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Karita Saravesi

MYCORRHIZAL RESPONSES TO DEFOLIATION OF WOODY HOSTS

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Abstract

Mycorrhizal fungi are important contributors to the functioning of boreal forests, since they act in the bilateral carbon and nutrient transport between above- and belowground parts of the ecosystem. In ectomycorrhizal (ECM) symbiosis of woody host plants, both fungal and plant partners depend on resources provided by the other. A single tree may simultaneously host several ECM fungal partners, which greatly enhance the host's nutrient uptake. At the same time nearly 20% of host primary production is allocated to mycorrhizal fungi.

Although fungi depend on host-derived carbon, it is poorly understood how reduced carbon availability, e.g., due to herbivory, affects the ECM fungal symbionts. In this thesis I studied the impact of simulated insect defoliation or mammal browsing on mycorrhizal fungi of boreal woody hosts. Quantitative and qualitative changes in biomass partitioning in different fungal compartments were detected. None of the experiments showed that defoliation or shoot clipping treatments reduced the intensity of ECM colonisation, while treatments often shifted fungal composition towards less biomass producing ECM morphotypes. Above- and belowground diversity in ECM symbionts tended to decrease due to shoot or foliar damage. In addition, in some cases defoliation also reduced fungal biomass in fine roots and decreased ECM sexual reproduction by reducing the number of sporocarps produced.

Defoliation induced a similar response pattern in the host and in ECM fungi with a stronger response to increasing severity of treatment (e.g. degree of removed foliage or repeated years of defoliation). This was also confirmed when relating the effects of host and ECM fungal symbionts to defoliation using present and previously published data. The present results suggest that belowground adaptation of boreal trees to the changing environment is mediated by changes in fungal community or biomass partitioning. The lack of response in the intensity of ECM colonisation further emphasises the importance of the symbiosis to boreal trees.

Keywords: belowground carbon allocation, Betula pubescens, colonisation, defoliation, dual mycorrhizal, ectomycorrhiza, fungal community, herbivory, host tree, morphotype, Pinus sylvestris, Salix repens, sporocarp

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Oulu, May 2008 Karita Saravesi

List of original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Kuikka K*, Härmä E, Markkola AM, Rautio P, Roitto M, Saikkonen K, Ahonen-Jonnarth U, Finlay R & Tuomi J (2003) Severe defoliation of Scots pine reduces reproductive investment by ectomycorrhizal symbionts. Ecology 84: 2051–2061.

II Saravesi K, Markkola AM, Rautio P, Roitto M & Tuomi J (2008) Defoliation causes parallel temporal responses in a host tree and its fungal symbionts. Oecologia 156: 117–123.

III Markkola AM, Kuikka K*, Rautio P, Härmä E, Roitto M & Tuomi J (2004) Defoliation increases carbon limitation in ectomycorrhizal symbiosis of *Betula pubescens*. Oecologia 140: 234–240.

IV Saravesi K, Markkola AM, Rautio P & Tuomi J (2008) Simulated mammal browsing and host gender effects on dual mycorrhizal *Salix repens*. Manuscript.

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Contents

1 Introduction

Most boreal tree species maintain symbiotic ectomycorrhizal (ECM) fungi in their roots. ECM fungi are key components in forest ecosystems, since they act in the bilateral carbon and nutrient transport between above- and belowground parts of the ecosystem. In ECM symbiosis both fungal and plant partners depend on resources provided by the other. Boreal forest soils are characterised by low nutrient availability, especially nitrogen and phosphorus, due to low litter turnover, leaching and low pH (Persson *et al.* 2000, Wardle *et al.* 2004). High levels of complex polyphenolic compounds in coniferous needle litter further undermine nutrient availability by immobilising nitrogen into recalcitrant forms in forest humus compounds (Northup *et al.* 1995). In such conditions trees heavily rely on ECM fungal symbionts in their nutrient uptake and especially *Pinaceae* are regarded as obligately ectomycorrhizal (Read 1998). At the same time ECM fungi depend on host-derived carbon, since most of them have only a very limited capacity to obtain carbon by decomposing organic matter. A considerable proportion, nearly 20%, of a host's photosynthetic carbon is allocated to the ECM fungal guild (Hobbie & Hobbie 2006). Allocation of host plant-derived carbon to mycorrhizal fungi is important for the whole belowground forest ecosystem, since it provides a main pathway by which carbon enters the soil organic matter pool (Godbold *et al.* 2006).

