External Mycelia of Mycorrhizal Fungi
- responses to elevated N in forest ecosystems

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Till
Ella, min mormor, som var så vidsynt
och nyfiken på allt okänt,
och till, den likaledes i barndomen övergivne, Harry,
samt till den nedre bonden, som så oförmärkt strävar på.

Vem vördar daggmasken,
odlaren djupt under gräsen i jordens mull.
Han håller jorden i förvandling.
Han arbetar helt fylld av mull,
stum av mull och blind.
Han är den undre, den nedre bonden
där åkrarna klädas till skörd.
Vem vördar honom,
den djupe, den lugne odlaren,
den evige grå lille bonden i jordens mull.

Harry Martinsson (1904-1978)

”If we listed components of forests in order by their importance in ecosystem processes divided by how much we know about them, there can be no doubt that mycorrhizae would be right at the top.”

from Forests in Time, 2004

…about the purpose of this thesis…

Abstract

Most plants live in symbiosis with mycorrhizal fungi. Mycorrhizal roots constitute the interface between the plant and the soil, and almost every fine root of forest trees in nitrogen-limited boreal and temperate forests is colonised by ectomycorrhizal (EM) fungi. The mycelia of EM and ericoid mycorrhizal (ErM) fungi are very important for plant N uptake and N cycling in forest soils. Earlier laboratory studies have shown that elevated N levels have a negative influence on the growth of EM mycelia.

I have developed methods to quantify the biomass and the production of external mycorrhizal fungi in the field. In-growth mesh bags filled with sand were buried in forest soils to determine EM mycelial growth. I have also estimated the biomass of mycorrhizal mycelia in soil by measuring the amounts of fungal biomarkers in soil samples that degraded after depriving mycorrhizal fungi from its energy source, the C flow from tree roots, by root trenching or incubation of soil samples.

The production of EM extramatrical mycelia in spruce forest soils in southern Sweden was found to correspond to 125 to 200 kg ha^{-1} y^{-1} and the biomass of EM mycelia in soil was found to constitute a large proportion of the belowground biomass. External EM mycelia extending from the fine roots contributed most (~80%) to the total EM fungal biomass, with considerably smaller proportions on EM root tips (15-20%) and EM fruit bodies (<1%).

Fertilisation of spruce forests (~1,000 kg N ha^{-1} during a 10-year period) in SW Sweden was found to reduce EM mycelial production and biomass. The EM biomass also declined with higher nutrient availability along natural nutrient gradients in N Sweden. In the most nutrient-poor soils in these gradients ErM contributed significantly to the mycorrhizal biomass, and the production of EM mycelia was lowest in the most nutrient-rich soils. In oak forests in S Sweden EM mycelial production was lower in regions with higher N deposition (~20 kg N ha^{-1} y^{-1}) than in regions with only half that deposition. The biomass of AM fungi was found to be stimulated by high soil pH, both in the natural nutrient gradients and in oak forests.

EM mycelial production is thought to be controlled by belowground C allocation in the tree, which here is hypothesized to be regulated by forest tree N status rather than by soil N concentration. Low production of EM mycelia was observed to coincide with high nitrate leaching in at least two situations, indicating that EM mycelia may be important in retaining added N in forest soils.

Sammanfattning

De flesta växter lever i symbios med mykorrhizasvampar. I mykorrhizaroten, som utgör kontaktzonen mellan växten och den omgivande marken, sker ett utbyte av energi (kolhydrater) från växten till svampen, och näringsämnen (t.ex. kväve och fosfor) som tagits upp av mykorrhizasvampen överförs till växten. Från mykorrhizaroten utgår det externa mycelet, nätverket av svamphyfer, som utgör en förlängning av rotsystemet. De flesta av de vanligaste träden i skogar på våra breddgrader lever i symbios med ektomykorrhiza (EM)-svampar och många av de vanligaste svampfruktkroppar som man hittar i skogen är EM-svampar. Många gräs och örter i skogens fältskikt har arbuskulär mykorrhiza (AM) och många ljung- och risväxter i släktet Ericaceae har erikoid mykorrhiza (ErM). EM- och ErM-svampar är ofta anpassade till näringsfattiga markförhållanden, där kväve är det näringsämne som begränsar tillväxten hos träd och växter, och dessa svampar är speciellt bra på att ta upp kväve. Det är känt från laboratorieförsök att det externa mycelet hos EM svampar påverkas negativt av ökad kvävetillförsel. Kunskapen om hur EM-mycelet påverkas i naturliga skogsekosystem av ökade kvävehalter, från exempelvis kvävedeposition från luftföroreningar, har hittills varit mycket begränsad. Det beror bl. a. på att det tidigare inte har funnits metoder att skilja EM-svamparnas externa mycel från de nedbrytande svamparnas mycel.

Jag har därför utvecklat och utvärderat metoder för att studera mykorrhizamycel i fält. Produktion av EM-, och AM-, mycel har beräknats i inväxningspåsar fyllda med sand, som placerats ut i skogsmark. Biomassan hos mykorrhiza-mycel i jord har beräknats utifrån den mängd av kända biomarkörer för svamp som bryts ned efter att kolhydrattillförseln från trädens rötter skurits av, i fält, eller genom att jordprover inkuberats.

I granskogar i sydvästra Sverige producerades det mellan 125 och 200 kg EM-mycel ha^{-1} $år^{-1}$ och EM visade sig utgöra en stor del av den mikrobiella biomassan. Av den totala EM-biomassan utgjorde det externa mycelet den största delen (omkring 80 %), medan EM biomassan i rötterna endast bidrog med 15-20 % och fruktkropparna med mindre än 1 %.

Kvävegödsling av dessa granskogar (med omkring 1000 kg N ha^{-1} under en tioårsperiod) medförde i stort sett en halvering i EM-mycelproduktion och EM-biomassan. EM-biomassan minskade också med ökande kvävetillgänglighet i barrskogar längs naturliga näringsgradienter i norra Sverige. ErM bidrog till en stor del av mykorrhizabiomassan i de mest näringsfattiga jordarna, och EM-mycelproduktionen var minst i de mest näringsrika jordarna, i dessa naturliga näringsgradienter. I ekskogar i södra Sverige var EM-mycelproduktionen lägre i regioner med högre kvävedeposition (i Halland och Skåne; med en deposition på cirka 20 kg N ha^{-1} $år^{-1}$) än i regioner med hälften så mycket kvävedeposition (Öland och Småland, med en deposition på cirka 10 kg N ha^{-1} $år^{-1}$). AM-mycelbiomassan ökade med markens pH, både i de naturliga näringsgradienterna i norra och i ekskogarna i södra Sverige.

De metoder som har utvecklats för att mäta mycel av mykorrhiza-svampar visade sig vara användbara och borde vara lämpliga bioindikatorer vid studier om miljöbelastning på växt- och markekosystem. Produktionen av EM-mycel anses vara kontrollerad av trädens överföring av kolhydrater till rötterna vilken, utifrån studierna i den här avhandlingen, föreslås regleras av trädens kvävestatus (trädens behov av kväve) snarare än av kvävekoncentrationen i marken. Låg mycelproduktion av EM-svampar visade sig i flera av studierna sammanfalla med högt läckage av nitratkväve, vilket antyder att EM-mycel kan spela en viktig roll för att hålla kvar kväve i skogsmark och därmed minska risken för kväveläckage.

List of papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (Paper I to VI). The papers are appended at the end of the thesis.

I **Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field**
Wallander H, Nilsson LO, Hagerberg D, Bååth E. 2001.
New Phytologist 151: 753-760.

II **Production of external mycelium by ectomycorrhizal fungi in a Norway spruce forest was reduced in response to nitrogen fertilization**
Nilsson LO, Wallander H. 2003.
New Phytologist 158: 409-416.

III **Direct estimates of C:N ratios of ectomycorrhizal mycelia collected from Norway spruce forest soils**
Wallander H, Nilsson LO, Hagerberg D, Rosengren U. 2003.
Soil Biology & Biochemistry 35: 997-999.

IV **Soil N chemistry in oak forests along a nitrogen deposition gradient**
Nilsson LO, Wallander H, Bååth E, Falkengren-Grerup U.
Manuscript.

V **Total soil fungal biomass and growth of external ectomycorrhizal mycelia in oak forest soils along a nitrogen deposition gradient**
Nilsson LO, Bååth E, Falkengren-Grerup U, Wallander H.
Manuscript.

VI **Growth and biomass of mycorrhizal mycelia along short natural nutrient gradients**
Nilsson LO, Giesler R, Bååth E, Wallander H. 2005
New Phytologist 165:1 (in press).

Abbreviations

AM	Arbuscular mycorrhiza
DS	Dwarf shrubs vegetation type. The most nutrient poor end of natural nutrient gradients, with a field layer dominated by ericaceous dwarf shrubs (Paper VI)
EM	Ectomycorrhiza
ErM	Ericoid mycorrhiza
IER	Ion-exchange-resin (Paper IV)
SH	Short herb vegetation type. Intermittent part of natural nutrient gradients, with a field layer dominated by short herbs. (Paper VI)
TH	Tall herb vegetation type. The most nutrient rich end of natural nutrient gradients, with a field layer dominated by tall herbs. (Paper VI)

Content

1. MYCORRHIZAL SYMBIOSIS

1.1 Mycorrhizal symbiosis in boreal and temperate forests

Mycorrhiza is the mutualistic association through which the plant receives nutrients and water, while the fungal symbiont receives energy in the form of carbohydrates derived from photosynthesis (Smith & Read, 1997). Mycorrhizal symbiosis has evolved independently at many times, and fossil records suggest that the earliest symbiosis co-evolved with the first land-living plants (Brundrett, 2002). The fungal symbiont can receive a considerable proportion, between 15 and 30%, of plant-assimilated C (Finlay & Söderström, 1992). Mycorrhizal fungi thus have a continuous and relatively abundant energy supply, making this group of microorganisms quite different from saprotrophic fungi which have to rely on energy (carbohydrates) from soil organic matter (SOM).

Mycorrhizal roots constitute the interface between most plants and the surrounding soil. The studies described in this thesis deal with three major mycorrhizal types: 1) ectomycorrhiza (EM), which dominates in forest soils in areas with seasonal or intermittent plant growth, 2) ericoid mycorrhiza (ErM), found in habitats with ericaceous plants, and 3) arbuscular mycorrhiza (AM), found on many roots of non-woody plants, and on tree roots, especially in tropical and subtropical forests, but also on some tree species in temperate forests (Johnson et al., 1999).

The distribution of major mycorrhizal associations in natural ecosystems has been suggested to follow regular patterns over large ranges of altitude and latitude (Read, 1991; Read & Perez-Moreno, 2003; Smith & Read, 1997). At the highest latitudes and altitudes ErM symbiosis is most common, while EM successively becomes dominating in the ensuing boreal and temperate biomes. Finally, AM associations become abundant in field layers of temperate deciduous forests, in grasslands and agricultural ecosystems and in tropical forest soils. These shifts in mycorrhizal associations may be explained by the change in factors limiting plant growth, from N at high altitudes/latitudes to P at low altitudes/latitudes.

In addition, mycorrhizal types may reflect the main form of N available in the soil pool, from organic N to ammonium and finally nitrate, along the latitudinal and altitudinal gradients.

Biome	Heathland	Boreal forest	Temperate forest	Grassland
Soil	Organic-Raw Humus (Mor) or peat	Surface Organic (Mor-Moder)	Brown Earth (Moder-Mull)	Mineral
Nitrogen Source	Organic-Protein Little mineralisation No nitrification	Organic - NH_4^+ Limited mineralisation	NH_4^+ - NO_3^- Mineralisation	NH_4^+ - NO_3^- Nitrification dominates
Mycchorrhizas	ErM (some EM)	EM (ErM understory)	EM (AM understory)	AM (scarce EM)
Fungal Symbiont Activity	Extensive abilities to degrade polymers	Considerable saprotrophic capabilities in both ErM and EM fungi	EM-fungi of reduced saprotrophic capabilities + AM largely non-saprotrophic	AM-fungi with little or no saprotrophic abilities

Figure 1.1 Distribution of mycorrhizal types with latitude and altitude (redrawn from Read, 1984, with permission). (Complementary data from Read & Perez-Moreno, 2003.)

1.2 Mycorrhizal structures, carbon and nutrient transfer

All types of mycorrhiza involve the formation of some kind of symbiotic structure, through which the main exchange of nutrient and carbon occurs. EM symbiosis involves formation of 'mycorrhizas'

(or EM fine roots), the symbiotic structures formed by the EM fungi and the roots of EM trees, found on almost all fine roots of most of the boreal and temperate trees species. A 'mantle', a sheath, is formed by multiple layers of EM fungal hyphae around the root tip. Fungal hyphae also grow between the epidermal root cells to form a 'Hartig net', where the transfer of carbon from the tree and nutrients from the fungus occurs (Smith & Read, 1997). An EM root is morphologically different from a non-EM root, and the EM mantle provides protection of the root from pathogens and from desiccation, and functions as a nutrient store of e.g. N, P and carbohydrates (Langley & Hungate, 2003; Högberg et al., 1996; Brunner, 2001).

1.3 External mycelia of mycorrhizal fungi

Fungal hyphae extend from the symbiotic structures on roots of EM, AM and ErM plants into the surrounding soil forming a mycelial network. These mycelia are referred to as 'external mycelia' (or just 'mycelia') in this thesis. One important function of the mycelia is the uptake of nutrients to be used by the fungus or for translocation to the plant symbiont. Filamentous fungi have a unique ability to translocate nutrients within the entire mycelium, and they can thereby efficiently use nutrient sources spatially distributed in patches around the soil.

Hyphae in different parts of the mycelia can be adapted to a specific functional role. Fine hyphae are found in nutrient-absorbing parts of the mycelia. Other parts can be specific for long-distance nutrient transport or for host root searching. 'Rhizomorphs' are thicker 'root-like' structures of the EM mycelia comprised of multiple, parallel hyphae that sometimes merge to form wide tunnel-like pathways. This specialisation of parts of the EM mycelia may influence soil properties, such as water-holding capacity, since hyphae can have either hydrophobic or hydrophilic properties (Cairney & Bourke, 1996; Unestam & Sun, 1995). EM mycelia extending from the EM fine root may have access to a 1000-fold larger soil volume than a non-EM tree root (Rousseau et al., 1994).

AM mycelia are less dense than those of EM fungi and it has

been suggested that they have a proliferating strategy different from that of EM fungi (Olsson et al., 2002), with e.g. 'runner-hyphae' important for AM fungi in finding new host plant roots. Olsson et al. (2002) calculated the distance between mycorrhizal hyphae and found it to be 250 μm for an AM and 20 μm for an EM species. In contrast to EM and AM hyphae, ErM hyphae probably extend only a few millimetres from the root surface (Smith & Read, 1997). EM and ErM fungi are important for the uptake of N and P, but also for the uptake of other nutrients, such as K, Mg and Ca, while AM fungi are mainly important for plant P supply (Smith & Read, 1997).

1.4 EM and ErM fungi are adapted to low N availability

Microbial communities tend to be more fungal-dominated in N-poor than in N-rich soil ecosystems (Högberg et al., 2003), maybe due to the filamentous properties and the hyphal network of fungi. The mycelium enables redistribution of resources within the fungus, from patches with relatively high to patches with lower nutrient or energy supply. The continuous supply of energy from the host plant to the fungal symbiont is another reason for the importance of mycorrhizal symbiosis in the most N-poor soil ecosystems, where N mineralisation is low (and where net mineralisation may not be detectable). EM and ErM fungi can use some of this abundant energy supply to produce proteolytic enzymes to hydrolyse, and take up, organic N compounds such as lignin and other polyphenolic compounds (Leake & Read, 1990; Bending & Read, 1996ab, 1997). Some EM fungal species are able to produce extracellular proteases for degradation of chitin, nucleic acids, polyphenols, etc. (Abuzinidah & Read 1986, 1989; Finlay et al., 1992). Most EM species are, however, probably less efficient degraders than saprotrophic fungi (Schimel & Bennett, 2004). Interestingly, the transfer of nutrients between wood-degrading and EM mycelia has been demonstrated in laboratory experiments (Lindahl et al., 2001), indicating that nutrient cycling in forest soils is probably very complicated.

2. NITROGEN IN FOREST SOILS

2.1 N cycling in forest ecosystems

Although N is one of the most common elements on earth and N_2 gas constitutes almost 80% of the air in the troposphere, N is the element that is most often in short supply and thus restricts tree growth in boreal and temperate forests (Tamm, 1991). N is the fourth most common nutrient in plant tissues after C, H and O. Nitrogen is removed from forests by harvesting, by natural forest fires, which occur in boreal ecosystems roughly every hundred years (Zachrisson, 1977), and by glacial events on longer time scales.

In contrast to the situation for most other common minerals the parent rock material contains only insignificant amounts of N. Atmospheric N_2, in spite of its inert properties, is the source of most N in the biosphere. Historically, the input of N to the biosphere has mainly been due to biological processes, either from plant symbionts or free-living, N-fixing microorganisms. A small fraction of N fixation is due to lightning (less than 5%). However, during the past century or so N fixation has increasingly been the result of human activities.

In soils, only a small fraction of the total N pool is available for plant uptake. The large N pools in forest ecosystems consist of plants, microbes and SOM. A large part of soil N is bound in recalcitrant forms. N cycling is mainly driven by biological processes and net N mineralisation rates in boreal forest soils can be very low (even zero), due to low temperatures and unfavourable moisture conditions. Another special feature of N is that inputs of N to soil ecosystems, and losses of N from soil ecosystems (by nitrate leaching or denitrification), are relatively small compared with the total pools of N and amounts of N cycled.

The traditional view of soil N cycling, originating from agricultural soil science, has often focused on mineralisation of organic N compounds and the subsequent release of inorganic N into a soil pool available for plant and microbial uptake. Heterotrophic microorganisms have been considered to be the main actors responsible for the N mineralisation process. More

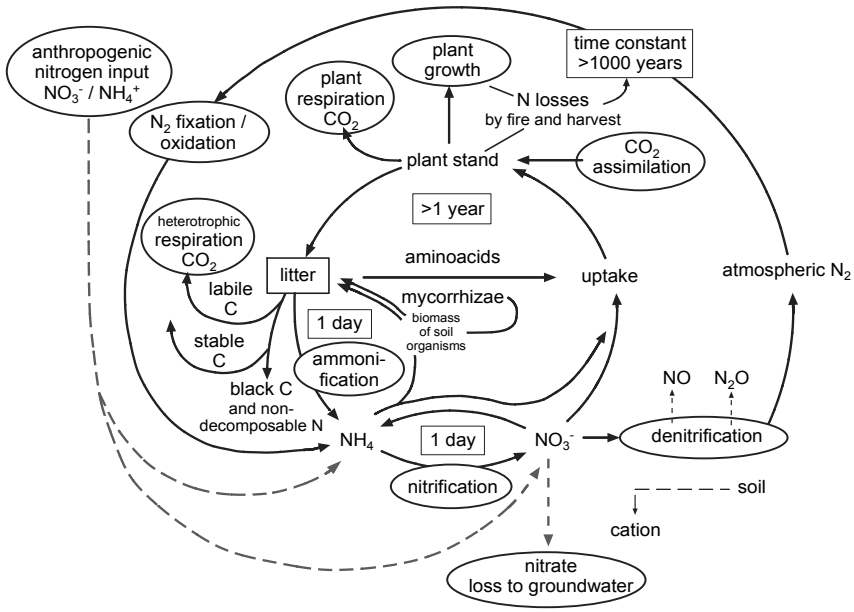

Figure 2.1 The ecosystem N cycle. Major pathways of N in forest ecosystems are shown (from Schultze, 2000, with permission).

recent research on forest soil processes has, however, shown that organic forms of N can also be taken up directly by plants and by mycorrhizal fungi (Näsholm et al., 1998). The proportion of the uptake of organic N in comparison with inorganic N in forest trees has, however, not yet been quantified (Schimel & Bennett, 2004). In accordance with this, Nordin et al., (2001) found that both the pools of organic N in soil solution and plant uptake of organic N forms were higher in nutrient-poor than nutrient-rich soils.

The majority of the studies on soil N cycling presented in the literature do not include active roots or mycorrhizas. Results from such experimental systems may be relevant mainly for nutrient-rich soil conditions, e.g. for agricultural soil, but perhaps to a lesser degree for very N-limited forest systems where mycorrhizal fungi take a more active part in N cycling. Apart from the

advantage of having a mycelial network (and a continuous supply of carbohydrates – as for mycorrhizal fungi), fungi have lower N needs (higher C/N ratios) than bacteria (Paul & Clark, 1996; Paper III), which is another reason why fungi are more important than bacteria in the cycling of N in nutrient poor-ecosystems.

2.2 Increased N input to forest soils

N availability can be increased due to human activities, as a result of N deposition, N fertilisation, or by land use. N availability can also vary naturally with latitude and altitude (Read 1991; Read & Perez-Moreno, 2003) or over shorter distances as a result of topographical and hydrological conditions (Giesler et al., 1998; Giesler et al., 2001; Högberg 2001). Time is also an important factor and ecosystems late in succession usually contain more N than these earlier in succession. Recently, it has been suggested that all forest, with time, eventually will become limited in growth by P (Wardle et al., 2004). Although the current anthropogenic N input is often relatively small compared with the total N pool and N transformation rates, the accumulated anthropogenic N input may, in time, become relatively large and in the same range as N uptake by plants (Nadelhoffer et al., 1999). During the past decades fixation of N by human activities, including production of fertilisers, cultivation of N-fixing crops and fossil fuel combustion, exceeds the N fixed naturally (Galloway et al., 2003).

2.2.1 Anthropogenic deposition of N

The deposition of N is considered to be one of the 'global change problems'. N deposition consists of oxidised N (NO_x-N) and reduced (NH_y-N) forms of N. Most oxidised N is generated by fossil fuel combustion and occurs mainly as NO and NO_2, which is easily, converted into nitric and/or nitrous acid. Reduced N forms originate mainly from agricultural activity and the amount is directly proportional to animal numbers and body mass (Asman et al., 1998).

