fears about future effects of CO₂ on plant productivity in forest ecosystems can be put aside.

The apparent complexity of ecosystem responses to CO₂ enrichment make general conclusions difficult, if not impossible, to make. The observed diversity in responses of mycorrhizal plants to elevated CO₂ levels may be related to interspecific differences (BassiriRad *et al.*, 2001), and for ECM fungi these may be considerable (Cairney, 1999). As pointed out by Cairney and Meharg (1999), we still do not know to what extent apparent differences in the response of mycorrhizal fungi to a CO₂-enriched atmosphere relate to differences in soil condition, nutrient availability or host-specific differences in C-availability to the mycorrhizal roots.

Will a changed C availability to ECM fungi lead to improved nutrient uptake by plants?

Carbon supply to the fungi may decrease due to grazing of foliage (Markkola, 1996; Saikkonen *et al.*, 1999) and shading, or environmental perturbations such as ozone exposure (Andersen & Rygielwicz, 1995). An increased C availability, on the other hand, could result from CO₂ enrichment. The photosynthetic rate of plants increases (Norby *et al.*, 1999), and this usually leads to increased allocation of C belowground (*e.g.* Ceuleman *et al.*, 1999). Interspecific differences in the response to elevated CO₂ have been shown for mycorrhizal fungi (Klironomos *et al.*, 1998; Gorisson & Kuyper, 2000), and differences among species may be expected to be considerable. Mycelia, extending from mycorrhizal roots and exploring the surrounding soil, mobilise and translocate nutrients found in organic matter (Bending & Read, 1995; Perez-Moreno & Read, 2000) and mineral sources (Bidartondo *et al.*, 2001). Nutrient substrates occurring in the soil are of great complexity (Bending & Read, 1995a), and ECM fungi are known to have different nutrient mobilisation capabilities (Leake and Read, 1997). For example, *P. involutus* appears to be able to remove P selectively from litter (Perez-Moreno & Read, 2000). A requirement for the tree response to elevated levels of CO₂ is that the expected increase in nutrient demand, which will follow if the tree increases its photosynthetic rate and biomass production, is met. If the ECM fungi growing on the tree roots produce larger amounts of mycelium under CO₂ enrichment this may improve the nutrient acquisition of the tree by increasing substrate colonisation. Another possible scenario is that the nutrient use efficiency may increase, *i.e.* the amount of biomass that can be produced per unit nutrient taken up. More energy is also available to the fungus under increased C-supply and this may be put towards acquiring nutrients, *e.g.* in form of an increased enzyme production.

Finally, the increased C-availability to the root system and fungi may give individual mycorrhizal root tips larger amounts of C. This has not been examined so far. However, there seems to be a general increase in the belowground biomass, in the size of root systems and in the number of root tips. Individual root tips may thus not experience increases in the amount of C at all, but rather there may be an overall increase. Higher C uptake by the plant may therefore simply increase the C cycling in the system (Rouhier *et al.*, 1994), possibly as a result of the rapid turnover of the extraradical mycelium (Fitter *et al.*, 2000).

We know well that ECM fungal communities change in response to perturbations such as N-deposition, fertiliser addition and increased atmospheric CO₂ levels (*e.g.* Cairney & Meharg, 1999). What we still do not know are the mechanisms bringing about these changes and what the functional ecological significance of the changes is. These highly interesting questions need to be further investigated in the future.

Conclusions

- Changes in C- and nutrient-availability affect the community structure of ECM fungi growing in symbiosis with Norway spruce trees (paper II, III). The shifts seen in community composition under elevated CO₂ treatment and after balanced nutrient additions are obvious and of the same magnitude, although the CO₂ treatment was only supplied for three years and the fertilisation treatment for 15 years.
- High variability among samples is a general feature of ECM community data (Horton & Bruns, 2001), and because of this individual ECM species responses can be difficult to distinguish in field studies (paper II, III). Nevertheless, a significant effect of the CO₂ treatment was detected (paper II).
- Despite high variability a clear effect was seen after fertiliser treatment (paper II, III). Some ECM fungal species became more common with balanced nutrient additions; *Cenococcum geophilum*, *Amphinema byssoides*, *Tylospora fibrillosa*, tomentelloid species, and others, *Piloderma byssinum* and *P. croceum*, became less common (paper III).
- Data suggest that the same ECM fungal species may respond similarly to both elevated CO₂ and balanced nutrient additions. However, new sampling procedures that can account for high spatial variation are required to fully determine species-specific responses.
- Although reductions in the numbers of ECM root tips and/or the degree of root colonisation have often been reported after fertilisation (Wallenda & Kottke, 1998), daily additions of balanced nutrients for 10 years did not cause these types of changes at Flakaliden. In addition, no decrease in ECM fungal species richness was found.
- The observed changes in ECM community structure in response to elevated CO₂ (paper II) probably reflect changes in the competitive ability of species in reaction to altered C availability. In laboratory experiments greater C-supply belowground increased the production of extraradical mycelium by *Hebeloma crustuliniforme*, increasing mycelial spread and root colonisation (paper IV). Under field conditions this could enable the fungus to increase in abundance.
- ECM fungi may receive 20% of the C fixed by host plants and are essential components of host nutrient acquisition. As a consequence of the vast physiological diversity that may exist among ECM fungi, changes in community structure of these organisms may potentially alter C and nutrient allocation and turnover within forest ecosystems.

- The natural abundance of ^{13}C can be used as a tool to distinguish between the two functional groups, namely ECM and saprotrophic fungi (paper I). However, some caution is necessary since there are overlapping values between the two functional groups.
- The natural abundance of ^{13}C can also be used to reveal the host-origin of carbon in mycorrhizal fungi in mixed forests (paper I). Generalist fungi, which can be associated with several different tree species, were found to receive most of their C from overstorey trees, as indicated by their high $\delta^{13}\text{C}$ values. This implies that large trees, which are able to fix more C, potentially subsidise smaller trees via a common ECM mycelial network.

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Acknowledgements

Many people have been involved in the work surrounding this thesis and I would like to thank you all. First, my main supervisor Roger Finlay for giving me the opportunity to work with mycorrhizal fungi, for supporting me - especially with the theoretical sides of science, with writing and thinking, and for encouraging me to try and find my own way!

Andy Taylor, my co-supervisor, for invaluable help both in the lab, field and in the office. Without you this thesis would have been something completely different. All friends and colleagues at the department for a nice time and good work environment, especially Björn Lindahl, Ignacio Rangel-Castro, Anna Rosling, Ulla Ahonen-Jonnarth, Eric Danell and Christina Wedén. My roommate Mårten Gustafsson for all patience – it can not be easy to share rooms with someone like me for four years! Katarina Ihrmark and Maria Jonsson who has helped me with the PCR-work and with all types of practical questions. My friend Hanna Johannesson, for all the times you were there to listen to problems and successes. Karin Backström – you are in control, lady!

My family: Mona, Johnny and Nina – you give me much appreciated breaks away from science. Anna, Nicke and Ludde, my second family who always take me in and share my moments. Åsa – Monday evenings at your place.....need I say more? My flatmates at Sturegatan (Markus, Lena, Ingela, Lina, Sarah, Andreas and Karin) who made living in Uppsala nice and easy. And last but not least: Edwing, gracias por estar ahi.....para mi!