1.1 ECM structures, function, and diversity

In boreal trees a vast majority of root tips, nearly 100%, are colonised by ECM fungi (Taylor *et al.* 2000). A multi-layered ECM mantle encloses one, or in some cases several, tips simultaneously inside a common sheath. ECM fungal colonisation induces short root ramification, leading to a multiplication of the number of root tips. Especially the genera *Suillus* and *Rhizopogon* are known to induce large short root clusters with a common fungal sheath (Agerer 1987–1996). An intimate contact zone, the Hartig net, where also nutrient exchange between the symbionts takes place, is formed between fungal and root epidermal or cortical cells. External mycelium extending from the ECM fungal mantle to the surrounding soil forms the most functionally important fungal compartment, and constitutes the main part of the ECM fungal biomass in the forest soil (Wallander *et al.* 2001). Host trees may be interconnected by ECM mycelial networks, which are also responsible for suggested nutrient and carbon transfer between hosts

(Simard *et al.* 2002) The structure of external mycelium varies significantly depending on fungal species: some ECM fungi produce sparse, simple mycelium contacting the surrounding substrates nearby, while others have extensive mycelium with large hyphal cords, rhizomorphs, which are specialised in long-distance translocation of resources (Agerer 2001). Especially many Basiodiomycetes also allocate a large proportion of fungal biomass to sexual reproduction in sporocarps.

Nutrient uptake by boreal trees occurs mainly through ECM fungi, which cover nearly all nutrient absorbing regions in roots isolating them from the soil solution (Bücking *et al.* 2002, Taylor & Peterson 2005). Soil nutrients and water are more efficiently taken up by ECM fungal hyphae than by bare tree roots, due to their larger volume, ability to access small soil pores and to grow beyond the root's nutrient depletion zone (Yanai *et al.* 1995, Simard *et al.* 2002). Further, through their high enzyme production ECM fungi may exploit organic forms of nitrogen and phosphorus mainly inaccessible to the host plant (Chalot & Brun 1998, Read & Perez-Moreno 2003). ECM fungal hyphae may also excrete oxalic acids, which mobilise inorganic nutrients such as phosphorus, potassium, calcium and magnesium by mineral weathering (Landeweert *et al.* 2001). Ability to use organic and inorganic nutrient sources shows, however, high variability among ECM taxa.

The diversity of boreal ECM fungal species is high including at least 700 species (Dahlberg 2002) with an increasing number of new ECM taxa constantly being identified especially among Ascomycetes (Tedersoo *et al.* 2006) but also in Basidiomycetes (Kõljalg *et al.* 2000). Such species richness is remarkable compared to the low number of host tree species in boreal forests. An individual tree may harbour several species of ECM fungal symbionts in its roots simultaneously (Saari *et al.* 2005) with some tens of fungal species inhabiting one monoculture stand (Dickie 2007). Identification of ECM species from belowground parts, root tips or soil mycelium, requires molecular methods (Gardes & Bruns 1993), but in ecological studies grouping of fungi into morphotypes, including several related species according to morphological characteristics in different fungal compartments, is often used (Agerer 1987–1996). While it has been traditionally assumed that ECM trees and ericoid mycorrhizal (ERM) dwarf shrubs in forest ecosystems harbour different fungal symbionts, recent reports on common fungal taxa and even the same strains forming ECM in the tree host and ERM in the ericoid host (Villareal-Ruiz *et al.*

2004, Vrålstad 2004) further adds to diversity of mycorrhizal symbioses in boreal woody hosts.

In addition, some predominantly ECM boreal tree genera, such as *Salix*, *Populus* and *Alnus*, may also support arbuscular mycorrhizal (AM) symbionts in their roots. (Lodge 1989, Cervantes & Rodriguez-Barrueco 1992). Studies with other dual mycorrhizal trees show that the dominant mycorrhizal type may change during growth and ageing of a plant with high AM colonisation in young seedlings but ECM dominance in older plants (Bellei *et al.* 1992, Chen *et al.* 2000). AM fungi belonging to Glomeromycetes form mainly internal structures and usually less external mycelia than many ECM fungal species (Olsson *et al.* 2002). Traditionally, ECM fungi are thought in greater extent to specialise in nitrogen uptake, while AM fungi mainly contribute to their host's phosphorus nutrition (Olsson *et al.* 2002).