More than half of 400 monitored forest plots in 23 European

countries were found to receive 14 kg N ha^{-1} y^{-1} or more, with the highest N deposition in central and eastern parts of Europe, and the lowest N deposition in northern Scandinavia (de Vries et al., 2003). Loads above 21-42 kg N ha^{-1} y^{-1} can lead to nutrient imbalance, increased shoot-root ratios, and drought stress, which may all influence tree health negatively (de Vries et al., 2003; Bobbink & Roelofs, 1995). In addition to the deposition of inorganic N, organic N species may also make considerable contributions (Neff et al., 2002), and deposition budgets based only on inorganic N have been suggested to underestimate the total N deposition by a third (Cornell et al., 2003).

Forests canopies have large, rough uptake surfaces and

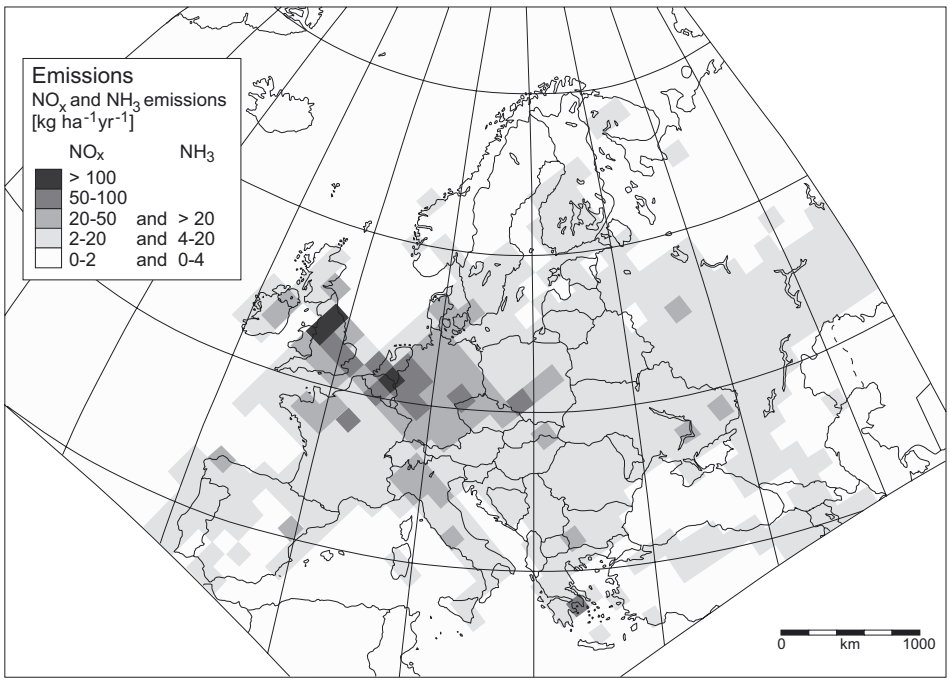

Figure 2.2 N emissions in Europe. Emissions of NO_x and NH_3 (from Schultze, 2000, with permission).

therefore receive relatively large amounts of N from deposition. N can be deposited wet, from rain, cloud droplets or from aerosols, or dry, from gases, solid particles or aerosols (Fowler et al., 1988). Conifers often have higher throughfall N than broadleaves at high levels of N input, which may depend on their greater surface area and rougher canopy, as well as the evergreen nature of conifers (Rothe et al., 2002). Trees were found to take up more N through their canopies at sites with low N deposition than at sites with high N deposition (Lövblad et al., 1995).

2.2.2 N fertilisation of forests

Fertilisation has been widely practiced in forestry in many places, including northern Sweden, during the recent decades. However, the fertilisation of Swedish forests decreased from a peak in the late 1980s, when 200,000 ha annually was fertilised, to the present level of ~30,000 ha (Högbom & Jacobson, 2002). A typical single dose of 150 kg N ha^{-1}, 10 years before harvest, normally increases stem production by 10-20 m^3. In the 1960s urea was commonly used by Swedish forest companies, but was replaced by ammonium nitrate, since this was found to increase yields more. Today, after noticing the acidifying effect of ammonium nitrate, other N fertilising compounds including dolomite are generally used.

2.3 Effects on increased N to forest soils

2.3.1 N mineralisation, nitrification and nitrate losses

Effects of N deposition include loss of species and reduced diversity of plants (e.g. Stevens et al., 2004; Falkengren-Grerup et al., 1998; Brunet et al., 1998), EM fungi (Taylor et al., 2000) and reductions in ErM plants (Strengbom et al., 2003), and N fertilisation resulted in loss of soil micro- and mesofauna (Lindberg & Persson, 2004). Many soil processes in N-limited forests are affected by increased N input. Net N mineralisation in incubated soil samples often increases (Persson et al., 2000; Falkengren-Grerup et al.,

1998), at least initially, as does tree uptake of N (Aber et al., 1998).

In situations where the availability of N is higher than the demand from plants and microorganisms, oxidation of ammonium to nitrate by microorganisms may increase. Nitrification rates are favoured by high pH and aerobic conditions. However, nitrification also occurs in acid soils, which may be explained by high nitrification rates at N- rich microsites (Schimel & Bennett, 2004). Nitrate is relatively labile in soils due to its negative charge, and movement down through the soil profile can increase under conditions of excess N in forest soils. Eventually nitrate may reach runoff water, such as ground-water or surface water, and N is lost from the system. Leaching of nitrate has been observed from many forest ecosystems after increased N input.

Denitrification is another way by which N can leave soil systems. This reduction of nitrate occurs mainly under low-oxygen situations, but does not necessarily demand water-logged conditions. Denitrification can probably also occur in 'hotspots', i.e. patches where conditions favour the reduction of nitrate (Azam et al., 2002). Through denitrification N is lost from the system as atmospheric N_2, or N_2O, the latter known as a potent greenhouse gas.

'N saturation' of forest ecosystems has been defined in various ways (e.g. Ågren & Bosatta, 1988, Aber et al., 1989, Binkley & Högberg, 1997). In the following text N saturation is used as a broad description of situations where for example tree production is not limited by N and/or where nitrate can be detected in soils at 50 cm or in runoff water. The most deleterious effects of N saturation and leaching of nitrate are impaired water quality, health risks to humans, loss of other nutrients than N, impaired forest health and increased emissions of greenhouse gases.

2.3.2 N retention capacity

Forest soils have surprisingly high N retention capacities for added N, often with small losses of N relative to the input (Aber et al., 1998; Magill et al., 2004). However, leaching of small amounts of

nitrate has been reported from some forests. The best variables to predict N leaching from forests are N deposition (Dise & Wright, 1995), the C/N ratio of the mor layer (Gundersen et al., 1998) and the ^{15}N enrichment factor of foliage relative to soil (Emmett et al., 1998). Strong correlations between nitrate mobility and foliar N concentration indicate that plant N status is critical for the N retention

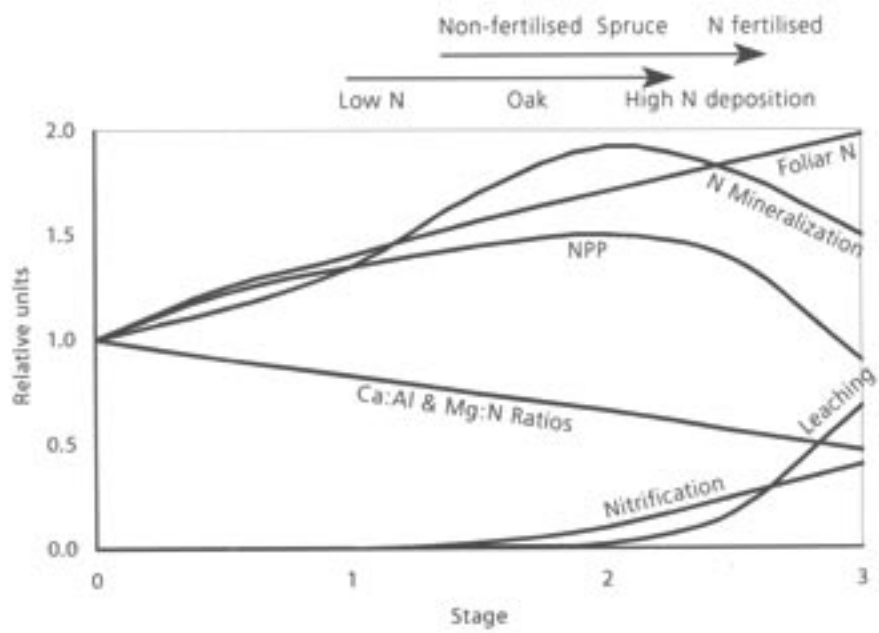

Figure 2.3. Changes over time in several ecosystem processes in response to long-term, chronic N addition. (Figure redrawn from Aber et al., 1998, with permission). The stage of N-saturation was assessed for (a) non-fertilised and (b) N-fertilised spruce forests at Skogaby (Paper II), and for (c) oak dominated deciduous forests in a N deposition gradient in southern Sweden (Paper IV and Paper V). a) Deposition is ~20 kg N ha^{-1} y^{-1}. b) Fertilised with 100 kg N ha^{-1} y^{-1}, for more than 10 years. c) Deposition ranges from 10 to 20 kg N ha^{-1} y^{-1}.

capacity in forest soils (Aber et al., 1998). The initial conditions of a forest stand, i.e. its stage in the N saturation scale, predicts its response to chronic N addition, but it has been suggested that the addition of large amounts of N will eventually cause a 'nitrate breakthrough', even in the most N-limited stands (Magill et al., 2004). The importance of tree N uptake for N leaching was demonstrated by more N leaching from older, mature forests than from younger stands, with their higher nitrogen demands (Goodale et al., 2003).

It has been suggested that forest ecosystems exposed to high N deposition respond in a non-linear way, passing through several stages where N limitation on tree growth ceases in the first stage (Fig. 2.3). In the second stage nitrification and some nitrate leaching are seen, which is followed by a decline in tree growth and further increases in nitrification and nitrate loss. Nitrate production and mobility have thus been suggested to have a key role in controlling the response of forest ecosystem to N deposition. Fixation of N by abiotic processes in forest soils and uptake by mycorrhizal fungi have been suggested to be the most important mechanisms for forest soil N retention capacity (Aber et al., 1998).

2.3.3 Response of deciduous and coniferous forests to elevated N deposition

Most studies on forest soil responses to elevated N input have been performed in coniferous forests. The few studies on deciduous forest have reported somewhat contradictory results. For example, Aber et al. (1998) and Magill et al. (2004) reported higher N retention capacities for added N in mixed deciduous stands than in pine plantations, and Rothe et al. (2002) found higher nitrate leaching from spruce than from adjacent beach stands. Kristensen et al. (2004), on the other hand, reported higher nitrate concentrations in deciduous forest soils than in coniferous forests soils. The soil C/N ratio and foliage N can be used to predict nitrate leaching from coniferous forests (Gundersen et al., 1998; Dise et al., 1998), but not from deciduous forests (Kristensen et al., 2004).

Deciduous forests are adapted to more fertile soils than coniferous forests (Kristensen et al., 2004; Persson et al., 2000). Interestingly, deciduous forests on similar soil types to reference coniferous forests responded differently to N deposition from deciduous forests in general (Persson et al., 2000; Rothe et al., 2002). These findings stress the importance of careful experimental design in comparative studies between forests of different types.

In addition to lower soil C/N ratios, higher soil pH, has been considered as the cause of higher net N nitrification rates in deciduous than in coniferous reference forests in a study of forests in a European N deposition gradient (Persson et al., 2000). Higher leaf litter pH and lower content of polyphenolics in deciduous than in coniferous forest soils could possibly affect other factors, such as the soil micro- and mesofauna. Soil animals are important, for example, for soil mixing and grazing on fungal hyphae. Moreover, deciduous forest soils are often not, or to a lower degree, podsolised than coniferous forest soils, which would affect N cycling. Field-layer plants, often more abundant in temperate deciduous forests than in coniferous forests, have been shown to be important for N retention, especially early in the season, and may act as a temporary storage pool of N (Olsson & Falkengren-Grerup, 2003).

2.4 How do mycorrhizal fungi respond to elevated N?

The response of EM fungi to elevated N has been reviewed by Wallenda & Kottke (1998). EM fruit bodies are often found to be reduced, while the EM fine root colonisation rate appears to be less affected (Wallenda & Kottke, 1998) with almost all roots colonised by EM fungi (Taylor et al., 2000) also after increased N input. The EM fungal community on root tips is often reported to change in response to N addition. Long-term N deposition has been found to reduce the number of EM species on roots (Lilleskov et al., 2002).

The response of EM mycelia to elevated N levels has been studied in numerous microcosm studies. Through such studies it was revealed that EM mycelia respond to increased N levels by

decreased growth (Arnebrant, 1994) and that the mycelium is more affected than EM biomass on fine roots (Wallander & Nylund, 1992). Many studies on EM mycelia, so far mostly performed in microcosm studies and pot cultures, have contributed to the large amount of knowledge that exists about EM symbiosis. Mechanisms for the uptake and assimilation and subsequent transfer of nutrients to the host plant by EM fungi, and the allocation of carbohydrates to different parts of the EM mycelia are examples of information obtained from experiments in the laboratory or in the greenhouse. The advantages of such studies are that interference from other groups of organisms may be avoided and that the effects of a particular form of treatment on the mycorrhizal symbiosis can be studied under controlled conditions. However, in nature multiple organisms and species co-exist in intricate combinations. It is therefore difficult to extrapolate the results from laboratory experiments, since the results may be strongly influenced by the properties of the single or few species used and the absence of a mixed EM community. Moreover, the EM species that can be cultivated under laboratory conditions are often those tolerating (or demanding) high N levels. In addition, in microcosm studies and pot cultures the use of very small tree seedlings as plant symbionts does not represent a C source, or a C sink, comparable to that of mature trees in forests. Although the results from studies of EM symbiosis performed in microcosms or in pot cultures may have good precision in quantifying biomass, flows of nutrients, etc., these microcosm studies may have little relevance for field conditions and natural ecosystems. To achieve a better understanding of the functional role of mycorrhizal fungi in natural systems it is therefore desirable to perform studies of mycorrhizal symbiosis under more realistic conditions in the field.

Field studies of EM fungal abundance in forests have up to now, however, been forced to rely on observations of EM fruit bodies or EM root tips. EM fruit bodies can be good indicators of the response of specific species of EM fungi to various kinds of treatments, nutrient conditions, pollutants, etc. However,

fruit bodies probably contribute only a fraction of the total EM biomass, and treatment effects estimated from biomass changes in fruit bodies can not be translated into changes in EM biomass on root tips and mycelia (Dahlberg, 2001). One reason for this is that many EM species do not produce fruit bodies, or produce only hypogenous fruit bodies (Köljalg et al., 2000).

Studies on EM root tips have provided important information, especially about EM community structures and the responses of individual EM species to various treatments. However, since EM root tip biomass tends to respond less than EM mycelia to N addition, the response of EM root tips can probably not be translated to response of EM mycelia. It is also known that different EM species have very disparate strategies for mycelial proliferation in soil (Agerer, 2001), and thus vary in the amount of mycelia produced. Therefore, to obtain better knowledge about EM mycelia in soil, it is necessary to develop methods to study these under realistic conditions in the field.

3. AIMS OF THE STUDY

- To develop and evaluate methods of studying the production of external mycelia of mycorrhizal fungi, and the biomass of mycorrhizal fungi, in forest soils under natural conditions in the field (Papers I, III & VI).

- To investigate whether the production and the biomass of mycorrhizal external mycelia are negatively affected by increased levels of N following:

  — nitrogen fertilisation (Paper II),

  — anthropogenic deposition of N (Paper V),

  — natural variations in soil N (Paper VI).

- To identify possible relationships between EM mycelial growth and leaching of N (Papers IV & V).

4. METHODS

4.1 Methods used to estimate the production of mycelia by mycorrhizal fungi

In order to estimate the production of EM (and AM) mycelia, fungal in-growth mesh bags filled with sand were buried in forest soils for at least three months, usually from early summer to late autumn. The fungal in-growth bags (approximately 5x5x1 cm in size) were constructed of nylon mesh with a mesh size (50 µm) allowing the in-growth of fungal hyphae, but not of roots (Paper I). The in-growth bags were filled with acid-washed sand in order to minimize the colonisation of mycelia by saprotrophic fungi, and they were buried at the interface between the organic horizon and the mineral soil. The effect of a local P source was studied by adding mineral P (apatite) to the sand in the mesh bag (Paper II).

Figure 4.1 Ingrowth mesh bags were used to estimate EM (and AM) mycelial production.

After harvest the in-growth bags were opened in the laboratory and the fungal colonisation of the sand in the mesh bags was observed under a dissecting microscope. The fungal colonisation was estimated visually and by analysis of the phosphoplipid fatty acid (PLFA) 18:2ω6,9 (and ergosterol, in Paper I) content (for EM fungi), and by using the neutral lipid fatty acid (NLFA) 16:1ω5 content (for AM fungi). AM fungi do not contain PLFA 18:2ω6,9 or ergosterol and are thus not included when using these biomarkers.

Two types of experiments were performed in order to determine whether fungal colonisation of the in-growth mesh bags was mainly by EM fungi or saprotrophic fungi (Paper I). In the first test, C allocation to roots and the EM fungal partner was obstructed in root-trenched plots, created by inserting plastic tubes (diam. 0.16 m; length 0.25m) into the soil. Fungal colonisation of mesh bags in root-trenched plots was insignificant, compared with that outside trenched plots. Almost no mycelial colonisation was observed visually after up to 12 months. The use of chemical biomarkers confirmed this observation, with only 4–17% mycelial colonisation found in root-trenched plots compared with the colonisation without trenching (with intact roots).

In a second control test the carbon isotopic composition of mycelia collected from in-growth mesh bags and fungal fruit bodies sampled in the same forests was analysed. The carbon isotopic composition of the mycelia was significantly different from that of fruit bodies of saprotrophic fungi, but the same as those of EM fungi from the same site (Fig. 4.2). Both these experiments thus showed that mycelia colonising the in-growth mesh bags mainly originated from EM fungi, and that saprotrophic fungi colonised the mesh bags to an insignificant degree.

The different techniques used to estimate EM mycelial production (visual estimates and the PLFA 18:2ω6,9) were fairly well correlated (Paper V & VI), but in some cases very high PLFA 18:2ω6,9 values were found without any corresponding high value of visual estimate. Fungal hyphal length has been used as an alternative

estimate of EM mycelial production in mesh bags (Wallander et al., 2004), with similar good precision to the results presented in this thesis.

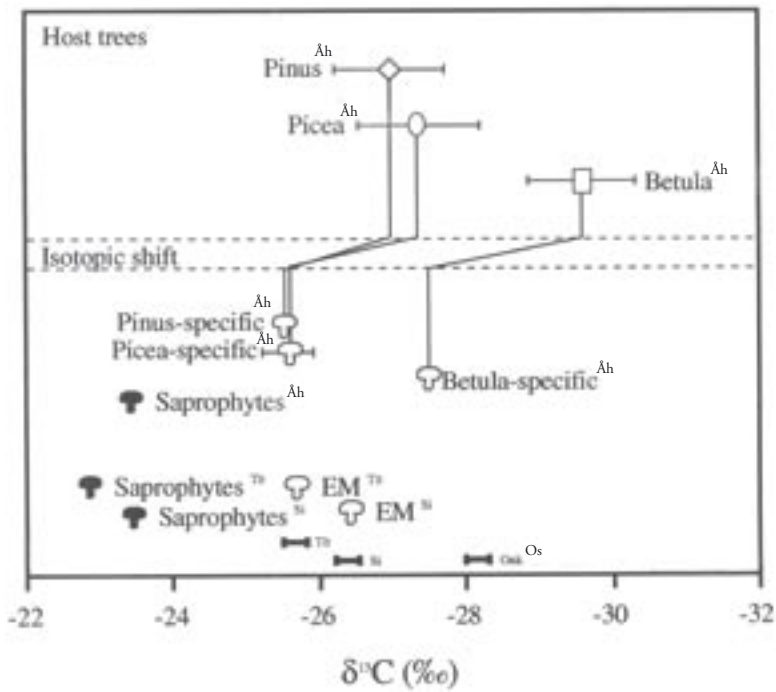

Figure 4.2 C isotopic composition of forest tree foliage, fruit bodies of EM and saprotrophic fungi (Åh) (From Högberg et al., 1999, with permission). Complementary data on fruit bodies of EM and saprotrophic fungi and mycelia from in-growth mesh bags (Paper I & V). Åh - Åheden spruce forest in northern Sweden (Högberg et al., 1999). Tö - Tönnersjöheden spruce forest, Si – Silvåkra pine forest (Paper I) and Os - Oskarshamn oak forest (Paper IV and V) in southern Sweden; Tö in Halland, Si in Skåne, and Os in Småland.

4.2 Methods used to estimate the biomass of mycorrhizal fungi in forest soils

Until recently, no method or biochemical marker has been available to distinguish between EM fungi and saprotrophic fungi in forest soils. Recently, molecular methods including sequencing of DNA extracted from forest soils have been used for identification of soil fungi (Landewert et al., 2003a). Molecular methods have been used for quantification of a few known fungal species in pot cultures (Landewert et al., 2003b). However, these methods seem not to be reliable yet for the quantification of fungal biomass in natural communities as the amplification rates for different species during the PCR reaction step may be different.