1.2 Carbon economy in ECM symbiosis

Carbon allocation dynamics in ECM symbiosis is affected by several factors, such as host photosynthetic activity, nutrient availability, symbiont identity, and sink strength created by the symbiont. ECM fungal activity and host photosynthesis are temporally tightly coupled, since photosynthetic carbon can be traced from ECM root tips within few days of fixation (Högberg *et al.* 2008). Further, even a slight change in, e.g., the host plant's light conditions may result in a lower rate of fungal growth or respiration (Lamhamedi *et al.* 1994, Heinemeyer *et al.* 2007). Photosynthesis is a strongly sink-regulated process, where unloading of carbohydrates from photosynthetic tissues mainly determines the rate of carbon fixation (Luxmoore *et al.* 1995). Belowground sink strength is largely increased by stimulation of short root ramification by ECM fungal colonisation, and thus, increases the number of active, meristematic root tips. Further, ECM fungal colonisation may enhance host photosynthesis even beyond nutritional benefits provided by the fungal symbiont (Dosskey *et al.* 1990, Rousseau & Reid 1990, Conjeaud *et al.* 1996). Thus, acting as a strong carbon sink in roots, ECM fungi generate a positive feedback to the host's carbon acquisition. However, in order to prevent parasitic exploitation the host may, at least to some extent, control the carbohydrate flow to ECM symbionts.

Carbon is transported from the host to fungal tissues as soluble sugars and organic acids (Nehls *et al.* 2007). The main carbon transport form in plant tissues is sucrose, which cannot be utilised by ECM fungi as such (Salzer & Hager

1991). Thus, sucrose must first be hydrolysed into simpler monosaccharides by the plant-derived acid invertase in the apoplastic space between fungal and plant cells in the Hartig net region (Salzer & Hager 1991). Regulation of the host's acid invertase activity may provide the first checkpoint for controlling carbon flow to the fungus (Nehls & Hampp 2000, Wright *et al.* 2000). Fungal cells take up monosaccharides from the apoplast via special transport proteins, after which the sugars are rapidly converted into fungal metabolites (Nehls *et al.* 2007). High monosaccharide concentration in apoplastic space stimulates fungal carbon uptake increasing the sink activity in ECM symbionts (Hampp *et al.* 1999). Monosaccharide transport from the apoplast may provide another mechanism by which carbon flow to ECM fungi is controlled. Some plant monosaccharide transporter genes have shown up-regulation in ECM symbiosis, and thus the host plant may compete with the fungus for carbohydrates (Grunze *et al.* 2004). Host competition of carbohydrates could operate in a case where carbon consumption exceeds the nutritional benefits provided by the fungal symbiont. Further, fine root longevity is closely related to soil nutrient availability (especially nitrogen), which implies that carbon allocation to fine roots continues as long as they are able to provide nutritional benefits (Burton *et al.* 2000). Theoretically, the host could selectively abort such ECM root tips in which the carbon costs exceed the benefits provided as mineral nutrients (Hoeksema & Kummel 2003).

Carbon consumption for ECM metabolism and biomass production varies between fungal species and even within the same species depending on its activity. Fungal biomass is an important determinant of the structural carbon cost of the ECM symbiosis. In laboratory studies ECM fungi producing extensive mycelium in soil have been shown to require more carbon from the host tree than fungi with lower fungal biomass (Colpaert *et al.* 1992, Gorissen & Kuyper 2000). Production of fungal biomass by ECM morphotypes has been linked to carbon costs also at community level, where morphotype assemblage was determined by host carbon availability (Godbold & Berntson 1997, Godbold *et al.* 1997, Saikkonen *et al.* 1999). However, fungal biomass production is only a part of the carbon costs of the ECM symbiosis for the host. Many ECM fungal species are able to produce extracellular enzymes for exploitation of organic nutrient sources (Read & Perez-Moreno 2003), which requires extra energy from the host. For example, species of *Lactarius* and *Russula* show high enzymatic activity, but do not produce high mycelial biomass in soil (Agerer 2001). Furthermore, ECM carbon consumption is greatly affected by fungal respiration, which may vary between and within the species and also show temporal variation depending on

fungal activity, e.g. in nutrient assimilation (Söderström & Read 1987, Bidartondo *et al.* 2001, Fransson *et al.* 2007a). To some extent respiratory carbon loss via ECM fungi seems to be related to fungal biomass (Fransson *et al.* 2007a, b). Finally, the amount of carbon allocated to ECM fungi depends on the age of the root. Carbon allocation to senescing ECM root tips has been reported to decrease (Durall *et al.* 1994), while at the same time ECM fungi prolong fine root life-span and thus maintain the activity of the belowground carbon sink for a longer period (Pregitzer 2002).