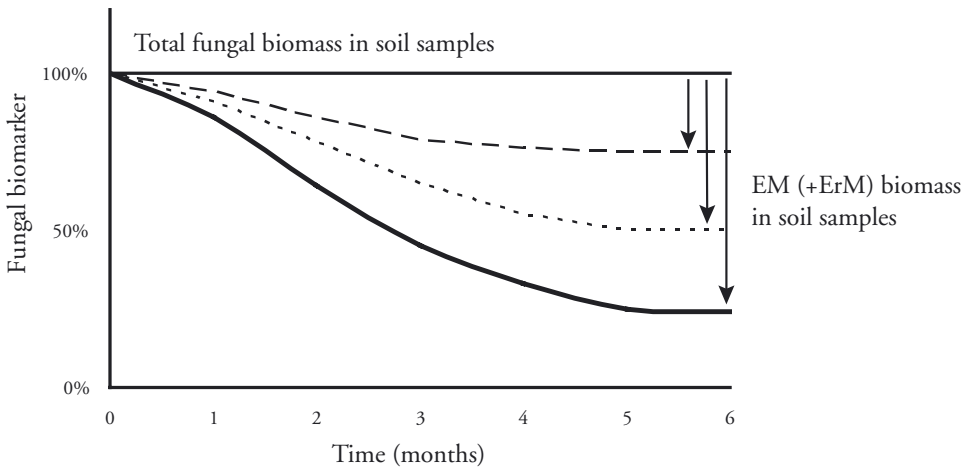

Figure 4.3 The method used to estimate mycorrhizal biomass in forest soil samples. The fungal biomarker (PLFA 18:2ω6,9 or ergosterol for EM [and ErM]) that degrade after root-trenching or during incubation of soils is assumed to originate from mycorrhizal fungi.

I have used known fungal-specific biochemical markers to estimate the biomass of mycorrhizal fungi in soil samples (Paper I, II & VI). The PLFA 18:2ω6,9 and ergosterol are biochemical markers often used for fungal biomass (Frostegård et al., 1993; Bossio et al., 1998; Larsen et al., 1998; Bardgett & McAlister, 1999; Montgomery et al., 2000). Another PLFA, and the neutral lipid fatty acid (NLFA), 16:1ω5, are often used as markers for AM fungi (Olsson, 1999). In the present studies it was assumed that mycelia (and the fungal biomarker) of mycorrhizal origin will degrade when active roots are not present in the system, since the mycorrhizal fungi are then deprived of its energy source, active tree roots, and the amount of the marker degraded was used as a measure of EM (and ErM) biomass in soil samples. C allocation to roots and mycorrhizal fungi was prevented by creating root-trenched plots in forests (as described in section 4.1.1) (Paper I & II) and by incubation of soil samples (Paper VI). The latter method is further described in Paper VI and by Bååth et al. (2004).

5. GROWTH AND BIOMASS OF EM MYCELIA IN FOREST SOILS

5.1 How large is the biomass of mycorrhizal mycelia?

I have estimated that the production of EM mycelia in in-growth mesh bags corresponds to 125 to 200 kg ha^{-1} per year in spruce forests in SW Sweden (at Skogaby), and that the biomass of EM mycelia in soil samples corresponds to 700 to 900 kg ha^{-1} in the upper 10 cm soil (Paper I). This can be compared with data from earlier studies estimating fungal biomass on EM roots to be 150 kg ha^{-1} (Kårén & Nylund, 1997), fine root biomass in the same soil horizon 2,700 kg ha^{-1} (Bergholm et al., 1994). These results, indicating a considerable contribution of EM fungi to the total belowground biomass are of a similar magnitude to those from an experiment of pine forests in northern Sweden where the flow of carbohydrate photosynthates to the roots was cut off by tree-girdling. Here, EM fungi were estimated to contribute one third to the soil microbial biomass (Högberg & Högberg, 2002).

By comparing with other studies of EM biomass from the same spruce forest at Skogaby, I have calculated that 80% of the total EM fungal biomass was external mycelia (Paper I) and 20% fungal biomass on EM roots (Kårén & Nylund, 1997). This is a similar partitioning to those found earlier in pot studies (Colpaert et al., 1992; Wallander & Nylund, 1992). The EM external mycelial biomass was also found to be much greater than that of EM fruit bodies, which was only 6 kg ha^{-1} in the same forest (Wiklund et al., 1994).

The fungal in-growth mesh bags used to estimate EM mycelial production enable analysis of the nutrient content of pure EM hyphae originating from a natural EM community. EM mycelia were found to have a very high C/N ratio, about 20, with very little variation in the data (Paper III). This is much higher than earlier C/N ratios reported for fungi, from 5 to 10 by Paul & Clark (1996), based on pure cultures of fungi grown on agar plates. The much higher C/N ratios observed in the present work thus stress the importance of studying fungi in the field under N-limited conditions.

Moreover, the low N demand by EM mycelia may be an additional explanation of the importance of EM fungi in N cycling in N-poor ecosystems. It is likely that not only EM fungi, but also many ErM and saprotrophic fungi, may have high mycelial C/N ratios.

5.2 Seasonal growth of EM mycelia

The seasonal patterns of EM mycelial growth found in the studies described in this thesis in coniferous (Paper I) and oak forests (Paper V) have many similarities. Almost no EM mycelial production was found in oak forests during the winter (November to April) and very low production was observed during spring and early summer in coniferous forests. Here, EM mycelial growth tended to start in mid or late summer, and the main production occurred during the autumn. A similar seasonal pattern was found in oak forests in southern Sweden (Paper V). However, in the oak forests EM mycelial growth rates were often similar during the summer (April to August) and the autumn period (August to November).

The period of EM mycelial production is similar to earlier found variations in ergosterol to chitin ratios of EM roots, with low values in January and high values in October (Wallander et al., 1997), and with the occurrence of EM fruit bodies. The seasonal patterns of EM mycelial growth also coincided with those of tree root growth in the same forest (Stober et al., 2000). Also in oak forests, seasonal patterns in EM mycelial growth can be correlated with root biomass. EM mycelial growth rates in oak forests were almost as high during summer as during autumn (Paper V) and oak root biomass is reported to have small seasonal variations (Büttner & Leuschner, 1994). More data are, however, necessary to confirm this relationship. The observed EM mycelial temporal production pattern thus probably follows the seasonal tree C allocation patterns and forest tree phenology.

Coutts and Nicoll (1990) found, however, a peak in EM mycelial growth rates during the summer, followed by a continuous decline during the autumn. This different

seasonal growth pattern for EM mycelia may possibly be explained by the use of small seedlings as plant hosts.

6. HOW ARE MYCORRHIZAL MYCELIA AFFECTED BY ELEVATED N?

6.1 Nitrogen fertilisation of spruce forests

Spruce forests in Skogaby, planted in 1967, in south-western Sweden have been experimentally fertilised with 100 kg N ha^{-1} y^{-1} since 1988. EM mycelial production was found to be reduced to about half in N-fertilised of that in non-fertilised forests (Paper II). There were, however, large variations between the two seasons investigated. EM mycelial growth almost disappeared in N-fertilised plots during the first of the seasons studied, while the reduction was less obvious in the second season. EM biomass in soil samples also decreased in N-fertilised plots, with similar interannual variations to those for EM mycelial growth.

Tree-girdling experiments enable separate estimates of autotrophic soil respiration and heterotrophic soil respiration. Interestingly, the decreases in autotrophic respiration (from roots and EM fungi) after N fertilisation of spruce forests in northern Sweden (Flakaliden) (P. Olsson, pers. comm.) were of a similar magnitude as the reductions in EM mycelial growth after N fertilisation at Skogaby (Paper II). A large proportion of the estimated autotrophic respiration should be due to EM, and both these methods (EM mycelial growth and autotrophic respiration) should reflect EM activity. These both studies thus show the negative effect of N fertilisation on EM fungi in spruce forest soils.

6.2 Nitrogen deposition in deciduous forests

Oak-dominated deciduous forests along an N deposition gradient in southern Sweden were studied in order to evaluate the influence of N deposition on soil N status, nitrate leaching (Paper IV), biomass of total soil fungi and EM mycelial growth (Paper V). Net N mineralisation and nitrification rates have earlier been found to increase with N deposition in this gradient (Falkengren-Grerup & Diekmann, 2003). Several of the measured variables, e.g. locally

estimated NO_3^- deposition (measured with ion-exchange resins; IER), grass N content, and $\delta^{15}N$ values of grass and organic soil were correlated with N deposition (Paper IV). Other data, e.g. soil NO_3^- (measured with IER) and grass N content, suggest that N deposition has resulted in increased soil N availability in these oak forests. Studies of the isotopic composition of N in soils and plants can be used to determine the N status of forests since many processes, such as nitrification, fractionate N so that the product (e.g. nitrate) becomes isotopically lighter (N depleted) compared to the remaining substrate (e.g. ammonium). Nitrification, as a result of input of N in excess of the needs for plants and microbes, followed by nitrate leaching would result in ^{15}N depletion in soils at lower horizons (Högberg, 1997). The $\delta^{15}N$ values down the soil profile did not indicate N saturation in any of the oak forest soils, even in the regions with high N deposition (Paper IV). Nitrate leaching (NO_3^- at 50 cm) was found only occasionally at a few sites and no soil chemical or environmental variable was found to correlate with these higher amounts of NO_3^- at 50 cm (Paper IV).

It was possible to detect lower EM mycelial growth in the regions receiving most N (20 kg N ha^{-1} yr^{-1}) compared to regions receiving less N (10 kg N ha^{-1} yr^{-1}) by deposition (Paper V), in spite of the relatively modest difference in N deposition. Total soil fungal biomass did, however, not differ in the N deposition gradient. Thus, production of EM mycelia using ingrowth mesh bags is probably a more sensitive method in detecting effects of elevated N inputs than estimations of total fungal biomass in soil samples. Amounts of AM fungi were mainly correlated to soil pH, and not to N deposition (Paper V). Some AM fungi are known to be inhibited by low pH (van Aarle et al., 2002), and a positive correlation has been found between AM plants and soil pH (Olsson & Tyler, 2004).

6.3 Natural nutrient gradients

Nutrient availability and tree productivity have been found to change along natural gradients over short distances (<100 metres).

In these short gradients trees are ectomycorrhizal, while the field layer vegetation shifts from being dominated by ericaceous dwarf shrubs (DS) at the nutrient-poor end to an increasing abundance of AM plants, with a short herb- (SH) and finally a tall herb- (TH) dominated field layer at the nutrient-rich end (Giesler et al., 1998, Giesler et al., 2002, Högberg, 2001). In these gradients the limiting factor for plant growth shifts from N to P (Giesler et al., 2002), and the dominating forms of N in the soil solutions shift from organic N, to ammonium and finally nitrate (Nordin et al., 2001).

Total fungal biomass has previously been observed to decrease along one natural nutrient gradient (Betsele) (Högberg et al., 2003). I found a decrease in total fungal biomass along this and three other natural nutrient gradients and this was due to a reduction in mycorrhizal rather than saprotrophic fungi (Paper VI). EM+ErM biomass was highest in the most nutrient-poor (DS) vegetation type and decreased along the gradients, and EM mycelial production was lowest in the most nutrient-rich (TH) vegetation type. Here, where pH is high and P may be the limiting factor for plant growth, both AM biomass in soil samples and production of AM fungi was highest.

Mycorrhizal communities thus change from being dominated by ErM, to EM, and finally to AM fungi along short natural gradients similar to original suggestions regarding altitudinal or latitudinal gradients over longer distances (Read, 1991; Smith & Read, 1997; Read & Perez-Moreno, 2003).

6.4 Factors influencing mycelial growth of mycorrhizal fungi

6.4.1 Nitrogen levels and root biomass

From the studies presented in this thesis it can be concluded that EM mycelial production decreases due to elevated N levels. This is irrespective of the cause of the N increase: fertilisation of forests (Paper II), deposition of N via airborne pollutants (Paper V), or natural variations in N (Paper VI).

Interestingly, EM mycelial growth in in-growth mesh bags was

well correlated to the biomass of tree roots (in the natural nutrient gradients at Betsele and Varjisån), while EM+ErM biomass in soil samples was well correlated to the total root biomass (Betsele), including field layer plants, which dominated root biomass in the most nutrient-poor soils (Paper VI). Furthermore, EM mycelial production and field measured soil respiration (Högberg et al., 2003) were correlated in the Betsele gradient, thus corroborating the suggested high contribution by EM to total soil respiration (Högberg et al., 2001). The biomass of a mycorrhizal fungus is thus likely to be linked to the host plants belowground C allocation and root biomass.

Figure 6.1 Vegetation types in the natural nutrient gradients (photos from Betsele). From left to right: dwarf-shrub (DS), short-herb (SH) and tall-herb (TH) vegetation type.

6.4.2 Soil pH

Two of the studies presented in this thesis confirmed previously found positive correlations between soil pH and AM biomass. The PLFA 16:1ω5 in soil samples (an indicator for AM fungi) was correlated with pH in oak forests in the N deposition gradient (Paper V) and also in the natural nutrient gradients (Paper VI).

The amount of total soil fungi (PLFA 18:2ω6,9 in soil samples) was not affected by the soil pH in the N deposition gradient (Paper V) Earlier studies have also shown the absence of a correlations between

the PLFA 18:2ω6,9 and pH, in soils from beech forests in a pH gradient (Bååth & Anderson, 2003), in alkaline-polluted conifer forests (Bååth et al., 1992) and in limed coniferous forests (Bååth et al., 1995).

No general or direct effect of pH was found on EM mycelial production. EM mycelial growth decreased along the natural nutrient gradients, where N availability and soil pH increased (Paper VI). Along the N deposition gradient, however, EM mycelial growth was lower in regions with high N deposition, and slightly lower soil pH, than in regions with low N deposition (Paper VI). Soil pH can thus either affect nutrient cycling, as in the natural nutrient gradients, or

be an effect of inputs of acidifying compounds from deposition. It is thus probably soil N availability and the N demand of the trees, and not soil pH, that mainly influences EM mycelial production.

7. HOW IS THE PRODUCTION OF EM MYCELIA REGULATED?

7.1 Carbon allocation to the tree roots

EM mycelial production was observed to be stimulated in N-rich patches in non-fertilised, but not in fertilised forests (Paper II). Similarly, Stober et al. (2000) found that hyphal length density in soil was stimulated by local additions of N in a nutrient-poor, but not in a nutrient-rich, forest soil. Based on these findings I hypothesize that EM mycelial production is probably not directly related to soil N concentration *per se*, but rather to the N status of the trees. However, more data from manipulative experiments are needed to confirm this hypothesis.

Increased N availability in N-limited forests is known to increase the shoot-to-root ratio of conifers (Seith et al., 1996; Flückinger & Braun, 1998) by reducing the allocation of C to the roots (Wallenda et al., 1996). The good correlations observed between tree root biomass and EM mycelial production (see section 6.4 and Paper VI) also suggest that root biomass and tree C allocation to the roots plays a crucial role in regulating EM mycelial growth. Moreover, EM mycelial production was negatively correlated to N availability and tree productivity in the natural nutrient gradients (Paper VI) indicating a decrease in C allocation to the roots and to EM fungi with increasing N availability.

The seasonal pattern of EM mycelial growth and tree root growth (Paper I & V), as earlier discussed (see section 5.2), with the highest EM mycelial growth during late summer and autumn, is another indication of the crucial role of tree C allocation to roots as a major regulator of EM mycelial production. C allocation patterns to the fungus according to tree phenology, in addition to the role of tree nutrient status, thus appear to be a major mechanism in regulating EM mycelial growth.

Apart from the regulation of EM mycelial growth by the trees, it has been suggested that EM fungi have the ability to direct their C use to mycelial growth or to assimilation of N,

depending on N availability (Wallander, 1995). EM fungi may, under situations with high soil N availability, use less C for mycelial growth by increasing the translocation of C and N to the host. The fungus could protect itself from high internal NH_4^+ concentrations by this mechanism, which may be induced by a need for efficient energy use. However, the importance of this ability of EM fungi, relative to the role of the tree, is not known.

7.2 Changes in EM species composition

Most EM species are more sensitive to N deposition than saprotrophic fungi (Arnolds, 1991; Peter et al., 2001). Changes in the composition of root-tip EM communities have been reported after long-term N deposition (Lilleskov et al., 2002; Taylor et al., 2000) or N fertilisation (Kårén, 1997). Changes in EM species composition could be caused by responses to C allocation to tree roots and EM fungi after elevation of N levels. Such shifts in the EM fungal community may reflect different strategies for C utilisation by EM species. EM fungi such as *Lactarius theiogalus*, *L. rufus*, *Tylospora spp* and *T. fibrillosa* are often found to increase with N availability (Lilleskov et al., 2002; Taylor et al., 2000; Kårén&Nylund, 1997). These EM species, found to increase with N, have smooth mantles and short external mycelia, characterised by Agerer (2001) as being of 'contact exploration type' (*Lactarius*) or 'short-distance exploration type' (*Tylospora*). These EM species probably expend less C on mycelial growth, due to their short external mycelium, than EM species found to decrease in N-rich soils (*Cortinarius spp*, *Piloderma spp*) which may need to use more C to build their more widespread mycelia.

The decrease in EM mycelial growth due to elevated N, as found for example in the studies presented in this thesis, may be caused by a general decrease in mycelial production by the existing EM fungal species, or may be the result of a change in EM fungal species composition to species that produce lower amounts of external mycelia, or both. Lilleskov et al. (2002) and

Taylor et al. (2000) found that normally, under low-N conditions, EM species specialised for the uptake of organic N (such as *Cortinarius* and *Piloderma spp*) dominate. Such changes in EM species composition, as a result of higher N availability, thus indicate functional changes in these EM fungal communities.

8. CONSEQUENCES OF DECREASED EM MYCELIA AFTER ELEVATED N INPUT

8.1 Is there a relation between EM mycelia and N leaching?

This thesis presents at least two situations where low EM mycelial production was found simultaneously with nitrate leaching. Firstly, an interannual variation was found in EM mycelial production and EM biomass in soil, with greater reductions in the N-fertilised spruce forests during the first of the two investigated seasons at Skogaby (Paper II). During the first season the reduction in EM mycelial growth was more obvious, and nitrate leaching was higher, compared with a smaller reduction in EM mycelial growth and lower amount of nitrate leaching during the second season. Secondly, EM mycelial production was negatively correlated with nitrate leaching, and sites with almost no EM mycelial growth had high nitrate concentrations at 50 cm depth in oak forests in the N deposition gradient (Paper V). Furthermore, preliminary data from spruce forests in southern Sweden and Denmark indicate similarly that stands with high nitrate levels at 50 cm soil depth often have poor EM mycelial production (Nilsson & Gundersen, unpublished data).

Reduced amounts of EM mycelia may thus result in increased nitrate leaching. It is, however, not possible to rule out the possibility that high levels of nitrate can cause EM mycelial growth to decrease, or that some other factors govern these processes. We have initiated a more comprehensive study in spruce forests in Denmark and southern Sweden, as well as laboratory experiments to evaluate a possible cause effect relationship between EM mycelia and nitrate leaching and to further evaluate the possible importance of EM mycelia in counteracting nitrate leaching from forest soils.

Another interesting aspect is the seasonal variation in EM mycelial growth and nitrate leaching. The lowest EM mycelial growth rates were found during the winter and spring (see section 5.2 and Paper I & V). Although little is known about EM mycelial longevity and turnover times, it can be assumed that the activity of EM mycelia

is low during winter and spring. Nitrate concentrations are usually highest during this time of the year, with the highest risk for nitrate leaching. Thus, a reduced amount of EM mycelia during the spring could reduce the N uptake capacity, and possible N retention capacity, by EM mycelia at the same time as nitrate concentrations are high.

Interestingly, Lovett et al. (2002) reported high nitrate concentrations and nitrification rates, and nitrate leaching from natural forest stands dominated by maple, but low nitrate concentrations and nitrification rates, and no leaching of N, from stands dominated by oak. This may indicate an important role of EM fungi for N cycling in forest soils, since oak trees are ectomycorrhizal, but maples are arbuscular mycorrhizal trees.

One possible explanation of the high N retention capacity could be that EM fungi retain added N in their biomass. Wallander et al. (2004) estimated the N content in living EM mycelia down to 70 cm soil depth to be 120 kg N ha^{-1} in spruce forests and 180 kg N ha^{-1} in mixed oak-spruce stands in south-eastern Sweden. The total N pool of EM mycelia and EM root tips in the upper 10 cm of soils was found to be around 20 kg N ha^{-1} in spruce forests in south-western Sweden (Paper II & III, which is in the same range as the annual N deposited in this forest. However, the N pools in living EM biomass in these soils did not increase after N fertilization, although N concentration in the mycelia increased from 2.5 to 3.3% (Paper II & III), indicating that living EM biomass can not solely explain the N retention capacity in forests with high N input. It is, however, possible that dead and decaying EM biomass or, as suggested by Aber et al. (1998), exudation of extracellular enzymes by EM fungi, contribute to the N retention capacity of forest soils.

Berntson and Aber (2000) recently suggested that 'slow', microbial, immobilisation processes would be replaced by 'fast', abiotic immobilisation processes in forests soils approaching a situation of being N saturated. Only after further N addition, and the loss of the slow immobilisation, did nitrate start to leach (Berntson & Aber, 2000). However, these experiments were performed in soil cores

without active roots or mycorrhizas. This makes the outcome of the experiments less relevant for most natural systems where mycorrhizal mycelia still not is considerably reduced e.g. by long-time high N input.

8.2 Uptake of other nutrients may be impaired

In this thesis I hypothesize that forest tree N status, rather than soil N concentration, regulates EM mycelial growth. Reduced EM mycelia, in response to elevated N input, may impair the uptake capacity for other nutrients than N (Smith & Read, 1997). However, under situations of elevated N input the effects of nutrient limitation on tree C allocation to the roots and EM mycelial production may depend on which nutrient that is growth-limiting.

Host trees experiencing P limitation for growth are found to increase C allocation to the roots (Ericsson, 1995) and to stimulate EM mycelial production (Ekblad et al., 1995). Under such conditions EM mycelial production was found to be stimulated by local addition of P (apatite) in N-fertilised spruce forests (Paper II) and in forest stands deficient in P (Hagerberg et al., 2003). The response to K limitation may, in contrast to P limitation, result in reduced C allocation to roots (Ericsson, 1995) and EM mycelia (Ekblad et al., 1995), and the addition of a local K source was found not to affect EM mycelial growth in K-limited forest stands (Hagerberg et al., 2003). The trees do not appear to have an inherent response to ameliorate K depletion by increasing the belowground allocation of C.

The severity in the effects of elevated N on the tree thus depends on which nutrient is growth-limiting. Interestingly, Lilleskov et al. (2002) found that EM fungal species better adapted for uptake of P increased in abundance in response to anthropogenic N deposition. This is another indication that the depletion of P is less serious than that of K. Reduced EM mycelial production in N-fertilised forests at Skogaby (Paper II) was found to be correlated with decreased uptake of Mg and Ca. This may indicate an important role for EM fungi in the uptake of these nutrients, as found earlier (Jentschke et al., 2000; Blum et al., 2002).