1.3 Host and ECM symbiont responses to aboveground herbivory

Generally, the growth of forest trees decreases after insect herbivory, browsing by mammals, or artificial defoliation (Krause & Raffa 1992, Reich *et al.* 1993, Kolb *et al.* 1999), the response to damage often being proportional to the amount of lost foliage (e.g. May and Carlyle 2003). After foliar damage plants tend to shift allocation from belowground to aboveground parts which may result in a decreased root to shoot ratio (Vranjic & Ash 1997). Such a shift in allocation after foliar damage may be a part of the plant's compensatory mechanism to obtain the most limiting resource (Bloom *et al.* 1985), which in the case of foliar damage would often be photosynthetic carbon. In the long run, plants also tend to maintain a rather constant ratio between shoot and root biomass, such that a decrease in one leads to a parallel reduction in the other (Brouwer 1983). In addition, defoliation usually induces carbon-based secondary metabolites in foliage (Roitto *et al.* 2003, Mumm & Hilker 2006), which further shifts allocation towards shoots.

Due to a reduced carbon flux belowground and consequent decline in carbon availability especially in roots, herbivory usually affects mycorrhizal fungal symbionts negatively. Hence, reduced carbon assimilation in the host tree due to insect or artificial defoliation has been found to negatively affect ECM symbionts by reducing the production of ECM sporocarps (Last *et al.* 1979), fungal biomass in the fine roots (Stark & Kytöviita 2005) and, in some cases, intensity of ECM colonisation in fine root tips (Gehring & Whitham 1991, 1995, Del Vecchio *et al.* 1993, Gehring *et al.* 1997, Rossow *et al.* 1997, Kolb *et al.* 1999). In most boreal tree species, studied so far, reduction in total ECM colonisation has not been detected (Markkola 1996, Saikkonen *et al.* 1999, Cullings *et al.* 2001), but instead defoliation has caused more qualitative changes in the ECM community. Saikkonen *et al.* (1999) showed a decrease in ECM morphotypes with high fungal

biomass in mantle or external mycelia in defoliated Scots pine, which suggests a greater carbon demand in these types compared to ECM fungi producing less fungal biomass. Defoliation-induced changes were also reported from an ECM community investigated by molecular methods, where differing carbon demand was proposed as one plausible mechanism causing the shifts in fungal species (Cullings *et al.* 2001, 2005). Further, ECM community change from Basidiomycetes to Ascomycetes, which typically have a lower biomass production, has been reported due to natural insect herbivores in *Pinus edulis* (Brown *et al.* 2001, Gehring & Whitham 2002). Variation in the host's carbon assimilation is thus likely to cause changes in ECM assemblage depending on carbon requirement and biomass production of the individual fungal symbionts (Fig. 1).

Fig. 1. Low-biomass (A) and high-biomass (B, C) ECM morphotypes in Scots pine roots collected from Hailuoto. Smoot type without extensive extraradical mycelia or rhizomorps (A), *Suillus* or *Rhizopogon* type enclosing several root tips inside a uniform mantle and with abundant rhizomorps (B), and a highly rhizomorphous type (C).

In addition to studies reporting herbivore-mediated effects on mycorrhizal symbionts, a number of studies have shown that also mycorrhizal status of the host can affect herbivore performance (Wardle *et al.* 2004). Both AM and ECM symbiosis typically increase host nutrient content, which especially in low nitrogen environments may attract insects to oviposit and feed more on mycorrhizal plants (Manninen *et al.* 1998, Goverde *et al.* 2000). However, mycorrhizal colonisation may also affect herbivores negatively by increasing the host's secondary metabolite production as a result of changes in carbon allocation (Gange & West 1994), although neutral effects of especially ECM colonisation have also been reported (Gehring *et al.* 1997, Manninen *et al.* 2000). Moreover, interactions between mycorrhizal fungi and aboveground herbivores seem to

depend on both insect and fungal species identity. Mycorrhizal colonisation may increase performance of specialist herbivores, but decrease the performance of generalists (Gange *et al.* 2002). Fungal species and community composition affects host plant response to herbivore attack (Gange *et al.* 2005, Bennet & Bever 2007) and may thus result in a variable response in herbivore performance (Goverde *et al.* 2000).