9. CONCLUSIONS

- It is possible to quantify the production and the biomass of ectomycorrhizal (EM) extramatrical mycelia in the field.
- EM extramatrical mycelia contribute substantially to the belowground biomass in forest soils.
- EM mycelia have high C/N ratios (around 20).
- EM extramatrical mycelia contribute more than root tips and fruit bodies to the total biomass of EM fungi.
- Raised N levels in forest soils, whether caused by anthropogenic activity (deposition or fertilisation) or by natural variations, result in reduced EM mycelial production.
- EM mycelial production is controlled by belowground C allocation in the tree, which is regulated by forest tree N status rather than by soil N concentration.
- Reduction in the EM extramatrical mycelia after elevated N inputs may increase N leaching from forest soils.
- Long-term field studies, over several seasons or vegetation periods, are necessary to increase our understanding of ecosystem processes.

10. FUTURE PERSPECTIVES

Estimates of EM mycelial growth (Paper I) were found to provide a sensitive method of studying the effects of small increases in N deposition (from 10 to 20 kg ha^{-1} y^{-1}) (Paper V). EM mycelia should be considered a potential indicator when studying the effects of e.g. N deposition in environmental monitoring programmes. The methods of estimating the biomass of mycorrhizal mycelia in soil samples (Paper V and Bååth et al., 2004) could perhaps be improved, especially if it can be combined with the use of stable isotopes, due to the large difference in $\delta^{13}C$ between mycorrhizal and saprotrophic fungi (Paper I).

The studies included in this thesis have mainly focused on the effects of elevated N input on mycorrhizal fungi. However, mycorrhizal fungi are also important in C cycling and this should be studied in natural environments in relation to global change. Microbial biomass has been suggested as an important precursor for recalcitrant C in SOM in forest soils (Ehleringer et al., 2000). One explanation to this could be the suggestion by Langley and Hungate (2003) that mycorrhizal colonisation substantially decreases decomposition rates of fine roots. The reason for this can be the higher N concentrations, and the presence of recalcitrant forms of N (such as chitin), in EM roots compared to non-mycorrhizal roots. Results presented in this thesis show that external EM hyphal contributes largely to the belowground biomass in forest soils. Therefore, the degradation and turnover rate of EM mycelia is probably important in total soil C turnover and soil C storage. More knowledge about the complicated interaction between C and N in soils would probably be obtained through a better understanding of mycorrhizal mycelia in a global change perspective.

This study also demonstrates the importance of ericoid mycorrhizas in the most nutrient-poor soils (Paper VI) and the need to increase our understanding of their functions in such ecosystems.

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Research

Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field

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Summary

• In-growth mesh bags were used to quantify the production of external mycelium of ectomycorrhizal (EM) fungi in the field.

• Colonization of the mesh bags was followed by visual estimation of the amount of mycelium, and by measuring fungal biomarkers (the phospholipid fatty acid (PLFA) 18 : 2ω6,9 and ergosterol). Mesh bags were placed inside and outside plots that were root isolated in order to estimate the amount of saprotrophic mycelium in relation to EM mycelium. The majority of mycelium in the mesh bags were EM, and this was confirmed by analysis of the $\delta^{13}C$ value in mycelia.

• Fungal colonization of mesh bags peaked during autumn. The total amount of EM mycelium produced in the mesh bags during a year was calculated to be between 125 and 200 kg ha^{-1}. The total amount of EM mycelium (including EM mantles) in the humus was estimated to be 700–900 kg ha^{-1}.

• The biomass of EM mycelium in the soil was in the same range as the biomass of fine roots and peaks of mycelial growth coincided with periods of maximum growth of fine-roots.

Key words: ectomycorrhiza, external mycelium, fungal biomass, phospholipid fatty acid (PLFA), seasonal growth, ergosterol.

Introduction

Ectomycorrhizal (EM) fungi are important for the uptake of nutrients, since the external mycelium extends into the soil and increases the soil volume exploited by the tree roots (Harley, 1989; Smith & Read, 1997). Although the importance of the external mycelium in plant nutrient uptake and carbon flow into the soil is obvious, the number of studies on the production of external mycelium in soil is small (Read, 1992). Most such studies have been performed on seedlings in the laboratory, while few studies have been performed in the field (Coutts & Nicholl, 1990; Lussenhop & Fogel, 1999). One reason for this is that no methods have been available to distinguish mycelia from EM fungi from saprotrophic fungal mycelia in soil.

In laboratory experiments, it is possible to follow the growth of the external mycelia from mycorrhizal roots by visual examination (Bending & Read, 1995). Another approach is to measure fungal-specific biochemical markers (Olsson, 1999). For EM fungi, the signature fatty acid 18 : 2ω6,9 (Olsson *et al.*, 1996), ergosterol (Nylund & Wallander, 1992) and chitin (Ekblad & Näsholm, 1996) have been used. None of these compounds, however, is specific to EM fungi. One therefore has to include a nonmycorrhizal control, where the biomass of saprotrophic mycelium can be estimated and subtracted from the total fungal biomass to calculate the EM fungal biomass. This approach has been used in several laboratory studies (Colpaert *et al.*, 1992; Ek, 1997). However, in soil the amount of saprotrophic fungi can be high compared with the amount of EM fungi, resulting in uncertain estimates of the biomass of the latter.

One way of reducing the background value of saprotrophic mycelium is to use a substrate with a low organic matter content, for example sand (Ekblad *et al.*, 1995; Ek, 1997) or perlite (Colpaert *et al.*, 1992; Colpaert *et al.*, 1999). EM fungi can easily colonize such substrates, since they will have a carbon source (the tree), and they are good at transporting substances over long distances. For most saprotrophic fungi, growth relies on the organic matter in the soil and they will thus not colonize low-organic-matter substrates to any large extent.

Sand has also been used as a substrate to study the growth of the external mycelium of arbuscular mycorrhizal (AM) fungi (Johansen *et al.*, 1996; Olsson & Johansen, 2000). Using the signature fatty acid 16 : 1ω5 to quantify the biomass of these fungi, the growth of the external mycelium was also studied in a field situation using a similar technique (Olsson & Wilhelmsson, 2000). Sand contained in plastic tubes with a nylon mesh that allowed the in-growth of fungal mycelium, but not that of roots, was placed in sand-dunes and the amount of 16 : 1ω5 was measured.

We have estimated the production of external EM mycelium in the field using nylon mesh bags filled with quartz sand in a similar way to that described above for AM fungi. However, since there is no easy way to differentiate between EM and saprotrophic fungi using biochemical markers or microscopy, nonmycorrhizal controls have to be used in the field. One way to obtain such controls is to inhibit the growth of EM mycelium from mycorrhizal roots by creating root-isolated plots by trenching the roots. The growth of the mycelia in trenched areas will then represent the growth of saprotrophic fungi, while outside the trenched plots it would represent the growth of both EM and saprotrophic mycelia. Such trenching experiments have been performed to compare respiration from soil with and without roots and associated microorganisms (Boone *et al.*, 1998; Buchmann, 2000). The origin of the mycelia in the mesh bags can be confirmed by analysing the $\delta^{13}C$ value of the mycelia, since saprotrophic and EM fungi have been shown to have distinctly different $\delta^{13}C$ values (Högberg *et al.*, 1999).

The aim of the present study was to ascertain whether in-growth mesh bags containing sand could be used as a simple indicator of EM fungal growth in the field. EM mycelial production was followed for a year. We also examined the extent to which only EM fungi contributed to the fungal biomass in the mesh bags by placing mesh bags inside and outside plastic tubes inserted into the soil (trenching). The values of $\delta^{13}C$ for the mycelium in the mesh bags were compared with those of fruit bodies of EM and saprotrophic fungi from the same forest.

Materials and Methods

Study sites

All 3 study sites were situated in south-west Sweden. The Silvåkra forest was planted in 1970 with Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.). The soil is sandy and the humus layer has an average thickness of 1 cm. The Skogaby experimental forest is a former *Calluna* heath land. The plots used in the present study were planted in 1966 with Norway spruce, replacing a first generation of Scots pine planted in 1913. The soil type is a poorly developed Haplic podzol with a silt loam texture down to 30 cm. The humus layer has an average thickness of 7 cm. More detailed descriptions of Skogaby can be found in Nilsson & Wiklund (1995). In Tönnersjöheden a Norway spruce forest was studied. The soil type and texture have been classified as podzol and sandy-silty till, respectively. Further details concerning the site are given by Olsson & Staff (1995).

Mesh bags and harvesting

Fungal in-growth bags were constructed of nylon mesh (50 µm mesh size, $10 \times 5 \times 2$ cm) by melting the edges together with a plastic bag sealer. The mesh size allowed the in-growth of fungal hyphae, but not that of roots. The bags were filled with 120 g acid-washed sea sand (0.36–2.0 mm, 99.6% SiO_2, Ahlsell, Sweden) and sealed. The mesh bags were placed horizontally at the interface between the organic horizon and the mineral soil.

The mesh bags were opened in the laboratory after being harvested. The sand was observed under a dissecting microscope and the fungal colonization was estimated visually (see below). The sand was then carefully mixed and samples were taken for the analysis of water, phospholipid fatty acid (PLFA) and ergosterol content. The samples were stored at −20°C until analysed. No degradation of PLFA and ergosterol was found at this temperature (unpublished).

Seasonal colonization of mesh bags

At the Silvåkra site, 30 mesh bags were buried in early spring. Three to five mesh bags were then harvested on five occasions during the year. In addition, six mesh bags were buried on two later occasions, and harvested after 1 or 2 months. At the experimental site in Tönnersjöheden 32 bags were buried in July. 16 bags were harvested in May and 16 in October the following year.

Root trenching

PVC tubes (16 cm diameter, 30 cm length) were forced down into the soil to a depth of 25 cm to isolate the soil from roots connected to the trees. At the Silvåkra site, three tubes were inserted at four locations (12 tubes in total). One tube was inserted around a Norway spruce seedling (approx. 50 cm high), one tube was used as a control, and one tube was filled with wood in different stages of decomposition in order to improve conditions for wood-decomposing fungi to colonize the mesh bags. One mesh bag was buried inside and one bag outside each tube in August. The mesh bags were harvested in December of the same year.

At the Skogaby site, three tubes were inserted in each of four plots (12 tubes in total). In April, one mesh bag was placed inside and one bag outside each tube. Mesh bags were collected after 6, 12 and 18 months.

Soil samples were taken from the humus horizon inside and outside the tubes at the Skogaby site. These samples were

stored at −20°C and later used for the analysis of water, PLFA and ergosterol content. Samples were also taken from the humus in Skogaby to determine the nitrogen content inside and outside the plastic tubes 12 months after inserting the tubes into the soil.

The amount of EM mycelium in the humus horizon and in the mesh bags was calculated by subtracting the fungal biomass outside the tubes from that inside the tubes.

Laboratory experiments

A laboratory experiment was performed to examine whether saprotrophic fungi from the humus horizon of forest soil and mycorrhizal fungi from cut roots would grow into sand-filled mesh bags. The soil (humus from the Skogaby site) was sieved (5 mm) and the roots not passing through the sieve were collected. The soil was then placed in plastic pots (size $5 \times 5 \times 5$ cm) and the roots were mixed with the soil in half of the pots. Similar, but smaller (15 g sand), nylon mesh bags to those used in the field study were buried in the pots. The pots were incubated for 145 d at 15°C. The amount of PLFA in the sand of the mesh bags was then analysed (see below). Four replicates were used in each treatment.

Analysis

The extent of fungal colonization of the sand in the mesh bags was estimated visually following harvesting. The degree of colonization was divided into five classes: no mycelia present; sparse mycelia present; mycelia present but no aggregation of the sand particles; plenty of mycelia present and some aggregation of the sand particles; and plenty of mycelia present and sand particles aggregated to a large extent.

The method of lipid extraction followed that of Frostegård *et al.* (1991). Extracted lipids were fractionated into neutral lipids, glycolipids and polar lipids on silicic acid columns (100–200 mesh, Unisil) by successively eluting with chloroform, acetone and methanol. The methanol fraction (containing the phospholipids) was subjected to mild alkaline methanolysis to transform the fatty acids into free methyl esters. These were analysed on a GC according to the method of Frostegård *et al.* (1993). The PLFA 18 : 2ω6,9 was used as an indicator of fungal biomass. The total amount of PLFAs was also analysed to enable the estimation of the proportion of 18 : 2ω6,9.

Ergosterol was determined according to Wallander & Nylund (1992). Dry sand (10 g) or 0.5 g of humus was extracted with 4 ml 10% KOH in methanol and 1 ml cyclohexane. The chromatographic system consisted of a C_{18} reverse-phase column (Nova-Pak, 0.39 cm × 7.5 cm) preceeded by a C_{18} reverse-phase guard column (Waters, Milford, USA). Extracts were eluted with 100% methanol at a flow rate of 1 ml min^{-1} and monitored at 282 nm. Conversion factors of 2 nmol 18 : 2ω6,9 mg^{-1} fungal biomass (Olsson, 1998) and 3 μg ergosterol mg^{-1} fungal biomass (Salmanowicz & Nylund, 1988) were used to calculate fungal biomass.

Humus (5 g f. wt) was extracted in 50 ml 0.2 M $CaCl_2$ for 1 h on a rotary shaker. The extract was filtered and analysed for $NH4^+$ and NO_3^- by flow injection analysis (Falkengren-Grerup *et al.*, 1998).

The ^{13}C abundance in fruit bodies collected from the field sites and in mycelia extracted from the mesh bags was analysed at the Department of Forest Ecology, SLU, Umeå, Sweden, using an online continuous flow CN analyser coupled to an isotope mass spectrometer. Results are expressed in the standard notation ($\delta^{13}C$) in parts per thousand relative to the international standard, Vienna Pee Dee Belemnite (Högberg *et al.*, 1999). Water was added to a specific volume of sand from the mesh bags. Mycelia and rhizomorphs present in the sand floated and were collected on a nylon mesh and dried at 70°C for 24 h. Fruit bodies of the following genera of EM fungi were analysed (number of species): *Russula* (2), *Lactarius* (3), *Cortinarius* (2), *Thelephora* (1), *Cantharellus* (1) and *Chroogomphus* (1). Fruit bodies of the following saprotrophic fungi were analysed (1 species in each genus): *Hypholoma*, *Mycena*, *Marasmius*, *Paxillus* (saprotrophic species), *Lycoperdon*, *Stropharia*, *Pholiota* and *Auriscalpum*. Portions of the upper part of the fruit bodies were dried at 70°C for 24 h and then ball milled.

Results

Field measurements

All estimates of fungal colonization of the sand in the mesh bags (visual, ergosterol and 18 : 2ω6,9) showed similar growth patterns at the Silvåkra site (Fig. 1). No growth was detected during the spring months (March–May), but the amount of fungi in the mesh bags increased to maximum levels on the last harvesting occasion in the autumn. More rapid colonization between July and September than between March and June was also seen when mesh bags were placed in the soil at different times during the year. Thus, mesh bags placed in the humus during spring or in July had approximately the same fungal content when harvested in September, indicating that the EM fungal growth was highest in the autumn.

The amount of the PLFA 18 : 2ω6,9 in mesh bags varied between different sites and sampling times. Values were highest in the October samples from Tönnersjöheden (0.91 ± 0.007 nmol g^{-1}) while they were lower in the April samples from the same site (0.23 ± 0.02 nmol g^{-1}). In Skogaby, mean values varied between 0.1 and 0.5 nmol g^{-1} at different harvest times with the lowest value in the spring harvest. In Silvåkra, the amount of 18 : 2ω6,9 was 0.20 ± 0.03 nmol g^{-1} in mesh bags harvested in April after 12 months in the forest soil. This value was similar to that in

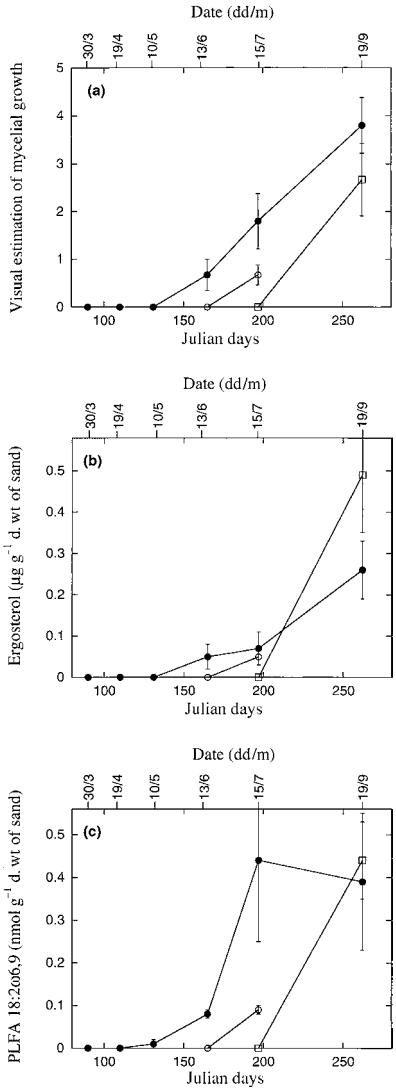

Fig. 1 Colonization of sand in mesh bags at Silvåkra, south-west Sweden estimated by: (a) visual estimation (b) ergosterol analysis and (c) phospholipid fatty acid 18 : 2ω6,9 analysis. Different symbols indicate mesh bags buried at different dates during the year. Bars indicate ± SE, $n = 3–6$.

Table 1 Fungal colonization of sand in mesh bags located inside and outside trenched plots in Silvåkra[1]

Treatment	Ergosterol (μg g^{-1} sand)	18 : 2ω6,9 (nmol g^{-1} sand)
Outside	0.23 ± 0.03[a]	0.19 ± 0.12[a]
Inside (empty)	0.07 ± 0.02[b]	0.030 ± 0.005[b]
Inside with spruce	0.13 ± 0.02[b]	0.040 ± 0.009[b]
Inside with wood	0.07 ± 0.01[b]	0.066 ± 0.019[b]
ANOVA ($P <$)	0.001	0.001

[1]Treated plots were either containing pieces of wood, a living spruce tree or were empty. Fungal biomass was estimated using the PLFA 18:2ω6,9 and ergosterol indicators. Different letters within each column indicate statistically different values (ANOVA/LSD).

mesh bags that were harvested in December, after 4 months in the forest soil (0.19 nmol g^{-1}, Table 1).

Root trenching

Trenching had a negative effect on fungal colonization of the mesh bags. Almost no mycelia could be observed under the microscope in bags collected inside the tubes after up to 12 months in Skogaby and 3 months in Silvåkra. These results were confirmed by the analysis of chemical biomarkers, which gave low values (4–17% of that without trenching) at Skogaby after 7 months (Table 2) and at Silvåkra (16–30%) after 2 months (Table 1).

After 18 months some of the mesh bags inside tubes at Skogaby became heavily colonized with mycelium (Table 2). At this time, mycorrhizal roots were also observed inside trenched plots, and in one case a fruit body of *Laccaria amethystina* (Hooker) Murill was also present. When the tubes were removed from the soil after 22 months, the soil inside all tubes contained abundant mycorrhizal roots.

The proportion of the PLFA 18 : 2ω6,9 in humus inside tubes in relation to that outside tubes was low after 7 months (28%) but increased with time to 152% after 18 months (Table 2). The proportion of 18 : 2ω6,9 to the total amount of PLFA in the humus was constant outside trenched plots (11.9% ± 1.0%) while it decreased in the trenched plots to 5.3% ± 0.7% after 7 months. The proportion increased to 7.0% ± 1.0% after 12 months and to 12.4% ± 1.6% after 18 months, which was similar to the value outside trenched plots.

The addition of wood or including a small spruce tree inside the tubes had no significant effect on the fungal colonization of the mesh bags in Silvåkra (Table 1).

The water content in the mesh bags and in the humus did not differ significantly between trenched and untrenched areas. The water content of the sand varied between 1.1% and 2.1% while the water content of the humus varied between 73% and 87%. Extractable nitrogen (ammonium + nitrate) was substantially higher inside than outside trenched areas (Table 3). The nitrate concentration was low, less than 5% of the ammonium concentration.

Table 2 Proportion (% ± SE) of fungal biomass in mesh bags and in humus collected inside relative to outside trenched plots in Skogaby after different incubation times in the field[1]

Type of sample	Biochemical marker	7 months	12 months	18 months
Sand from mesh bags	18 : 2ω6,9	17 ± 4	44 ± 23	50 ± 6
Sand from mesh bags	Ergosterol	4 ± 1	55 ± 18	84 ± 21
Humus	18 : 2ω6,9	28 ± 10	55 ± 14	152 ± 88
Humus	Ergosterol	53 ± 28	nd	nd

[1]Fungal biomass was estimated using the PLFA 18 : 2ω6,9 and ergosterol indicators. nd, not determined. One sample with a *Laccaria amethystina* fruit body connected to the mesh bag contained 10 times more 18 : 2ω6,9 and ergosterol compared with the mesh bag outside the trenched plot and was excluded from the data set.

Table 3 $CaCl_2$-extractable (0.2 M) NH_4^- and NO_3^- in soil collected from the organic horizon inside and outside trenched plots[1]

Locations	NH_4^- (μg g^{-1} d. wt)	NO_3^- (μg g^{-1} d. wt)
Inside	60 ± 13	0.7 ± 0.1
Outside	3.9 ± 0.5	0.2 ± 0.01
ANOVA (P <)	0.0001	0.0001

[1]ANOVA was carried out on logarithmically transformed data.

Table 4 $\delta^{13}C$ values (‰) for mycelium extracted from sand in mesh bags buried in the field, and from fruit bodies of ectomycorrhizal (EM) and saprotrophic fungi collected in Silvåkra Tönnersjöheden

Site	Mycelia	EM fruitbodies	Saprotrophic fruitbodies
Silvåkra	–26.4 ± 0.2	–26.5 ± 0.6	–23.1 ± 0.5
Tönnersjöheden	–25.5 ± 0.2	–25.7 ± 0.3	–23.6 ± 0.3

Estimation of EM biomass and production

The amount of EM mycelia present in the humus layer per unit area could be estimated by subtracting values inside from values outside trenched plots assuming that the decrease in fungal biomass inside the trenched plots was due to necrosis of mycorrhizal mycelium. If it is assumed that all EM mycelia decomposes during 7 months inside trenched plots, the amount of EM mycelia in the humus layer of the Skogaby site was estimated to be 900 kg ha^{-1} based on 18 : 2ω6,9 analysis and 700 kg ha^{-1} based on ergosterol analysis (EM mycelia present in the mineral soil is not included in these estimates). The amount of ectomycorrhizal mycelia produced in the mesh bags during a 7-month period at the Skogaby site was estimated to be 210 kg ha^{-1} based on 18 : 2ω6,9 analysis and 125 kg ha^{-1} based on ergosterol analysis.

^{13}C abundance

The $\delta^{13}C$ value of mycelia collected from the mesh bags was −26.4‰ at the Silvåkra site and −25.5‰ at the Tönnersjöheden site (Table 4). The mean value of $\delta^{13}C$ for fruit bodies of mycorrhizal fungi varied between −25.7‰ and −26.5‰, while the mean value for fruit bodies of saprotrophic fungi varied between −23.1‰ and −23.6‰. Thus, the mycelium in the mesh bags had significantly different $\delta^{13}C$ values from that in fruit bodies of saprotrophic fungi ($P < 0.001$), but the same as in EM fungal fruit bodies.

Laboratory experiments

No visible mycelia were observed in the sand of mesh bags placed in pots with soil, irrespective of whether excised mycorrhizal roots were added to the soil or not. The amount of 18 : 2ω6,9 in the mesh bags was low (0.06 ± 0.01 nmol g^{-1} sand) in both cases. This value is similar to values found in mesh bags collected inside root-isolated plots in the field.

Discussion

There are two main factors involved in using the in-growth of fungal mycelia in mesh bags as an estimate of EM fungal growth in the field. The first is to what extent EM fungi only will grow into the sand of the mesh bags and not saprotrophic soil fungi, such as wood-decomposing fungi. The second is to what extent the use of sand as a substrate instead of normal soil/humus will affect the estimate of the EM fungal growth rate.

There were several pieces of evidence indicating that the mycelia in the mesh bags were to a large extent, although not exclusively, of EM fungal origin. Most important, trenching reduced the amount of mycelial growth into mesh bags (Tables 1 and 2). Since trenching will deprive EM fungi of their food base, while presumably not affecting soil-dwelling fungi, this indicated that the mycelia found in mesh bags outside trenched areas were of EM fungal origin. The difference between inside and outside the trenched plots decreased over time (Table 2). This was an effect of roots, and thereby EM fungal mycelium, entering the trenched plots from below the plastic tube. In most trenched plots, mycorrhizal roots and even fruit bodies of mycorrhizal fungi were found after 18 months. The fact that once mycorrhizal roots had entered the soil tubes the mesh bags became colonized with fungal mycelium is, however, a further indication that the mycelia were mainly of EM fungal origin.

Frostegård *et al.* (1996) found a decrease in fungal biomass (as indicated by the PLFA 18 : 2ω6,9), when incubating a

coniferous forest humus in the laboratory for 18 months without any plants, while in an agricultural soil the fungal content was stable. They suggested that this was due to the presence of high amounts of EM fungi in the forest humus compared with the agricultural soil. These EM fungi would lack a carbon source during incubation. Deprived of their normal carbon source (the tree), they could not compete with the normal saprotrophic flora. They would therefore die and the mycelium would be degraded. We also found this in the present field study, since the fungal biomass present in the humus inside the trenched plots decreased during the experiment (Table 2). In the laboratory, the growth of *H. longicaudum*, decreased after excision of the pine shoot, and all external mycelia had degenerated after a few months indicating that EM fungi do not sustain their biomass without carbon from the plant (unpublished). EM mycelia from excised mycorrhizal roots could not colonize the mesh bags, which also demonstrates the importance of the host trees as carbon source.

Contrary to our results, Harmer & Alexander (1985) found no reduction in fungal biomass in the soil as a result of trenching. The reason for this discrepancy might be the higher nitrogen availability in the soil of the Sitka spruce forest studied by them (2 kg extractable NH_4-N ha^{-1}) compared with the forest in our study (1 kg extractable NH_4-N ha^{-1}, according to Nilsson & Wiklund (1995)). It has earlier been shown that high nitrogen concentrations reduce the growth of EM extramatrical mycelium (Wallander & Nylund, 1992; Arnebrant, 1994). Also, in experimental plots in Skogaby, where nitrogen has been added for several years (100 kg ha^{-1} yr^{-1}), we found no difference in fungal biomass in humus collected inside and outside tubes (unpublished).

Similarly to Harmer & Alexander (1985) we found an accumulation of inorganic nitrogen inside trenched areas. This is probably the result of a lack of nitrogen uptake by the EM fungi, since trenching disconnected the EM mycelia from the host trees. Since this nitrogen was not immobilized in the soil, it is an indication that saprotrophic soil organisms in this type of forest humus were carbon limited, as already indicated by Aldén *et al.* (2001).

The $\delta^{13}C$ value for mycelia in the mesh bags confirmed that the mycelia were of EM fungal origin (Table 4), since the mycelia had values similar to those of the mycorrhizal fruit bodies, but clearly separated from the values for saprotrophic ones. Högberg *et al.* (1999) and Hobbie *et al.* (1999) recently reported $\delta^{13}C$ values for fruit bodies of a large number of mycorrhizal and saprotrophic species, and showed that these two groups were clearly separated, with values similar to those found by us.

The use of root-isolated areas to ascertain whether the mesh bag technique could be used to indicate EM fungal growth assumes that the trenching treatment does not influence the growth of saprotrophic fungi, and that mycorrhizal fungi are the only fungal group that is inhibited by the trenching treatment. It may be argued that wood-decomposing fungi, which have localized food sources in the soil from which the mycelium grow (Boddy, 1993), would also be inhibited in a trenched plot if they were detached from the food source. We found, however, no indication that these fungi colonized the mesh bags since the $\delta^{13}C$ value of the mycelia in the mesh bags was clearly separated from values for fruit bodies of wood-decomposing fungi (Table 4). Furthermore, the addition of wood to trenched plots at the Silvåkra site did not stimulate fungal colonization of the mesh bags (Table 1). It therefore appears likely that these fungi prefer to grow in the organic part of the soil, and avoid the mineral horizon as well as sand-filled mesh bags.

To what extent sand can be used, as a growth medium similar to normal soil/humus is also important in evaluating the mesh bag technique. This is difficult to ascertain in the present study. However, the extensive colonization of sand by EM fungi in laboratory studies (Ekblad *et al.*, 1995; Ek, 1997), as well as in our field studies indicates that sand is suitable as a substrate for EM fungal growth. The concentration of ergosterol in the mesh bags was also similar to that found in laboratory systems using pine seedlings inoculated with *Paxillus involutus* (Fr.) Fr. in pot cultures (Ekblad *et al.*, 1995).

Including a small spruce tree inside the trenched plots had no significant influence on the amount of ergosterol or the PLFA 18 : 2ω6,9 in the mesh bags from Silvåkra (Table 1). It is therefore likely that small trees in the understory contribute little to the production of external EM mycelia in a forest soil. According to Högberg *et al.* (1999), EM fruit bodies in a mature coniferous forest received almost all their carbon from large overstory trees while smaller understory trees contributed very little.

Colonization was more intense during the later part of the growing season (July–September, Fig. 1), when many of the mycorrhizal fruit bodies developed on the site, compared with earlier in the season. This was not due to a long lag time before colonization of the mesh bags took place, since we obtained the same results with mesh bags buried for only 2 months in the autumn as with those incubated in the soil from March. Mesh bags that were placed in the same forest over the winter months (December–March) did not contain any visible amount of fungal mycelia (unpublished). The autumn as the period of maximal fungal growth was also supported in a study by Wallander *et al.* (1997) who found that the ergosterol : chitin ratio of mycorrhizal roots collected from a *Pinus sylvestris* forest varied seasonally from low values in January increasing to high values in October (Wallander *et al.*, 1997). Maximal growth of EM mycelia appears to coincide with maximal growth of fine roots since Stober *et al.* (2000) found that growth of fine roots, in the Skogaby site peaked in October during three subsequent years.

The fungal biomass of EM external mycelia and mycorrhizal roots in Skogaby was estimated to be between 700 and 900 kg ha^{-1}, assuming that the difference in fungal mycelium inside and outside root-isolated plots was due to the death

of EM mycelia. Kårén & Nylund (1997) estimated the fungal biomass of mycorrhizal roots in the same forest to be 150 kg ha^{-1}, based on ergosterol measurements of mycorrhizal root tips. This would suggest that a very large proportion (approx. 80%) of the biomass of EM fungi in forest ecosystems is in the form of external mycelia. Our field data thus confirm results found in laboratory experiments, where the proportion of the external mycelium has been estimated to be between 60 and 85% in a variety of ectomycorrhizal fungi (Colpaert *et al.*, 1992). The biomass of EM fruit bodies in Skogaby was estimated to 6 kg ha^{-1} yr^{-1} (mean value during 1989–93) (Wiklund *et al.*, 1994). This can be compared to the total amount of EM fungal biomass (ectomycorrhizal mantles and external mycelia) in the organic horizon of the Skogaby site, which was approximately 1000 kg ha^{-1}. The total amount of fine roots in the same horizon was 2700 kg (Bergholm *et al.*, 1994). EM fungal biomass may thus constitute a large proportion of the below-ground biomass associated with the trees.

In the present study we have demonstrated that it is possible to estimate the growth of EM fungal mycelia in forest soil with the use of sand-filled mesh bags. Although the use of conversion factors to estimate fungal biomass based on analysis of biochemical markers may be uncertain (Bermingham *et al.*, 1995) and the growth rate in the mesh bags may not be directly extrapolated to growth rates in the soil, the method should be appropriate for estimating relative changes in EM fungal growth. The most reliable measurements would be obtained by always including mesh bags from root-isolated areas as controls for nonEM mycelial colonization. However, this will make the technique more laborious, and for comparative purposes this is probably not necessary. One further advantage of the in-growth mesh bag technique is that AM and EM fungal growth can be measured simultaneously, since different PLFA markers can be used for AM and EM fungi (Olsson, 1999).

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II

New Phytologist

Research

Production of external mycelium by ectomycorrhizal fungi in a norway spruce forest was reduced in response to nitrogen fertilization

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Summary

• A field study was carried out to evaluate the influence of N fertilization on the growth of the external mycelium of ectomycorrhizal (EM) fungi in a Norway spruce forest in SW Sweden.

• Nylon mesh bags filled with sand were buried in the soil for 6–18 months and the ingrowth of mycelium was used as an estimate of EM mycelial growth. Root-isolated, trenched plots were used to estimate background growth of saprotrophic fungi.

• Mycelial growth of EM fungi in N-treated plots was reduced to *c.* 50% of that in nonfertilized plots. Local addition of apatite stimulated the EM mycelial growth in N-treated plots.

• The negative influence of N on the growth of external EM mycelium observed earlier in laboratory studies was confirmed in the present field study. The growth of EM mycelia was not directly related to N concentration in the soil but rather to the N status of the trees, although other factors induced by the N treatment may also have influenced EM mycelial growth.

Key words: ectomycorrhiza, fungi, extramatrical mycelium, nitrogen fertilization, forest soil, nitrate, apatite.

Introduction

Boreal and temperate forests are generally limited by the availability of nitrogen (Tamm, 1991). Ectomycorrhizal (EM) fungi have adapted to these conditions and are efficient in their uptake and subsequent transport of N to tree roots (Smith & Read, 1997). Elevated N levels have a negative impact on the growth of many species of EM fungi; shown both in the field and in laboratory studies, and reviewed by Wallenda & Kottke (1998).

Total number and biomass of EM fruit bodies have been found to decrease in several field studies due to N fertilization of forests (Menge & Grand, 1978; Ohenoja, 1978; Wiklund *et al.*, 1995) or the deposition of airborne N compounds (Termorshuizen & Schaffers, 1991). Some EM species (e.g. *Paxillus involutus* and *Lactarius rufus*) may on the other hand increase their production of fruit bodies in forest soils to which N has been added (Laiho, 1970; Ohenoja, 1978). EM fruit body production does, however, not necessarily reflect the activity of EM root tips and EM extramatrical mycelium (Gardes & Bruns, 1996; Jonsson *et al.*, 2000; Dahlberg, 2001).

EM colonization of root tips tends to be influenced less by the addition of N than fruit body formation (Menge & Grand, 1978; Ritter, 1990; Brandrud, 1995). In some cases the EM colonization of root tips has been reported to be reduced shortly after N fertilization (Menge *et al.*, 1977; Tétrault *et al.*, 1978; Arnebrant & Söderström, 1994). However, recent studies suggest that the main effect of N addition is a shift in the EM fungal community, favoring N-tolerant species, while the frequency of root tips colonized by mycorrhizal fungi remains high following N addition (Kårén & Nylund, 1997; Jonsson *et al.*, 2000; Taylor *et al.*, 2000).

The external mycelium is important in increasing the surface area available for uptake and therefore a reduction in the amount of EM mycelia may reduce the uptake capacity for elements other than N (Read, 1992; Wallenda *et al.*, 2000). The production of extramatrical mycelium by EM fungi is

often reduced in response to N addition in laboratory studies (Wallander & Nylund, 1992; Arnebrant, 1994).

It is difficult to estimate the growth of EM mycelia in the field, because existing methods do not separate fungal mycelia produced by EM fungi from those produced by other groups of fungi. These problems can be avoided by the method recently described by Wallander *et al.* (2001), using fungal ingrowth bags filled with sand. These are buried in forest soil for about 6 months or more and the nylon mesh allows fungal hyphae, but not roots, to enter. Analysis of carbon isotopes revealed that these mesh bags were colonized by mycorrhizal but not saprotrophic fungi (Wallander *et al.*, 2001).

When nitrogen supply exceeds the requirement for the growth of forest trees, other mineral nutrients, for example phosphorus or potassium, may become limiting (Aber *et al.*, 1989). Phosphorus deficiency results in increased allocation of carbon from shoots to roots by trees, while the opposite is true for K (Eriksson, 1995). Laboratory experiments have also shown that the production of extramatrical EM mycelia increased considerably under severe P starvation (Wallander & Nylund, 1992; Ekblad *et al.*, 1995) but decreased under K limitation (Ekblad *et al.*, 1995). Thus, the phosphorus status of forest trees may also influence the production of extramatrical mycelia in the field.

The objective of the present study was to investigate the influence of N fertilization on EM mycelial production under field conditions. We especially wanted to examine whether the decrease in mycelial production by EM fungi earlier found in laboratory studies after N fertilization would also be observed under field conditions. Additionally, we wanted to ascertain whether local amendment using a P containing mineral (apatite) would stimulate EM mycelial production and how this was related to N fertilization of the forest.

Materials and Methods

Site description

The field study was conducted at the Skogaby Experimental Forest in SW Sweden. This former heathland (dominated by *Calluna vulgaris L.*) was planted with Norway spruce (*Picea abies*) in 1966, replacing a first generation of Scots pine planted in 1913. The site is located at an altitude of 95–115 m above sea level, where a maritime climate prevails with a mean precipitation of 1100 mm yr^{-1} and an annual mean temperature of 7.5°C. It has a 200-d vegetation period from the beginning of April to the beginning of November. The soil type is a poorly developed Haplic podzol with a silt loam texture down to 30 cm. The humus layer had an average thickness of 7 cm when examined in 1987 (Bergholm *et al.*, 1994; Nilsson & Wiklund, 1995).

Atmospheric deposition of nitrogen at Skogaby is around 15 kg N ha^{-1} yr^{-1}. In the nitrogen-fertilized plots 100 kg N and 114 kg S ha^{-1} yr^{-1} were added as ammonium sulfate, divided into three applications per year, mainly during June and July. From the start of the treatment (1988) to the first year of our study (1998) 1000 kg N ha^{-1} was added to the N plots. During 1998, fertiliser was applied on May 22nd, June 25th and July 20th; and during 1999 June 3rd, June 21st and July 12th. Four replicates of each N-fertilized and nonfertilized plot were established 1987 and each plot had an area of approximately 2000 m^2 (Bergholm *et al.*, 1994).

Experimental design

To estimate the production of external EM mycelium in the field we followed the method described by Wallander *et al.* (2001). Fungal ingrowth bags (50 × 50 × 10 mm in size) were made of nylon mesh (50 μm mesh size). The bags were filled with 60 g acid-washed sea sand (0.36–2.0 mm, 99.6% SiO_2, Ahlsell, Sweden), or sand mixed with apatite (Kemira, 50–250 μm; to a final apatite concentration of 1% by weight), and sealed.

In order to estimate the background production of mycelium by saprotrophic fungi we established root-isolated, trenched, plots. Here, in the absence of active roots, we assumed EM fungi to have no significant ability to produce mycelia. Plastic tubes (16 cm in diameter, 30 cm in length) were forced into the soil to a depth of 25 cm. Three tubes were inserted into each of the four nonfertilized and N-treated plots at Skogaby by the end of March 1998. The mesh bags were buried at the interface between the organic horizon and the mineral soil, at *c.* 5 cm depth on two occasions, April 5th 1998 and April 14th 1999 (Table 1), one bag inside, and one in the vicinity but outside, each root-isolated plot.

Harvest of fungal ingrowth mesh bags and soil sampling

The mesh bags buried in the spring of 1998 were collected after 6 months, 12 months and 18 months and the mesh bags buried in the spring of 1999 were harvested after 6 months (Table 1). Soil samples were taken from the humus layer in the vicinity outside as well as inside trenched plots in N-treated and in nonfertilized plots in April and October 1999 (Table 1). The samples were stored at −20°C and later used for the analysis of phospholipid fatty acid (PLFA) content. Similar samples were also taken on April 14th 1999 to determine the N content of the humus inside and outside root-isolated plots in N-treated and nonfertilized plots.

Fungal biomass

After harvest of the mesh bags the sand and the sand amended with apatite were observed under a dissecting microscope and the fungal colonization was estimated visually. The degree of colonization was divided into five classes: 0, no mycelia present; 1, sparse mycelia present; 2, mycelia present but no

Table 1 Schedule of the field study to determine the production and content of EM mycelia in N-treated (N) and nonfertilized (C) plots showing mesh bag burial and harvest times and humus soil sampling times and number of replicates. Root-isolated plots were created by inserting plastic tubes into the soil by the end of March 1998. Fertilizer (ammonium sulfate) was applied on May 22nd, June 25th and July 20th 1998, and June 3rd, June 21st and July 12th 1999; 33 kg N ha^{-1} on each occasion

	1998 April 5th	October 20th	1999 April 14th	October 13th	October 26th
In-growth mesh bags	1st burial occasion sand + sand w. apatite		2nd burial, sand + sand w. apatite	4 + 4	
C plots	12 + 12		4 + 4		
C plots, root-isolated	12 + 12		4 + 4		
N treated plots	12 + 12		4 + 4		
N treated plots, root-isolated	12 + 12		4 + 4		
Harvest		1st	2nd	3rd	4th
C plots		4 + 4	4 + 4	4 + 4	4 + 4
C plots, root-isolated		4 + 4			
N treated plots		4 + 4	4 + 4	4 + 4	4 + 4
N treated plots, root-isolated		4 + 4			
Duration		6 months	12 months	18 months	6 months
Soil samples					
C plots			4	4	4
C plots, root-isolated			4		
N treated plots			4	4	4
N treated plots, root-isolated			4		

aggregation of the sand particles; 3, plentiful mycelia present and some aggregation of the sand particles; 4, plentiful mycelia present and sand particles aggregated to a large extent.

The sand from the mesh bags was carefully mixed and 10 g of sand were used for the analysis of the PLFA content. The humus samples were treated in the same way as the sand. The samples were stored at −20°C until analyzed. The method of lipid extraction followed that of Frostegård *et al.* (1991). Extracted lipids were fractionated into neutral lipids, glycolipids and polar lipids on silicic acid columns (100–200 mesh, Unisil, Clarkson, Chromatography Products Inc., PA, USA) by successive eluation with chloroform, acetone and methanol. The methanol fraction (containing the phospholipids) was subjected to mild alkaline methanolysis to transform the fatty acids into free methyl esters. These were analyzed on a gas chromatograph with a flame ionisation detector and a 50-cm HP5 capillary column, according the method of Frostegård *et al.* (1993). The PLFA 18 : 2ω6, 9 was used as an indicator of fungal biomass (Frostegård & Bååth, 1996).

To estimate the amount of EM mycelia produced in the in-growth mesh bags and the amount of EM biomass in the humus we subtracted values of fungal biomass obtained inside root-isolated, trenched plots from the values obtained outside. Values from October 1998 (6 months after trenching) were used as estimates of background saprotrophic mycelial production in the mesh bags, because EM mycelium and roots in several cases had entered the tubes on sampling occasions during 1999. For that same reason values from April 1999 (12 months after trenching) were used to estimate the saprotrophic background in the soil samples. In order to calculate the amount of EM biomass in humus samples we assumed that the biological marker for fungi (PLFA 18 : 2ω6,9) that represented the biomass of EM origin, in root-isolated, trenched plots had degraded by 12 months. This is probably a minimum value because all EM mycelia may not have degraded. A conversion factor of 2 μmol of the PLFA 18 : 2ω6,9 g^{-1} fungal biomass (Olsson, 1998) was used to calculate EM biomass from PLFA values.

C : N ratio and carbon isotopic composition of the mycelia

When mycelia were abundantly present in the mesh bags, water was added to a subsample of the sand to make a slurry. Mycelia and rhizomorphs present in the sand floated to the surface and could be collected on a nylon mesh and dried at 70°C for 24 h. The C (both ^{12}C and ^{13}C) and N contents of the mycelia were determined at the Department of Forest Ecology, SLU, Umeå, Sweden, using an online, continuous flow CN analyzer coupled to an isotope mass spectrometer. Results regarding the carbon isotopic composition are expressed in the standard notation ($\delta^{13}C$) in parts per thousand relative to the international standard, Vienna Pee Dee Belemnite (Högberg *et al.*, 1999).