1.4 Aims of the study

Although mycorrhizal fungi depend on host plant-derived carbon, it is poorly understood how reduced carbon availability affects especially ECM fungal symbionts, which are important contributors in the functioning of the boreal forest ecosystem. Only a limited number of studies report the impact of host foliar damage on related ECM symbionts, and the existing data have shown partially divergent results. This thesis aims to further elucidate general trends arising in belowground fungal symbionts as a response to aboveground simulated herbivory, and more specifically report treatment effects on quantitative and qualitative changes in biomass partitioning in different fungal compartments. In addition, I tested the hypothesis that the biomass produced by an ECM symbiont correlates with its carbon cost, which in turn could affect ECM morphotype composition depending on the host's carbon availability.

In the first study (I), the effect of Scots pine needle removal on related ECM symbionts was tested in natural conditions, with special emphasis on ECM sexual reproduction. The extent and structure of the aboveground ECM community was studied, and an estimate of fungal biomass allocation between ECM sporocarps, soil mycelium and ectomycorrhizas was provided for defoliated and non-defoliated trees. The main belowground ECM morphotypes were identified using molecular methods.

The two following studies (II, III) were conducted with Scots pine and white birch seedlings in an experimental field, which enabled examination of various defoliation treatments on host seedlings' biomass partitioning in relation to fungal biomass in roots and ECM morphotypes. Defoliation treatments were repeated on one to three years with two defoliation intensity levels in white birch and three different seasonal timing treatments of defoliation in Scots pine. Morphotypes in both studies were divided into only two categories, high-biomass and low-biomass types, the abundance of which was assessed in all treatments.

In the last study (IV), the effect of shoot clipping on dual mycorrhizal, dioecious *Salix repens* was examined in a natural seashore stand. ECM morphotype community and AM structures and their interactions were investigated in male and female hosts. Further, host growth and flowering frequency of both genders were assessed in the following year.

The results from the present studies are discussed in comparison with the previous studies, and the discrepancies that have arisen, especially in changes in ECM colonisation, are reviewed. Finally, relative effects of defoliation or shoot clipping on host plants and ECM fungi were calculated from the present and previous available data. This was done in order to examine, whether the response of ECM symbionts to simulated or natural herbivory was dependent on and of similar magnitude than the host plant response.

2 Material and methods

2.1 Study sites

Two experiments (I, IV) were conducted on natural sites on the coast of the island of Hailuoto in the Bothnian Bay. The sandy soils on the primary seashore succession area are young and nutrient-poor. Due to the young age of the soil, no podsol structure is yet seen. The area was chosen because of the sparse vegetation and low number of ECM species. The study area of *Salix repens* (IV) situates in Mäntyniemi (65°04' N, 24°37' E) on the dune slack behind the shore dune ridge, where vegetation is uneven and is dominated by patches of *Salix repens* and to a lesser extent mountain crowberry (*Empetrum nigrum ssp. hermaphroditum*). A few herbs, grasses and sedges (*Hieracium umbellatum, Rumex acetosella, Leymus arenarius*, *Deschampsia flexuosa*, *Juncus balticus*) are also present, but ground vegetation is rare or absent. Single tree seedlings (*Pinus sylvestris, Betula pubescens*) also occur. Soil pH is low, on average 4.8 (Oriol Grau, upubl.). The study site of young Scots pine (I) is situated in Virpiniemi (65°03' N, 24°36' E) on slightly older ground on a dune slack behind the dune zone. Vegetation is still patchy, consisting of dwarf shrubs (*Empetrum nigrum ssp*. *hermaphroditum*, *Vaccinium uliginosum*) lichens (*Cladonia* spp., *Cladina* spp., *Stereocaulon* sp.) and mosses (*Racomitrium canescens*, *Polytrichum piliferum*, *P*. *juniperinum*). Sparsely distributed young Scots pines are the dominant tree species. The humus layer is patchy and very thin (0.5–1.0 cm), if present. The mineral soil is acidic (pH 4.8), and organic matter and total nitrogen content are low (0.2% and < 0.01% of soil dry mass, respectively).