N content in humus samples

Vessels containing 5 g (f. wt) humus and 50 ml 0.2 M $CaCl_2$ were shaken for 1 h on a rotary shaker. The extract was filtered and analyzed for NH_4^+ and NO_3^- by flow-injection analysis

(Falkengren-Grerup *et al.*, 1998). In order to express the N content in relation to the organic matter content, the loss of ignition was estimated by heating soil samples to 600°C for 4 h.

Statistics

The effects of N fertilization on EM colonization of mesh bags, EM biomass in humus samples and on N concentration of the humus and EM mycelia were tested with the Students *t*-test or analysis of the variance (ANOVA). Values from the visual estimations of fungal colonisation were tested statistically with the nonparametric Mann–Whitney *U*-test.

Results

Visual estimation revealed that mesh bags buried in nonfertilized plots were well colonized with mycelia after 6, 12 and 18 months with a mean degree of colonization varying from 3.0 to 3.5 (using the 0–4 classification scale) (Fig. 1a). Significantly less mycelial colonization was observed in N-treated plots at all times (mean degree of colonization between 0.0 and 0.5).

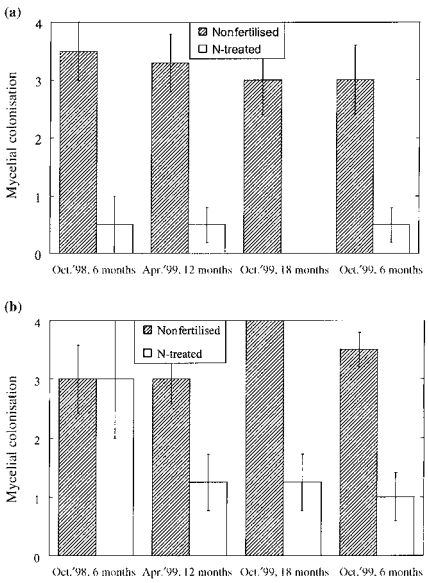

Fig. 1 Fungal colonization of (a) sand and (b) sand amended with apatite (1%) in mesh bags in nonfertilized and N-treated plots based on visual estimation. Bars indicate ± SE, $n = 4$. Mann–Whitney *U*-test: N-treatment (a); $P < 0.001$, apatite amendment N-treated plots (a,b); $P < 0.01$, apatite amendment nonfertilized plots (a,b); (ns).

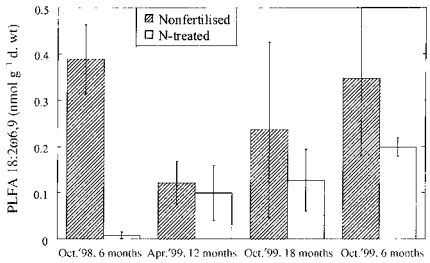

Fig. 2 EM mycelial colonization of sand in mesh bags in nonfertilized and N-treated plots based on the analysis of PLFA 18 : 2ω6,9. Bars indicate ± SE, $n = 4$. (ANOVA: N-treatment; $P = 0.02$, time; ns, N-treatment * time; ns).

Results of the phospholipid fatty acid 18 : 2ω6,9 of EM origin confirmed a significant reduction in the colonization of mesh bags in N-treated compared with nonfertilized plots (Fig. 2) ($P = 0.02$). The mean production of EM mycelia in N-treated plots was on average only 50% of that in the nonfertilized plots. N fertilization appeared to have a greater negative influence on EM mycelial production during 1998 than during 1999. However, the differences between the seasons were not statistically significant and the visual estimation did not reveal any interseasonal differences.

Visual estimation of mesh bags buried in root-isolated, trenched, plots revealed no colonization by fungal mycelia in N-fertilized plots (mean degree of colonisation 0.0 ± 0.0 on all harvesting occasions) and almost no colonization in nonfertilized plots (mean degree of colonization 0.5 ± 0.5 after 6 months and 0.0 ± 0.0 after 12 months). The PLFA 18 : 2ω6,9 was also low in mesh bags collected from trenched plots (0.071 ± 0.017 nmol g^{-1} d. wt in nonfertilised plots and 0.065 ± 0.009 nmol g^{-1} d. wt in N-treated plots after 6 months 1998). However, after 18 months roots and mycelium had entered some of the root-isolated plots, especially the nonfertilized plots where the PLFA 18 : 2ω6,9 contents increased to 0.281 ± 0.032 nmol g^{-1} d. wt (mesh bags buried in 1998) and to 0.471 ± 0.050 nmol g^{-1} d. wt (mesh bags buried in 1999). In N-treated plots PLFA 18 : 2ω6,9 increased 18 months after trenching to 0.220 ± 0.012 nmol g^{-1} d. wt (1998) and 0.217 ± 0.107 nmol g^{-1} d. wt (1999).

The visual estimates of fungal colonization of apatite-amended mesh bags revealed stimulated EM mycelial production by local additions of a P-containing mineral in N-fertilized plots ($P < 0.01$) (Fig. 1a,b). Local addition of apatite had no effect on fungal colonization in nonfertilized plots (Fig. 1a,b).

The proportion of PLFA 18 : 2ω6,9 to total PLFAs that could be attributed to saprophytic fungi in the humus samples

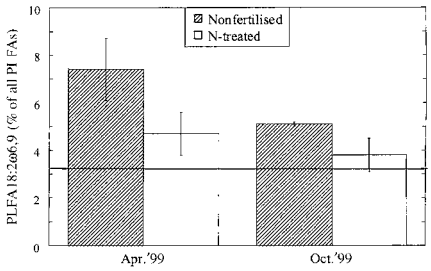

Fig. 3 Fungal biomass in soil samples from nonfertilised and N-treated plots expressed as the phospholipid fatty acid 18 : 2ω6,9 (% of total PLFAs). The horizontal line indicates the background due to saprotrophic fungi obtained in root-isolated plots and the biomass of EM fungi is found above the line. Bars indicate ± SE, $n = 4$ (April 1999), $n = 8$ (October 1999). (ANOVA for EM fungi: N-treatment; $P = 0.06$, time; $P = 0.02$, N-treatment * time; ns).

Table 2 C : N ratio of EM mycelia and rhizomorphs collected from ingrowth mesh bags in nitrogen treated (N) and nonfertilized (C) plots

	C : N ratio	*n*
C plots	20.1 ± 0.8	4
N treated plots	14.8 ± 0.3	2
t-test	$P = 0.013$	

inside the root isolated plots was 3.3 ± 0.02 in nonfertilised plots and 3.2 ± 0.24% PLFA 18 : 2ω6,9 (of total PLFAs) in N-treated plots (Fig. 3). The proportion of PLFA 18 : 2ω6,9 to total PLFAs that could be attributed to EM fungi (additional to the background) in nonfertilized plots in April 1999 was 4.1% (18 : 2ω6,9 of total PLFAs) and in October 1999 1.8% (18 : 2ω6,9 of total PLFAs) (Fig. 3). The EM biomass in humus samples in N-treated plots decreased, although not significantly ($P = 0.06$), to about 35% of that in nonfertilized plots (Fig. 3). Converted to biomass, EM biomass in the humus samples corresponded to *c.* 800 kg ha^{-1} in nonfertilized plots and 300 kg ha^{-1} in N-treated plots in April 1999, and to about 370 kg ha^{-1} and 120 kg ha^{-1}, respectively, in October 1999.

The nitrogen content of mycelia collected from in-growth mesh bags increased in N-treated plots and the C : N ratio was 20.1 ± 0.8 in nonfertilized plots and 14.8 ± 0.3 in N-treated plots (Table 2). The carbon isotopic ($\delta^{13}C$) value was –26.3 ± 0.4 ($n = 4$) in nonfertilized plots and –25.9 ± 0.0 ($n = 2$) in N-treated plots.

The amount of ammonium extracted by 0.2 M $CaCl_2$ was significantly higher in N-treated plots (57.2 μg g^{-1} organic matter, OM) than in nonfertilized plots (4.6 μg g^{-1} OM) when determined in April 1999 ($P < 0.001$) (Table 3). Nitrate concentrations were generally low, but were higher in N-treated plots (1.7 μg g^{-1} OM) than in nonfertilized plots (0.2 μg g^{-1} OM) ($P < 0.001$). Trenching increased the ammonium concentrations in N-treated plots (to 93.3 μg g^{-1} OM) ($P < 0.01$) and in nonfertilized plots (to 68.7 μg g^{-1} OM) ($P < 0.001$). Nitrate levels were unaffected by trenching in N-treated plots (1.5 μg g^{-1} OM), but increased (to 0.8 μg g^{-1} OM) after trenching in nonfertilized plots ($P < 0.001$).

Discussion

Almost all fungal mycelia colonizing the ingrowth mesh bags in this study were of ectomycorrhizal (EM) origin, when calculated as the difference between the fungal biomass in mesh bags collected outside, and those collected inside, the root-isolated, trenched plots. Here, only negligible amounts of fungal mycelia were produced. Furthermore, the carbon isotopic ($\delta^{13}C$) value in mycelia collected from mesh bags confirmed its EM origin, because the values correspond to values found in fruit bodies of ectomycorrhizal fungi in similar forests (Hobbie *et al.*, 1999; Högberg *et al.*, 1999; Wallander *et al.*, 2001).

The present study clearly demonstrates that nitrogen fertilization of a spruce forest caused a significant decrease in the production of external EM mycelium. This decrease may either be an effect of reduced production of external mycelia by individual species or an effect of a changed EM community induced by the N treatment, favoring species that produce lower amounts of external mycelia. The reduction in amounts of EM mycelia produced in N-treated plots, about 50% of the production in nonfertilized plots, was of the same magnitude as that previously found in laboratory studies. In an experiment in a semihydroponics system Wallander & Nylund (1992) found a decrease in the external mycelial biomass

Table 3 NH_4^+ and NO_3^- extracted by 0.2 M $CaCl_2$ from soil samples collected from the organic horizon in nitrogen treated (N) and nonfertilized (C) plots in April 1999. Soil samples were collected both inside and outside root-isolated plots 12 months after trenching of roots. Means ± SE ($n = 12$)

	NH_4^+ (μg g^{-1} OM)	NO_3^- (μg g^{-1} OM)
C plots	4.6 ± 0.4	0.2 ± 0.0
N treated plots	57.2 ± 12.3	1.7 ± 0.2
t-test (treatment)	$P < 0.001$	$P < 0.001$
C plots, root-isolated	68.7 ± 15.1	0.8 ± 0.1
t-test (root isolation)	$P < 0.001$	$P < 0.001$
N treated plots, root-isolated	93.3 ± 10.0	1.5 ± 0.2
t-test (root isolation)	$P < 0.01$	ns

of *Suillus bovinus* to 20% and of *Laccaria bicolor* to 35% with N in excess (100–200 mg N l^{-1}) of that in control (1–10 mg N l^{-1}) 8 wk after N addition started. Arnebrant (1994) found that growth of the mycelium of *S. bovinus* was reduced to 30% and that of *Paxillus involutus* to about 80% of that in control when N was added to the peat substrate at concentrations of 1–4 mg N g^{-1} d. wt.

In our study we found a tendency to a decrease in the amount of EM mycelia in N-treated plots according to phospholipid fatty acid analysis of soil samples. An indirect indication of reduced amounts of fungal mycelium in response to N addition at Skogaby has also been found by Lindberg *et al.* (2001), who noticed a reduction in the abundance of fungivorous collembolans and mites in N-treated compared with nonfertilized plots.

Interestingly, high soil ammonium levels *per se* did not appear to cause any decrease in EM mycelial growth, because EM mycelia and roots entered many of the root-isolated plots after 18 months of trenching (Wallander *et al.*, 2001). This colonization occurred although the inorganic N concentration in these trenched plots was higher than the concentration found in N-treated plots (Table 3), where the growth of EM mycelia was severely inhibited. Thus, it is probably not the N concentration in soil but rather the N status of the trees that regulates growth of EM mycelia. Other factors induced by the N treatment may also have influenced growth of EM mycelia and composition of the EM community, as discussed thoroughly by Kårén & Nylund (1997). From some other studies, designed to evaluate the effect of local patches of high N on mycelial growth in forest soils, conclusions on the importance of tree nutrient status may be drawn. Stober *et al.* (2000) found that hyphal length and density was stimulated by local additions of N in a nitrogen-deficient forest soil, but not in a nitrogen-sufficient site. Moreover, Brandes *et al.* (1998) found increased EM hyphal density when nitrogen and phosphorus were added to mycelial compartments containing sand in a laboratory system with low N availability. On the other hand, Read (1991) found no increase in biomass of EM mycelium in patches with added inorganic N in forest humus in a laboratory system, probably because the humus was originally rather rich in N.

The reduction in EM mycelial production found in our study may, in part, be an effect of reduced growth of mycorrhizal fine roots. Kårén & Nylund (1997) found that the fungal biomass in EM root tips decreased, although not significantly, from 150 kg ha^{-1} in nonfertilized plots to 110 kg ha^{-1} in N-treated plots at Skogaby during 1992 and 1993. However, this was not due to a lower colonization rate by EM fungi, but to reduced biomass of fine roots following the additions of nitrogen. In any case, the more pronounced reduction in EM mycelia found in this study suggests that the influence of N on the production of external EM mycelia is probably much greater than the effect on mycorrhizal short roots (Kårén & Nylund, 1997). This has also been found in laboratory studies (Wallander & Nylund, 1992).

The influence of N fertilization on EM fruit bodies at Skogaby was very rapid and vigorous (Wiklund *et al.*, 1995), as the production of EM sporocarps decreased from 6 kg ha^{-1} y^{-1} (mean values 1989–93) in nonfertilized plots to 0 kg ha^{-1} y^{-1} in N-treated plots. Fruit body production may be a good early indicator of the effects of N on EM fungi, but the sporocarps represent only a small fraction of the EM biomass compared with fine roots and external mycelia (Wallander *et al.*, 2001). In conclusion, under field conditions, N fertilization affects the production of EM fruit bodies drastically; the production of EM mycelia is also severely affected, while the fungal biomass of EM root tips seems to be affected to a lesser extent.

Tree growth was initially stimulated by N fertilization at Skogaby, but after about 8 yr of nitrogen treatment tree growth in these plots started to decline compared with nonfertilized plots. At this time, tree growth was considered to be limited first by P and thereafter probably by K or Mg (Nilsson *et al.*, 2001). Furthermore, Rosengren-Brinck & Nihlgård (1995) found an accumulation of N in old needles in N-treated plots, which they interpreted as an indication of N saturation. Our results support, to some extent, the idea that the limiting factor for tree growth has shifted from N to P, because local addition of apatite stimulated the growth of EM mycelia in N-treated plots (Fig. 1a,b). Hagerberg *et al.* (2003) found a similar increase in colonisation by EM mycelia of apatite-amended mesh bags in a forest with poor P status, while this was not the case in forests with a good P status.

A considerable variation in NO_3 leaching from N-treated plots at Skogaby has been reported for the 2 yr of the present study; 20 mg N l^{-1} in the runoff during 1998 and 2 mg N l^{-1} during 1999 (Bergholm in: Högberg *et al.*, 2001). This coincided with a variation in the production of EM mycelia, although time showed no significant effect in our study. The considerable loss of nitrate from the N-treated plots during 1998 could thus be the result of poor growth of the EM mycelia during this year. Although other factors may be important, the role of EM mycelium in preventing nitrate leaching should also be considered in future studies.

Needle concentrations of Mg and Ca have decreased in N-treated plots to 55–75% that in nonfertilized plots at Skogaby, and are negatively correlated to nitrate leaching (Nilsson *et al.*, 2001). Although this decrease in Mg and Ca concentrations in N-treated plots is probably associated with nitrate leaching, the EM mycelium may also be important for the uptake of Mg and Ca, as suggested by Jentschke *et al.* (2000) and Blum *et al.* (2002).

The technique of using in-growth mesh bags enables determination of nutrient content in naturally occurring forest soil EM mycelia. We found a C : N ratio of about 20 in EM mycelia in nonfertilized plots at Skogaby, and similar C : N ratios are also reported from other forests in south Sweden

(Wallander *et al.*, 2003). The C : N ratio of EM mycelia in ingrowth mesh bags decreased to 15 in N-treated plots at Skogaby. Despite the increase in EM mycelial N content after N fertilization, the EM mycelia contained less nitrogen based on area in N-treated (3.0 kg N ha^{-1}) than in nonfertilized plots (3.8 kg N ha^{-1}), due to the decease in EM mycelial biomass.

In conclusion, for the first time it has been shown that N fertilization of a spruce forest has a negative influence on the mycelial growth of EM fungi in the field. This reduction was not directly related to N concentration in the soil. The N status of the trees is one possible explanation of reduced production of mycelia by EM fungi, but other factors may also be of importance. We found that local amendment with a P-containing mineral may stimulate the growth of EM mycelia in soils in N-treated forests, which have probably moved from being N-limited to P-limited as a result of the N fertilization. Our continued studies on EM mycelia in the field will include investigations of the potential of EM mycelia to retain nitrogen in forests exposed to increased input of N through deposition.

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Short communication

Direct estimates of C:N ratios of ectomycorrhizal mycelia collected from Norway spruce forest soils

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Abstract

Direct estimates of C:N ratios of ectomycorrhizal (EM) mycelia growing in situ in forest soils have been obtained for the first time. The mycelial samples were collected from sand-filled mesh bags that were buried in the soil and incubated for 12–18 months in two Norway spruce forests in southern Sweden. At harvest the mesh bags were heavily colonized and the mycelia were extracted from the sand with water. The collected mycelia had earlier been identified as belonging to EM fungi based on their C isotopic composition. The mean value of the C:N ratio for mycelia was 20.2 ± 0.8 ($n = 25$). EM mycelia collected at different soil depths (5, 15 and 30 cm) had similar C:N ratios. C:N ratios of microbial biomass obtained by fumigation–extraction of similar soils have usually been lower (6–13) so possible differences in the extraction efficiency of C and N from bacteria and fungi are discussed.

Keywords: Ectomycorrhizal fungi; Microbial biomass; Microbial C; Microbial N; *Picea abies*

Soil microbial biomass is an essential component of terrestrial ecosystems since it is directly or indirectly responsible for nutrient cycling and serves as a source and a sink of nutrients such as N, which often limit growth in boreal forests (Tamm, 1991). Variations in C:N ratios of soil biomass have often been attributed to differing properties of fungal and bacterial biomass since fungi are reported to have a higher C:N ratio (5–15) than bacteria (3–6) (Paul and Clark, 1996; Harris et al., 1997; Levi and Cowling, 1969).

Ectomycorrhizal fungi make up a considerable proportion of the microbial biomass in coniferous forests and are probably the dominant fraction of fungi in these soils. Wallander et al. (2001) estimated the ectomycorrhizal biomass in the humus layer of a Norway spruce (*Picea abies* (L.) Karst.) forest in southern Sweden to be 800 kg ha^{-1}. In the same forest Persson et al. (2001) estimated the total microbial biomass to be 1100 kg ha^{-1} using the fumigation–extraction (FE) technique. This suggests that over 70% of the microbial biomass could be ectomycorrhizal extramatrical mycelia.

Direct estimates of C:N ratios of microbial biomass in soil are scarce due to problems in obtaining clean samples without adhering soil particles. Different extraction techniques have therefore been developed. These include the fumigation–incubation method (Jenkinson, 1988) and the FE technique (Brookes et al., 1985). Attempts to obtain direct estimates of C:N ratios in bacteria have been made in aquatic ecosystems. Fagerbakke et al. (1996) estimated microbial C and N of single cells with X-ray microanalysis and found that bacteria from brackish water had higher C:N ratios (5–6) than bacteria collected from marine water (3.5–4). This difference was interpreted as being the result of different nutrient availabilities in the two environments. When similar bacteria were grown under different nutrient regimes in the laboratory, C:N ratios were found to vary between 3.8 and 9.5 (Vrede et al., 2002).

Our aim was to obtain direct estimates of C:N ratios of ectomycorrhizal mycelia growing in situ in Norway spruce forests in southern Sweden. One Norway spruce site was situated in Tönnersjöheden Experimental Forest, located in the southwest of Sweden (56°41′N, 4°57′E), where the annual mean temperature was 6.5 °C and the annual precipitation 1000 mm. The soil type and texture have been classified as podzol with a sandy-silty till texture.

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Table 1
Site characteristics and C:N ratios for EM mycelia collected from sand-filled mesh bags incubated in the soil of two forest sites

Site	Soil depth from the surface (cm)	pH (H_2O)	C:N ratio of soil	C:N ratio of EM fungi	Number of replicates
Tönnersjöheden	5	3.5[a]	30.4[a]	21.9 ± 1.3	12
Jämjö	5	4.6[b]	21.4[b]	18.9 ± 1.3	5
Jämjö	15	4.7[b]	16.4[b]	18.7 ± 0.8	4
Jämjö	30	5.1[b]	16.9[b]	18.5 ± 1.5	4
Jämjö (all samples)	5–30	–	–	18.5 ± 0.7	13
All samples from both sites	5–30	–	–	20.2 ± 0.8	25

[a] Olsson and Staaf (1995).
[b] Thelin et al. (2002).

A more detailed description of the site was given by Olsson and Staaf (1995). The other spruce site was located at Flakulla, Jämjö in southeastern Sweden (56°53′N, 15°16.5′E). The mean annual temperature was 6.9 °C and the mean annual precipitation 550 mm. Soil type was determined as dystric cambisol and the soil texture was a silty loam (Thelin et al., 2002). In Jämjö, samples were obtained from three different soil layers (5, 15 and 30 cm) while in Tönnersjöheden all samples were obtained from one soil layer (5 cm). A total of 25 samples of clean (although adhering soil bacteria on the EM hyphae may have been included) EM mycelia without contaminating soil particles were obtained by extracting mycelia from sand-filled mesh bags that had been buried in the soil for 12 (Jämjö) or 18 months (Tönnersjöheden) (Wallander et al., 2001). The C isotopic composition of the samples had earlier been determined and compared with values of fruit bodies of saprotrophic and mycorrhizal fungi collected at the sites (Wallander et al., 2001; Hagerberg and Wallander, 2002; Håkan Wallander pers. comm.). These estimates confirmed that the mycelia in the mesh bags were of mycorrhizal origin. In our study, the C:N ratio of mycelia collected from the same mesh bags was determined (Table 1) using an online continuous-flow CN analyser at the Department of Forest Ecology, Swedish University of Agricultural Sciences, Umeå, Sweden (Högberg et al., 1999). pH and C:N ratios of the different soil layers are also reported in Table 1 (data from Thelin et al., 2002; Olsson and Staaf, 1995).