Two other experiments with seedlings of Scots pine (II) and European white birch (III) were conducted in an experimental field at the Botanical Gardens of the University of Oulu (65°00' N, 25°30' E). Here the soil is a moderately fertile peat-mineral soil mixture (pH 5.3; total nitrogen 30.9 mg g^{-1} soil organic matter) limed and fertilised with a NPK fertiliser 7 years before the experiments. In the experimental field, nursery-grown seedlings were planted in rows 0.5-1 m apart from each other one year prior to the treatments. All other vegetation was removed by harrowing the topsoil yearly.

2.2 Study species and experimental setups

Scots pine (*Pinus sylvestris* L.) is one of the most common forest trees in the boreal zone, being the dominating tree species in 65% of the forest area in Finland (Finnish Forest Research Institute 2005). Like most boreal coniferous tree species, Scots pine also follows a determined seasonal growth pattern, where the number of needle pairs and internodes are determined according to the growth conditions of the previous season (Kozlowski *et al.* 1991) and resource allocation to different plant parts changes periodically. Shoot elongation is, however, affected by current season conditions, e.g. precipitation. Early in the growing season elongating shoots form a strong sink that is mainly fed by photosynthetically active previous years' needles, while stored carbohydrates play a less important role (Hansen & Beck 1994, Lippu 1998). After shoot elongation a major proportion of resources is allocated to developing needles, which gradually shift from sinks to carbon sources towards the end of the season (Ericsson 1978, Troeng & Linder 1982). Root growth mainly occurs after shoot and needle growth late in the growing season (Iivonen *et al.* 2001).

White birch (*Betula pubescens* Ehrh.) is another common boreal tree species. Unlike conifers, boreal deciduous trees show a less distinctive seasonal growth pattern and the number of leaves is not determined in the previous season (Kozlowski *et al.* 1991). Instead, their growth depends relatively more on environmental factors of the current season, affecting the number of leaves produced in long shoots. Thus, deciduous trees may show a greater capacity for compensatory growth than conifers, e.g., after herbivore damage. *Salix repens* L. is a dioecious, clonal shrub, which is very common especially in coastal dune areas. *S. repens* shows high plasticity in shoot growth varying from very low (up to 10 cm) to higher growth forms (80 cm) (Ranwell 1960). In the present, study *S. repens* mostly grew low shoots, most likely due to harsh environmental conditions, such as heavy coastal winds and moving sand. Further, under the influence of moving sand *S. repens* forms clearly defined hummocks in the experimental area (IV).

Tree roots in the present studies were highly mycorrhizal by ECM fungi with over 93% of root tips colonised in Scots pine trees (I), 89% in Scots pine seedlings (II), 98% in white birch (III) and 72% in *Salix repens* (IV). From the young Scots pine stand in the seashore succession area, altogether 15 ECM morphotypes were identified from roots and 12 ECM species were producing sporocarps (I). Based on the sporocarp and molecular data from roots, *Suillus* and

Rhizopogon spp. dominated in the ECM community. As expected, at the younger succession stage the number of ECM morphotypes in *Salix repens* roots was lower with only six characterised morphotypes (IV). Unlike other species studied, *S. repens* may form dual mycorrhizal symbiosis with both ECM and AM fungi, although ECM fungi typically are the dominant symbionts in the roots (van der Heijden & Vosatka 1999). The dominant ECM coloniser in *Salix* roots was *Cenococcum geophilum*, one of the few ECM species which may be identified in the roots on the basis of morphology. In the experimental field the fungal inoculum for planted seedlings was initially provided by the soil of the nursery from where they originated (II, III). During the experiments only some *Thelephora terrestris* and *Hebeloma* sp. were fruiting at the site, suggesting that the ECM community in the experimental field consisted of fungal species typical for disturbed soils. Additional inoculum was likely contributed by the young Scots pine-dominated stand surrounding the experimental field and having typical boreal ECM community of an urban area (Tarvainen *et al.* 2003).