We found that C:N ratios in EM mycelia from Norway spruce forests ranged between 14.1 and 29.0. The mean value (±SE) was 20.2 ± 0.8 ($n = 25$, Table 1). Similar values were found (20.1 ± 0.8, $n = 4$) in another Norway spruce forest in Skogaby in southern Sweden by Nilsson and Wallander (2003). This supports previous suggestions that fungi have a higher C:N ratio than bacteria. Values of C:N ratios for total microbial biomass (including both bacteria and fungi) obtained by the FE technique usually lie between those obtained for fungi alone (this study) or bacteria alone (Fagerbakke et al., 1996). The FE technique to estimate C:N ratios is dependent on the efficiency of the extraction method used, which makes it difficult to compare with the more direct method used in our study and in the study by Fagerbakke et al. (1996). In a Swedish coniferous forest Persson et al. (2001) found microbial C:N ratios to be around 10, while Martikainen and Palojärvi (1990) found values between 6 and 14 in Finnish coniferous forests using the FE technique. Studies by Högberg and Högberg (2002) also indicated that ectomycorrhizal fungi have a higher C:N ratio than bacteria in a coniferous forest in northern Sweden. In plots where the trees had been girdled, and growth of EM fungi was substantially reduced, the C:N ratio of microbial biomass was reduced from 9 to 6 (Högberg and Högberg, 2002).

Although fungi are thought to make up most of the microbial biomass in forest soil, the values of C:N ratios of microbial biomass obtained using the FE technique are usually more similar to those of bacteria than EM fungi. One reason for this may be that an unknown fraction of the EM mycelia in our study was composed of extracellular material which would not be included in the C:N ratios based on the FE technique. Bakken (1985) calculated a C:N ratio of 3.7 in cellular material of bacteria isolated from soil, but he also found a large proportion of extracellular material which was assumed to have a much larger C:N ratio (Lars Bakken, pers. comm.). Another possibility is that the FE technique is more efficient in releasing C and N from bacteria than from fungi. Ladd et al. (1977) pointed out that microbial N obtained after FE is more closely related to cytoplasmic constituents of the soil biomass than to cell wall N, which has a much higher C:N ratio. Consequently, McGill et al. (1981) used different C:N ratios for structural (30) and metabolic (3) biomass components when modelling N mineralization. Fungi usually have a lower proportion of cytoplasm than bacteria and fungal mycelia growing in nitrogen-poor substrates are reported to translocate cytoplasm to the active hyphal tips, resulting in a large proportion of vacuolated hyphae (Cooke and Rayner, 1984; Levi and Cowling, 1969).

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VI

Article in press

Growth and biomass of mycorrhizal mycelia in coniferous forests along short natural nutrient gradients

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Summary

- Total fungal biomass, the biomass of ectomycorrhizal and ericoid mycorrhizal (EM+ErM), and arbuscular mycorrhizal (AM) fungi, as well as the production of EM and AM fungi were estimated in coniferous forest soils along four natural nutrient gradients. Plant community changes, forest productivity, soil pH and N availability increase over relatively short distances (<100 metres) along the gradients.
- The amounts of the phospholipid fatty acid (PLFA) 18:2ω6,9 were used to estimate total fungi (not including AM), and the PLFA 16:1ω5 to estimate AM fungi in soil samples. The decrease in the PLFA 18:2ω6,9 during incubation of soils was used to estimate EM+ErM biomass. The production of AM and EM mycorrhiza was estimated using ingrowth mesh bags.
- Total fungal biomass was highest in soils with the lowest nutrient availability and tree productivity. The biomass of ErM+EM was also highest in these soils. We found tendencies that EM mycelial production was lowest in the soils with the highest nutrient availability and tree productivity. The production of AM fungi was highest in nutrient-rich soils with high pH.
- Our results suggest that mycorrhizal communities change from being ErM-, thereafter to EM- and finally to AM-dominated along these gradients. The observed changes in mycorrhizal type in the short nutrient gradients follow similar patterns to those suggested for altitudinal or latitudinal gradients over longer distances.

Keywords: Arbuscular mycorrhiza, ectomycorrhiza, ericoid mycorrhiza, mycelium, nitrogen, pH

Introduction

The distribution of major mycorrhizal associations in natural ecosystems is suggested to follow regular patterns over large altitudinal and latitudinal gradients (Read, 1991; Smith & Read, 1997). At the highest latitudes and altitudes ericoid mycorrhizal (ErM) symbiosis is most common, while EM successively becomes dominating in the ensuing boreal and temperate

biomes. Finally, AM associations become most abundant in field layers of temperate deciduous forests, in grasslands, agricultural ecosystems and in tropical forests soils. These shifts in mycorrhizal associations may be explained by the change in factors limiting plant growth, from N to P, and in dominant N species, from organic N to ammonium and finally nitrate, along the latitudinal and altitudinal gradients.

Strong links between plant communities and soil nutrient conditions have been found in regional surveys of Fennoscandinavian boreal forests (Dahl et al., 1967; Lahti & Väisänen, 1987), and similar shifts in plant community composition, growth-limiting factors, and forms of N have also been seen in short natural nutrient gradients in boreal forests studied in northern Sweden (Giesler et al., 1998, Giesler et al., 2002, Nordin et al., 2001; Högberg, 2001). In these short nutrient gradients trees are dominated by EM, while the field layer changes from being dominated by ericaceous plants at the nutrient-poor end of the gradient towards an increasing contribution of arbuscular mycorrhizal (AM) plants at the nutrient-rich end. Along one such gradient (Betsele) a considerable decrease in total fungal biomass in the soil was found, and it was suggested that this was the result of a reduction of mycorrhizal rather than saprotrophic fungi (Högberg et al., 2003). The densities of EM fine roots in the O horizon have been found to decrease along this and another natural gradient (Varjisån) (Hoffland et al., 2003), and the EM community structure on root tips was seen to change when investigated along the Betsele gradient (A. Taylor, pers. comm.).

The aims of the present study were to ascertain whether a decrease in total fungal biomass is a general phenomenon, also occurring in other short natural nutrient gradients, and to test the hypothesis that ericoid and ectomycorrhizal fungi are responsible for the decrease in total fungal biomass in the soil along these gradients. For the first time both mycorrhizal biomass in soils and the production of mycorrhizal mycelia was determined in forest soils where nutrient availability increased naturally. To do this we used the phospholipid fatty acid (PLFA) 18:2ω6,9 to estimate total fungi in soil samples. AM fungi do not contain this PLFA and were thus not included in this estimate, and another PLFA (16:1ω5) was used to estimate AM fungi in soil samples. We also applied a soil incubation technique to estimate EM+ErM (ecto- and ericoid mycorrhizal) biomass in soil samples (Bååth et al., 2004). An ingrowth mesh bag technique was used to estimate the production of EM mycelia and AM fungi (Wallander et al., 2001).

Material and methods

Study sites and vegetation types

Four natural nutrient gradients in northern Sweden were studied: Betsele (B), Flakastugan (F), Kryddgrovan (K) and Varjisån (V) (**Table 1**). The sites include two or three different vegetation types reflecting different site productivity, and thus different nutrient availability for plant growth, within less than 100 metres. At the poor, uppermost end of two of the gradients (B and V) there is an open Pinus sylvestris L. forest with a field layer dominated by ericaceous dwarf shrubs such as Vaccinium vitis-idaea L. With increasing nutrient availability the forest shifts to a forest dominated by Picea abies Karst. and with an increase in short herbs intermingled with ericaceous dwarf shrubs in the field layer (all 4 gradients). At the rich, lower end of the gradient, dense tall herbs replace the previous field layer vegetation (all 4 gradients). Three vegetation types have been defined along the gradients, a dwarf shrub type (DS), a short herb type (SH) and a tall herb type (TH). The DS and SH vegetation types are found in groundwater recharge areas, while the TH vegetation type is found in groundwater discharge areas (Giesler et al., 2002).

Two of the sites, Betsele and Varjisån, include all vegetation types and are situated along gently sloping transects. The Kryddgrovan and Flakastugan sites only include SH and TH vegetation. The Flakastugan site is situated in a toe slope area but with no distinct ground-

Table 1 Sites and vegetation types sampled along four nutrient gradients in northern Sweden. Levels of soil pH, tree productivity, soil inorganic N (Ni) and soil nitrate (NO3-) are given. More information about the sites can be found in Giesler et al. (1998; 2002). NA = not applicable, ND = not detected.

Site	Soil pH			Productivity[a]			Ni(µM)[b]			NO_3^-(µM)		
	DS	SH	TH	DS	SH	TH	DS	SH	TH	DS	SH	TH
Betsele(B)	3.5	5.3	6.3	2.8	3.6	6.0	20	100	190	10	10	160
Flakastugan(F)	NA	4.3	4.8	NA	3.3	4.8	NA	25	20	NA	0	0
Kryddgrovan(K)	NA	4.5	7.3	NA	2.5	4.0	NA	25	390	NA	0	90
Varjisån(V)	4.1	4.4	6.4	1.9	3.3	4.8	ND	25	260	ND	0	80

water discharge area. Here, the TH vegetation occurs in smaller local areas surrounded by SH vegetation. The soil pH in the humus layer and the inorganic soil N generally increase with increasing productivity within each site, but there are site-specific differences (**Table 1**).

Estimation of total fungal biomass and fungal biomass of mycorrhizal origin

Soil samples were taken from the humus layer at all sites in October 2000 using a soil auger (0.04 m diameter). Three composite samples, each including five sub-samples, were taken from each vegetation type and site giving a total of 30 humus soil samples. The water content (estimated after drying at 105 ˚C for 24 h) was determined from one sub-sample.

Field-moist humus soil samples (~50 g f. wt) were weighed and placed in 100 ml plastic jars (three bulk samples per vegetation type per site). The jars were incubated in darkness at 20 °C for 6 months and the soil moisture content was maintained at a constant level throughout this period. About 1 g of soil was taken as a sub-sample at the start of the experiment and after 3 and 6 months. The sub-samples were immediately frozen until required for analysis of fatty acids. Analysis of the phospholipid fatty acid (PLFA) 18:2ω6,9 in the soil samples before incubation was used as an estimate of total fungal biomass and the loss of PLFA 18:2ω6,9 during incubation was used as an estimate of the EM+ErM biomass, since we assumed that mainly PLFA 18:2ω6,9 of EM+ErM origin would decompose in the absence of living, active roots (Bååth et al., 2004). This will of course be valid for all root-dependent fungi, including e.g. dark septate fungi. However, since little is known about the abundance of these fungi (Jumponen & Trappe, 1998), we have for simplicity described this fraction of the total soil fungi as EM+ErM. AM fungi are not included in this fraction, since they do not contain the PLFA 18:2ω6,9 (see below).

Estimation of mycelial production by mycorrhizal fungi

In order to estimate the production of external EM mycelium in the field we followed the method described by Wallander et al. (2001). Ingrowth mesh bags (approx. 5×5 cm) were made of nylon mesh (mesh size 50 µm) which allows colonisation by fungal hyphae, but not by roots. The ingrowth bags were filled with 40 g acid-washed sand and sealed. At the Betsele and Flakastugan sites ingrowth mesh bags with sand mixed with 0.5 % apatite mineral (Kemira) was also used in order to evaluate the effect of adding a local P-source to the ingrowth mesh bags. This did, however, not affect the ingrowth of mycelia.

The mesh bags were placed at the interface between the humus layer and mineral soil in mid June 2000. Five mesh bags containing sand (and pair-wise with those, another five containing sand mixed with apatite at the Betsele and the Flakastugan site) were installed at the location of each vegetation type (DS, SH, and TH). The mesh

bags were harvested after four months (October 2000) and stored frozen until further analysis.

All fungal hyphae colonising the ingrowth mesh bags were considered as originating from EM mycorrhizal fungi according to previous results (Wallander et al., 2001; Nilsson & Wallander, 2003). In this case ErM is not included in the measurements, since mycelia of ErM fungi probably do not colonise the mesh bags to any significant degree, as external ErM hyphae only extend some millimetres from the root surface (Smith & Read, 1997). Fungal colonisation of the sand from the field-incubated mesh bags was estimated visually under a dissecting microscope. The degree of colonisation by EM mycelia was divided into classes: 0, no mycelia present; 1, sparse mycelia present; 2, mycelia present but no aggregation of the sand particles; 3, plenty of mycelia present and some aggregation of the sand particles; 4, plenty of mycelia present and sand particles aggregated to a large extent. The degree of fungal colonisation was also estimated using fatty acids as biomarkers.

Analysis of fatty acids

About 10 g of sand from the fungal ingrowth mesh bags, and 0.5 g of soil from the incubation experiment, were analysed for their fatty-acid content. The method of lipid extraction followed that of Frostegård et al. (1991). Extracted lipids were fractionated into neutral lipids, glycolipids and polar lipids on silicic acid columns (Bond Elut, Varian Inc., Palo Alto, CA, USA) by successively eluting with chloroform, acetone and methanol. The chloroform fraction (containing the neutral lipids) and the methanol fraction (containing the phospholipids) were subjected to mild alkaline methanolysis to transform the PLFAs and NLFAs into free fatty-acid methyl esters. These were analysed on a gas chromatograph with a flame ionisation detector and a 50 cm HP5 capillary column according to Frostegård et al. (1993b). Thirty-two PLFAs from the initial soil samples (before starting the incubation experiment) were used in a principal component analysis (PCA). The PLFA 18:2ω6,9 was used as an indicator of total fungi and EM+ErM fungi in soil samples and as an indicator of EM mycelial production in mesh bags. The amount of PLFA 18:2ω6,9 in soil is positively correlated to ergosterol (Frostegård & Bååth, 1996). Ergosterol is a fungal specific sterol (Weete, 1974) and both ergosterol and PLFA 18:2ω6,9 are commonly used as indicators of fungal biomass in soil (Frostegård et al., 1993a; Bossio et al., 1998; Larsen et al., 1998; Bardgett & McAlister, 1999; Montgomery et al., 2000; Ruzicka et al., 2000). AM fungi do, however, not contain PLFA 18:2ω6,9 (Larsen et al., 1998; Olsson & Johansen, 2000) and is thus not included in total fungi, EM+ErM fungi and EM mycelial production. Instead, the PLFA 16:1ω5 was used as an indicator of AM in soil samples, and the neutral lipid fatty acid (NLFA) 16:ω5 was used for estimation of the production of AM fungi in ingrowth mesh bags. Both the PLFA and the NLFA 16:ω5 have been used as indicators of AM, however, the use of the NLFA 16:ω5 is often more sensitive (Olsson, 1999). Therefore, we used the NLFA 16:ω5 as an indicator for AM in ingrowth mesh bags, while the PLFA 16:ω5 was used as indicator for AM in soil samples in order to facilitate comparison with other PLFAs in the PCA.

Statistics

The individual PLFAs (mol %) from the analysis of non-incubated soil samples were subjected to PCA after standardizing to unit variance. Resulting factor scores of the first and second principal components (PC1 and PC2) were tested in two-way Analysis of variances (ANOVA). The effects on fungal biomass of site and vegetation type (mean values per vegetation type at each site) were tested in a two-way ANOVA, and the effects on fungal biomass of vegetation type were tested separately for each site in one-way ANOVAs. Pair-wise comparisons (Tukey's HSD) were performed to test for differences between the vegetation types. Since a paired t-test showed no statistical difference between fungal colonisation of the ingrowth mesh bags regarding the different substrates (sand, or sand mixed

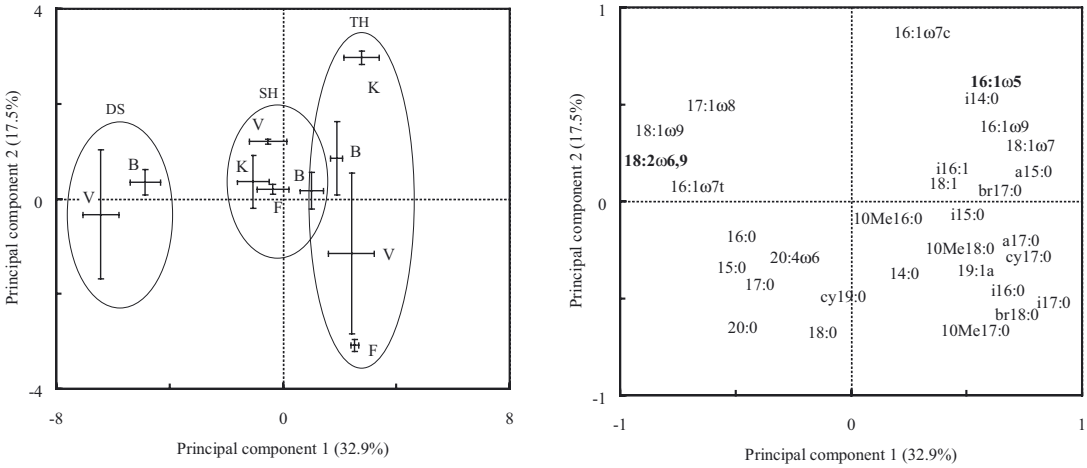

Fig 1 The phospholipid fatty acid pattern in soil samples from the humus layer ng four nutrient gradients. PLFA data were subjected to principal component analysis. (**a**) Scores (mean values ± SE) from the different vegetation types along the gradients (sites). ANOVA (PC1): vegetation type, $P<0.001$; site, ns; vegetation type x site, ns. ANOVA (PC 2): vegetation type; ns, site; ns, vegetation type x site; ns. Vegetation types: dwarf shrub (DS), short herb (SH) and tall herb (TH). Sites: Betsele (B), Flakastugan (F), Kryddgrovan (K) and Varjisån (V). (b) Loadings of the individual PLFAs from the PCA of the PLFA data.

with apatite) mean values of each pair of mesh bags with the different substrates were used.

Results

PLFA patterns in soil samples

The PCA of soil PLFA data indicated a systematic change at all sites, with the vegetation types in the gradients differentiated clearly ($P<0.001$) along PC1 (accounting for 32.9% of the variation), irrespective of the site (**Fig. 1a**). The vegetation types were separated from left to right along the PC1 axis in the order DS, SH and TH (**Fig. 1a**). The loadings of individual PLFAs (Fig. 1b) identified the 18:2ω6,9 and 18:1ω9, another eukaryotic PLFA (Bååth, 2003), as most important for PC1. This indicates that a change in fungi was the most important change in the microorganism community. Several PLFAs (i.e. a15:0, i16:0, a17:0) related to gram-positive bacteria

Table 2 Total fungal biomass in soil samples (initial amount of the PLFA 18:2ω6,9, nmol g-1 d.wt) and relative fungal biomass of EM+ErM origin in soil (PLFA 18:2ω6,9 degraded during incubation) (within brackets). Means ± SE are given.

Site	Betsele	Flakastugan	Kryddgrovan	Varjisån
DS	322±25[a] (57%±8)			325±69 (57%±3)
SH	115±26[b] (31%±10)	111±46 (44%±13)	134±4 (33%±6)	303±21 (19%±5)
TH	70±5[b] (21%±6)	57±4 (28%±3)	98±23 (50%±13)	127±26 (38%±9)
ANOVA	P=0.001	ns	ns	P=0.03

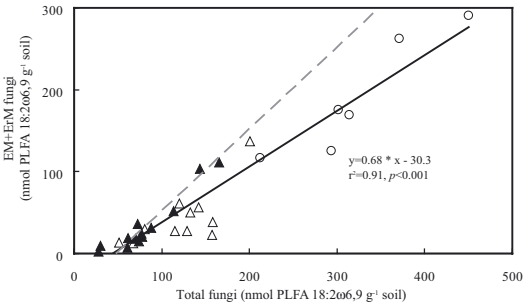

Fig 2 Fungal biomass of ectomycorrhizal and ericoid mycorrhizal (EM+ErM) origin in soil samples relative to total fungal biomass. Total fungal biomass was estimated as the concentration of the PLFA 18:2ω6,9 in soil, and EM+ErM biomass was estimated as the decrease in the PLFA 18:2ω6,9 during incubation of soil samples. Samples are from DS (circles), SH (open triangles) and TH (filled triangles) vegetation. The dashed line represents the situation when EM+ErM fungi constitutes all fungi apart from a background level of other (saprotrophic) fungi corresponding to 50 nmol PLFA 18:2ω6,9 g-1 d.wt soil.

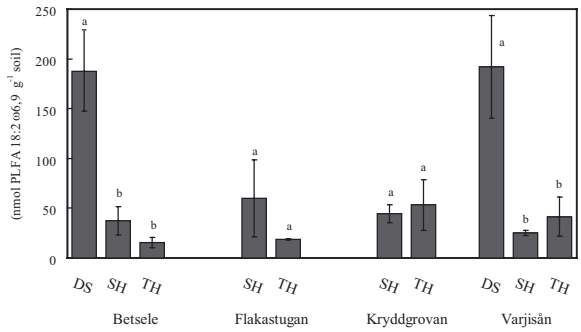

Fig 3 Ectomycorrhizal and ericoid mycorrhizal (EM+ErM) fungi in soil samples along four natural nutrient gradients. EM+ErM fungi was estimated as the decrease in the PLFA 18:2ω6,9 during incubation of soil samples. Vegetation types: dwarf shrub (DS), short herb (SH) and tall herb (TH). Means ± SE. Statistical analyses (ANOVA) were performed per site. Vegetation types with the same letter (within each site) do not differ significantly ($P>0.05$).

were found to the right along PC1, indicating that those bacteria are more abundant in TH than DS vegetation type soils. The PLFA 16:1ω5 (indicator of AM) was also found to the right along PC1.