Both Scots pine and white birch were defoliated manually with scissors by cutting the whole needle pairs or leaves from the base to mimic insect herbivory (I, II, III). In Scots pine the developing current needle class was always left intact. In experiments I, III, and IV defoliation was done in the middle of the growing season. At that time Scots pine has a fully elongated annual shoot with newly developing needles, white birch has matured the early leaves and *Salix repens* is already shedding seeds. In experiment II, Scots pine seedlings received three different seasonal defoliation treatments: Early season defoliation was conducted before the current year shoot elongation (late May-early June), middle season defoliation was conducted after the current shoot elongation period but before current needle growth had started (mid-June) and late season defoliation was conducted after the cessation of current shoot and needle growth (early August). Seasonal timing, intensity and repetition treatments in consecutive years varied depending on the experiment (Table 1). Mammalian browsing was simulated in *Salix repens* (IV) by manually clipping away half of each shoot.

To further examine effects of defoliation on fungal fruiting, a separate experiment was done with Scots pine seedlings among the young Scots pine trees (I) in the same natural stand in Hailuoto. Thirty similar-sized (~1 m in height) seedlings were selected, of which every other seedling (n = 15) was randomly allotted to be defoliated by removing all mature needles in the middle of the growing season. The rest of the selected seedlings were left intact (n = 15).

Table 1. Summary of the study plants and treatments. Defoliation intensity is reported as degree of mature foliage removed. AD = apical damage on each shoot.

Paper	I	II	III	IV
Target plant	*Pinus sylvestris*	*Pinus sylvestris*	*Betula pubescens*	*Salix repens*
Plant age	young trees	seedlings	seedlings	mature
Study site	natural stand	experimental field	experimental field	natural stand
Defoliation				
Intensity	0 or 100%	0 or 100%	0, 50 or 100%	0 or 50% AD
Repetition (yrs)	two	one, two, or three	one or two	one
Seasonal timing	middle	early, middle, or late	middle	middle
No. of treatments	three	ten	seven	four
Replicates per treatment	15	15	15	10
Blocking	no	yes	no	yes

2.3 Plant parameters

In the experimental field (II, III), plant biomass was measured from excavated seedlings after drying (60° C, 48 h) by separately weighing shoots, leaves/needles, stem, coarse roots and fine roots (< 2 mm in diameter in pine, < 1 mm in birch). Shoot length was measured in Scots pine (I, II) from main annual shoots, and in *Salix repens* (IV) from lateral branches of shoots. In *Salix* also the number of vegetative shoots per patch was estimated (IV). Root biomass in Scots pine trees in the natural stand (I) was assessed from soil cores collected around studied trees. Reproductive investment was estimated as number of female cones in Scots pine (I) and as number and proportion of flowering shoots in *Salix repens* (IV). Foliar nitrogen and carbon content was determined with a CHN analyser in dried (< 60 °C) and pulverised samples in experiments I, II and IV. Non-structural carbohydrates (glucose, fructose, sucrose and starch) were quantified from pulverised fine roots of white birch (< 1 mm in diameter; III) and Scots pine (< 2 mm in diameter; I) colorimetrically according to Beutler *et al.* (1978).

Data in experiments II and III were analysed by factorial ANOVA including number of repeated defoliation years, defoliation intensity or seasonal timing as fixed factors and block as the random factor. Planned contrasts were designed to compare the control group with the individual defoliation treatments (III) or with pooled treatments (II), when the ANOVA model did not reveal any significant effects. Factorial ANOVA was also used in experiment IV, where host clipping

and gender were assigned as fixed factors and block as the random factor. In experiment I, data were analysed by repeated-measures ANOVA with defoliation year as a within-subjects and defoliation treatment as between-subjects factor. Differences between control and defoliated trees within each year were tested by t-tests (significance levels corrected by means of Dunn-Sidák method). The relationship between the host and fungal parameters were analysed using the Pearson correlation coefficient or linear regression.