The second principal component (PC2) (accounting for 17.5% of the variation) did not significantly separate samples from the different vegetation types or the different locations. However, the TH vegetation differed according to site, with Flakastugan positioned at the lower end of the axis, Betsele and Varjisån around zero, and Kryddgrovan at the upper end. This corresponds to soil pH in TH vegetation, being lowest at Flakastugan and highest at Kryddgrovan (**Table 1**). PC2 identified the PLFAs i14:0, 16:1ω9, 16:1ω7c, 16:1ω5 and 18:1ω7 as those increasing most, and i16:0, i17:0, br18:0 and 10Me17:0 decreasing most along PC2. Many of the PLFAs increasing along this component were therefore mono-unsaturated PLFAs, often associated with Gram-negative bacteria, while those decreasing most, mainly branched PLFAs, are reported to be common in Gram-positive bacteria. The PLFA 16:1ω5 was also one of the main PLFAs influencing PC2, indicating that AM fungi were more abundant in TH sites with higher pH compared to TH sites with lower pH.

Total soil fungi and EM+ErM fungi in soil samples

Total soil fungi (i.e. initial amount of the PLFA 18:2ω6,9 in soil samples) decreased significantly along the gradients (Table 2 and 3). Mean values (± SE) for the relative amounts of the PLFA 18:2ω6,9 at all sites were 12.9±0.1 mol% in DS, 5.0±0.3 in SH, and 3.2±0.3 in TH vegetation.

The EM+ErM biomass in soil samples (amount of PLFA 18:2ω6,9 that degraded during incubation of soils) compared to total soil fungi (initial amount of PLFA 18:2ω6,9) was 57±0% in DS, 32±5% in SH, and 34±6% in TH vegetation (mean values for all sites). The EM+ErM biomass

in soil samples was linearly related to the total soil fungal biomass (r2=0.91, P<0.001) (**Fig. 2**). The equation for the relationship was: EM+ErM biomass=0.68×total fungi – 30.3 (with an x-axis intercept at 50 nmol PLFA 18:2w6,9 g-1 d.wt).

The absolute amounts of the PLFA 18:2ω6,9 related to EM+ErM biomass (amount of PLFA 18:2ω6,9 that degraded during incubation of soils), differed significantly between vegetation types, with higher amounts in DS (190±2 nmol PLFA 18:2w6,9 g-1 d.wt) than in SH (42±7) and in TH (32±9) vegetation (mean values for all sites) (**Fig. 3; Table 3**).

Production of external mycelia by ectomycorrhizal (EM) fungi

The production of external mycelia by EM fungi, determined by visual estimates of the colonisation of the mesh bags, was lowest in soils in the most nutrient-rich soil, TH (1.2±0.6), compared to SH (2.9±0.1) and DS (2.3±0.8) (mean values for all sites), when all sites were included in the analysis (**Table 3**). For the individual gradients this pattern was found at the Betsele, Flakastugan and Kryddgrovan sites, but not at Varjisån (**Fig. 4a**). However, the production of EM mycelia determined using the PLFA 18:2ω6,9 content in the mesh bags, did not differ significantly between the vegetation types (DS, 0.24±0.12; SH, 0.19±0.04; TH, 0.13±0.03 nmol g-1 sand; mean values for all sites) (**Table 3**) including all the sites in the analysis, although the trend was similar as by visual estimates. Only in the gradient at the Betsele site did the EM mycelial growth in mesh bags differ significantly with the lowest EM mycelial colonisation in the most nutrient-rich soil (TH) (Fig. 4b). A tendency towards a negative correlation between estimated tree productivity (Table 1) and EM mycelial growth (PLFA) in mesh bags was found (r2=0.31, P=0.09) (**Fig. 5**).

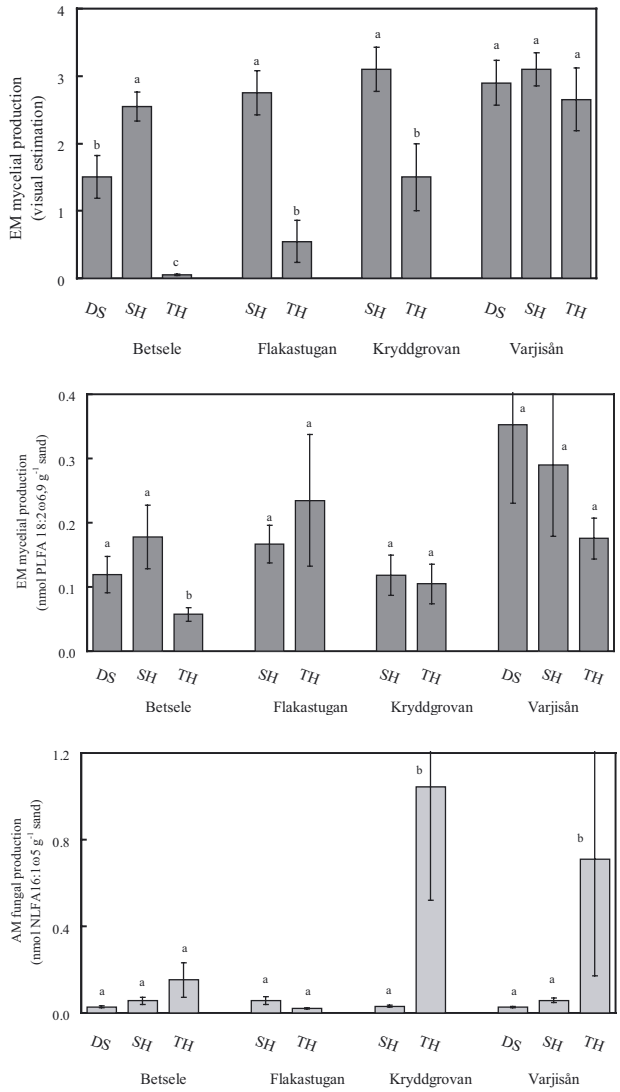

Fig 4 Production of mycelia by EM fungi in ingrowth mesh bags filled with sand, measured by (a) visual estimation (classified according to a scale from 0, no mycelia; to 4, abundant mycelia) and by (b) PLFA 18:2ω6,9 content. (c) Production of arbuscular mycorrhizal (AM) fungi in ingrowth mesh bags filled with sand, measured as the NLFA 16:1ω5 content. Vegetation types: dwarf shrub (DS), short herb (SH) and tall herb (TH). Means ± SE. Statistical analyses (ANOVA) were performed per site. Vegetation types with the same letter (within each site) do not differ significantly (P>0.05).

Table 3 Results of ANOVA analyses of total fungal biomass, EM+ErM fungal biomass in soil samples, and mycelial production of EM and AM fungi in ingrowth mesh bags.

Variable	Site	Vegetation type	Pair-wise comparisions[a]		
Total fungi in soil	*P*=0.05	*P*<0.001	DS[a]	SH[b]	TH[c]
Em+ErM fungi in soil	(ns) *P*=0.60	*P*<0.001	DS[a]	SH[b]	TH[b]
EM mycelia produced (visual)	(ns) *P*=0.09	*P*=0.03	DS[a]	SH[a]	TH[b]
EM mycelia produced (PLFA 18:2ω6,9)	(ns) *P*=0.09	(ns) *P*=0.23	DS	SH	TH
AM mycelia produced (NLFA 16:1ω5)	(ns) *P*=0.18	(ns) *P*=0.54	DS	SH	TH

The two methods used to analyse the production of EM mycelia in the ingrowth mesh bags, visual estimation and PLFA 18:2ω6,9 determination, were positively related (r2=0.44, P=0.05). When two extremely high PLFA 18:2ω6,9 values (out of five) in mesh bags from TH vegetation at the Flakastugan site were excluded, a much stronger relationship was found between the two methods used to estimate EM mycelial production in ingrowth mesh bags (r2=0.76, *P*<0.001) (**Fig. 6**).

Biomass and production of arbuscular mycorrhizal fungi

The relative amounts of the PLFA 16:1ω5 (AM) found in soil samples were 2.3±0.2 mol % (DS), 3.4±0.3 (SH) and 3.4±0.1 (TH). Almost no biomarker for AM fungal production (NLFA

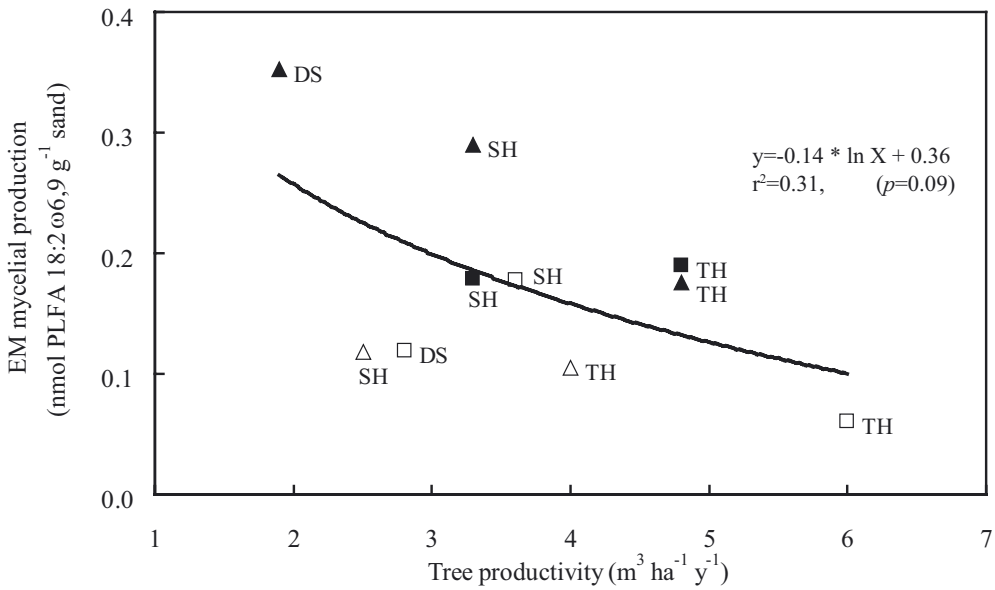

Fig 5 Production of EM mycelia (PLFA 18:2ω6,9) relative to estimated tree productivity. Vegetation types: dwarf shrub (DS), short herb (SH) and tall herb (TH). Sites: Betsele (B), Flakastugan (F), Kryddgrovan (K) and Varjisån (V).

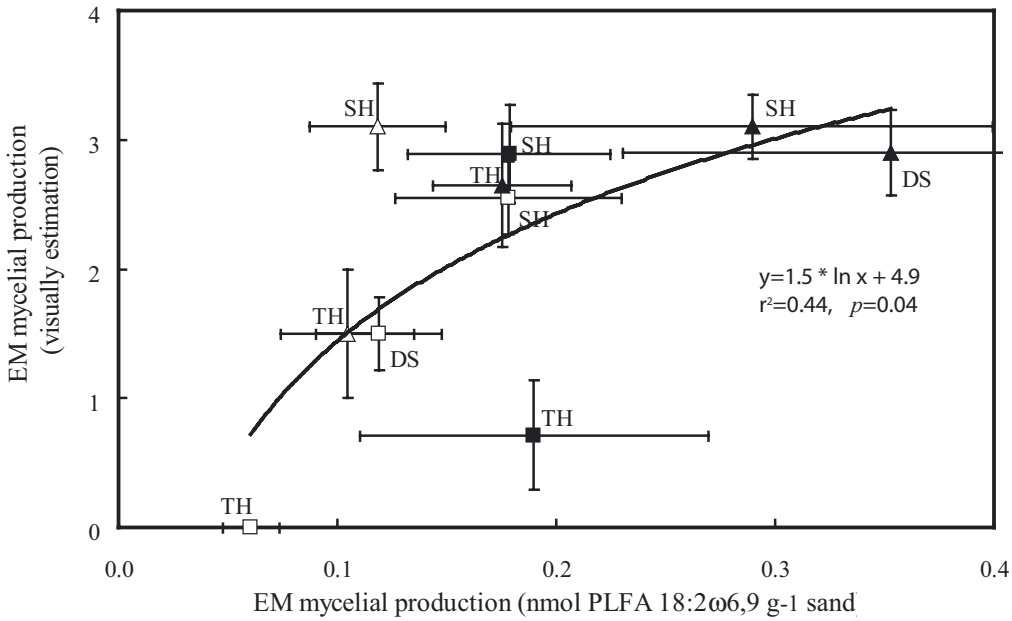

Fig 6 Production of EM mycelia estimated visually, relative to estimation by the PLFA 18:2ω6,9 content (r_2 = 0.44, P=0.05). Vegetation types: dwarf shrub (DS), short herb (SH) and tall herb (TH). Means ± SE.

16:1ω5) was found in the ingrowth mesh bags except for some mesh bags in the TH vegetation. The mean production of AM fungi including all the gradients corresponded to 0.03±0.00 nmol NLFA 16:ω5 g-1 sand in the DS, 0.05±0.01 in the SH and 0.48±0.24 in the TH vegetation. Some of the ingrowth mesh bags in soils in the most nutrient-rich soil (TH) at the Kryddgrovan and the Varjisån sites contained high amounts of the NLFA 16:ω5 and the production of AM fungi differed significantly between the vegetation types at these two sites (K: P<0.001; V: P=0.02) with a similar trend at Betsele (**Fig. 4c**).

Discussion

Total soil fungal biomass decreased along the four short natural nutrient gradients studied (Table 2 and 3). Also, in the principal component analysis, the vegetation types were clearly differentiated along the PC1 axis irrespective of the site (**Fig. 1a**). The PLFAs associated with fungi, 18:2ω6,9 and 18:1ω9 (Bååth, 2003), were the PLFAs most important for PC1 (**Fig. 1b**), indicating that a change in fungi was the most important change in the microorganism

community along the gradients. This is in agreement with the decrease previously observed in one of the gradients (Betsele) (Högberg et al., 2003).

This decrease in fungal biomass along the gradient at Betsele was suggested to be due to a decrease in biomass of ecto- and ericoid mycorrhizal fungi (Högberg et al., 2003). However, they did not measure mycorrhizal biomass. The present study is the first attempt to estimate the biomass of mycorrhizal fungi in ecosystems where nutrient availability changes naturally, and our results support their previous suggestion. We found that EM+ErM biomass, estimated as the decrease in the PLFA 18:2ω6,9 during incubation of soil samples, was higher in the most nutrient-poor soils (in the DS vegetation) than in the more nutrient-rich parts of the natural gradients (SH and TH) (**Fig. 3, Table 3**). We cannot distinguish between EM and ErM fungi in the soil samples with the method used, since hyphae from both fungal groups or from other root dependent fungi are expected to degrade during the incubation of the soil samples in the absence of their host plants. There are, however, some indications that ErM contribute a relatively large proportion to the EM+ErM biomass in the DS vegetation. The amount of total roots at the Betsele site was high in the DS vegetation (~3700 kg ha-1) compared with the SH vegetation (~1650 kg ha-1), and as much as 90% of the roots was ericoid in the DS vegetation compared with less than 50% in the SH vegetation (root data from Högberg et al., 2003). As EM+ErM biomass at the Betsele site was about five times higher in the DS than in the SH vegetation (**Fig. 3**), this indicates that ErM may constitute a large part of the EM+ErM biomass found in the DS vegetation.

The observed tendency of lower EM mycelial production in the most nutrient-rich soils (TH vegetation) than in more nutrient-poor soils (DS and SH vegetation) (**Fig. 4a,b**), is probably an effect of increasing nutrient availability and not a direct effect of, for example, pH. EM mycelial production is known to decrease in response to N addition when studied in the laboratory (Wallander & Nylund, 1992; Arnebrant, 1994) and in the field (Nilsson & Wallander, 2003). The decrease in EM mycelial production was less apparent in the natural gradients studied here than in a field study in spruce forests after N fertilisation, where EM mycelial production was reduced to about 50% compared with non-fertilised forests (Nilsson & Wallander, 2003). It must, however, be kept in mind that those forests had been exposed to a very heavy fertilisation regime (100 kg N ha-1 y-1) for more than 10 years.

Interestingly, the production of mycelia in mesh bags by EM fungi (Fig. 4a,b) was highest in the middle of the gradient at the Betsele site (SH), where the highest tree root biomass was also found (~750 kg ha-1). In the DS vegetation both EM mycelial production (Fig. 4a,b) and tree root biomass (~450 kg ha-1) were lower. In addition, EM mycelial production also followed earlier observed field respiration measurements at Betsele well (~105 mg C h-1 m-2 in DS, ~145 in SH and ~95 in TH vegetation), thus corroborating the suggested high contribution by EM to total soil respiration (Högberg el al., 2001; root and respiration data from Högberg et al., 2003). The reason for the lower EM mycelial production at the most nutrient-poor end (DS) than in the middle (SH) of the gradient is not known. One explanation could be that roots of pine trees, the dominating tree species here, are more commonly found in deeper soil layers than spruce tree roots, which prefer more shallow soil layers. The earlier studies of fine tree root biomass in Betsele (Högberg et al., 2003) and in Varjisån (Hoffland et al. 2003) shows a similar pattern as our estimates of EM mycelial production, suggesting that EM mycelial production is strongly coupled to fine tree root biomass.

Raised N availability in N-limited forests is known to increase aboveground biomass and the shoot-to-root ratio of conifers (Seith et al., 1996; Flückinger & Braun, 1998) by reducing the relative allocation of C to the roots (Wallenda et al., 1996). Increased N availability along the nutrient gradients in our study have also resulted in higher tree productivity with a negative correlation to EM mycelial production (**Fig. 5**), indicating a relative

decrease in C allocation to the roots and to EM fungi. Interestingly, mycelial production by EM fungi has previously been found to be stimulated in N-rich patches in non-fertilised forests but not in N-rich patches in fertilised forests (Nilsson & Wallander, 2003), and similarly hyphal length densities increased after local N addition in a low-nutrient, but not a high-nutrient, soil (Stober et al., 2000). This indicates that forest tree N status, rather than soil N concentration per se, regulates the allocation of C to tree roots. Therefore, regulation of EM mycelial growth must be seen in relation both to the N availability of a soil, and the tree nutrient status and needs.

The reduction in EM mycelial production could also be mediated via shifts in the EM fungal species composition. When EM fungal community structure on EM root tips was studied along the gradient at Betsele, the observed shift of EM fungal species was regarded as being an effect of nutrient conditions rather than of the host tree species (A. Taylor, pers. comm.). Previously observed shifts in root-tip EM fungal species communities after long-term N deposition (Taylor et al., 2000; Lilleskov et al., 2002) or N fertilisation (Kårén, 1997) may reflect differences in strategies for C utilisation or in mycelial foraging strategies of EM fungal species. EM fungal species like Lactarius theiogalus, L. rufus, Tylospora spp and T. fibrillosa are often found to increase with N (Kårén & Nylund, 1997; Taylor et al., 2000; Lilleskov et al., 2002). These smooth-mantled EM fungal species, with short external mycelia, characterized by Agerer (2001) as being of 'contact exploration type' (Lactarius) or 'short-distance exploration type' (Tylospora), probably expend less C on mycelial growth, due to their short external mycelia, than EM fungal species found to decrease in N-rich soils (Cortinarius spp, Piloderma spp) which may need to expend more C on producing more widespread mycelia.

The second principal component (PC2) of the PCA appeared to be explained by soil pH in the TH vegetation, since a low pH was found at the Flakastugan site (4.8) and a high pH was found at the Kryddgrovan site (7.3). A further indication of pH being the main factor explaining PC2 is that the changes in the PLFA pattern were similar to changes previously found to correlate to soil pH. For example, Bååth & Anderson (2003) found that i14:0, 16:1ω5, 16:1ω7c, and 18:1ω7 increased, whereas 10Me17:0 and i16:0 decreased, with increasing soil pH.

The PLFA 16:1ω5 has been shown to be a good indicator of the presence of arbuscular mycorrhiza in soil (Olsson, 1999) although it can be confounded with PLFA 16:1ω5 emanating from bacteria. There was, however, a correlation between high levels of the PLFA 16:1ω5 in soil (AM biomass) and high levels of the NLFA 16:1ω5 in mesh bags (AM production), indicating that this confounding factor was probably of minor importance in the evaluation of the effect of the type of vegetation on AM in the present study.

High levels of AM biomass in soil samples (**Fig. 1**) and production in mesh bags (**Fig. 4c**) were only found in the TH vegetation, with the exception of the Flakastugan site, where very little AM biomass and production was detected. The highest levels of AM biomass and production were found at the Kryddgrovan site. The presence of high levels of AM only in TH, and not in DS, vegetation is consistent with the plant community switching from plants with ErM and EM (DS), to plants with AM (TH) (Giesler et al., 1998, Giesler et al., 2002). This switch in the plant community has previously been suggested to be due to a change from N limitation to more N-rich conditions and possible P limitation among field layer plants (Giesler et al., 1998, Giesler et al., 2002). Furthermore, some AM fungi are known to be inhibited by low pH (van Aarle et al., 2002), and a positive correlation has been found between AM plants and soil pH (Olsson & Tyler, 2004). This might explain the low values of AM in the Flakaliden site.

In conclusion, for the first time, both the biomass of EM+ErM and AM in soil samples, and the production of mycelia by EM and AM fungi were measured in ecosystems where nutrient availability changes naturally. The results of the

present study, and those of a previous study in N-fertilised forests (Nilsson & Wallander, 2003), indicate that tree nutrient status and N availability are the main factors governing the production of EM mycelia. In the short natural nutrient gradients mycelia of EM+ErM fungi were found to decrease with increased nutrient availability, while AM fungi were sometimes found in high amounts in soils with high pH and low P availability. The decrease in ErM and EM and the increase in AM along short natural nutrient gradients follow the patterns of regular shifts in dominating types of mycorrhizal associations, originally suggested for longer latitudinal and altitudinal distances (Read, 1991; Smith & Read, 1997). EM+ErM biomass in soil samples was correlated with the amount of total (including ericoid) roots, while EM growth was correlated with the amount of tree roots (which was estimated in two of the gradients previously studied). Our study thus suggests that ericoid mycorrhizas are important in nutrient-poor soils. Methods of distinguishing ErM from other fungi in the field therefore need to be developed in order to obtain knowledge on the abundance and functions of ErM fungi in nutrient-poor ecosystems.

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