2.4 Fungal colonisation, biomass and community structure

Various parameters in mycorrhizal fungi in the present experiments were investigated (Table 2). Root samples for determination of mycorrhizal colonisation and morphotype analysis were taken with a soil corer (3 cm in diameter into 15 cm depth) in natural stands (I, IV). Ten soil core samples were examined around young Scots pine trees. In each *Salix repens* patch six soil core samples were taken, out of which two to six were examined to reach a similar number of root tips (minimum 300 tips per patch). Soil core samples were kept in a freezer (–20 °C) until preparation. All samples were thawed at room temperature, after which soil samples were gently sieved through 2 mm mesh under running tap water. In the studies conducted in the experimental field (II, III) samples were collected around the root system of each seedling, and pooled to make a composite sample, then frozen (–20 °C). Roots were picked out under a stereomicroscope and classified as ectomycorrhizal (mostly short roots with a visible fungal mantle) and non-mycorrhizal (roots without mantle and sometimes with root hairs) to obtain the total colonisation of ECM fungi in roots.

ECM roots tips were further classified in morphotypes according to their morphology and colour (Agerer 1987–1996) and proportional colonisation of each morphotype of all root tips was counted. In the experimental field (II, III), the main aim was to separate morphotypes by the visible amount of fungal biomass, and hence individual types were divided only into two categories: thin-mantled types with sparsely attached mycelia, if any (referred as low-biomass types), and all thick-mantled types, or those types forming abundant hyphal bundles or rhizomorphs (referred as high-biomass types). Classification of morphotypes into thin- and thick-mantled types was confirmed in these experiments by measuring the diameter of ECM root tips. Molecular identification of the most common morphotypes (n = 8) was used in the study with young Scots pine trees (I). Fungal DNA was extracted from fresh individual

mycorrhizae and amplified by polymerase chain reaction (PCR) using the primers ITS1, ITS4, ITS1-F, and ITS4-B (Gardes & Bruns 1993). The PCR product was sequenced, and the fungi were identified by comparing the obtained sequence data with fungal sequences in the GenBank database using the BLAST program.

In dual mycorrhizal *Salix repens*, also AM structures from the roots were examined. Roots were stained by trypan blue method to assess the internal AM colonisation (Phillips & Hayman 1970). Roots were kept over night in 10% KOH, rinsed with water, and soaked in 30% H_2O_2 for 60 min to bleach the melanised roots. After this, roots were rinsed again with water, kept in 1% HCl for 2.5 hours and stained in trypan blue (90° C 60 min). After the staining procedure AM structures were monitored from a random subsample of the stained roots mounted on a slide. Whole root length was assessed under a light microscope under 100–400 × magnification. Due to the relatively thick and melanised roots of *Salix repens* at the site, I only detected AM spores, vesicles and lobed spores/vesicles from the roots, while other structures (e.g. single hyphae or arbuscules) could not be observed. Root length from each slide was measured using image analysis software (iSolution^{TM}). On average 43.6 ± 0.38 cm of root was examined per each treatment patch. Data are reported as number of AM structures (spores, vesicles) per examined root length (cm).

Ergosterol concentration was used as an estimate of fungal biomass in fine roots (I–III) and mineral soil (I, IV). Ergosterol is a component of fungal cell membranes and provides a quantitative estimate of living fungal biomass, although the amount of ergosterol may depend on the species and age of mycelium (Antibus & Sinsabaugh 1993). Ergosterol is present in higher fungi such as Basidio- and Ascomycetes, which typically form mycorrhizal associations with woody plants or are saprotrophic, while ergosterol has not been discovered in the AM symbiotic fungi Glomeromycetes *in vivo* (Fontaine *et al.* 2001, Olsson *et al.* 2003). Freshly collected fine roots and soil samples were first frozen in liquid nitrogen and stored in a freezer (−70° C). Ergosterol was analysed using a modified assay (Nylund & Wallander 1992, O. Kåren, personal communication). Free ergosterol was extracted with ethanol, followed by saponification with KOH for esterified ergosterol fraction; pentane was used for final extraction. Ergosterol was quantified with high performance liquid chromatography (HPLC) using a reverse-phase C18 column and methanol as the eluant. Commercial ergosterol (5,7,22-Ergostatrien-3ß-ol, Fluka AG) was used as a standard. Ergosterol concentration is reported as µg per mg of dry fine root / soil material.

In the natural Scots pine stand in Hailuoto, ECM sporocarps were examined (I). The number of sporocarps of each species was monitored twice a month during 1998–2001 from August to September or October depending on duration of fungal fruiting. Only sporocarps around the experimental trees (n = 30) at a distance of 1.5 m from the tree trunk were counted. Similarly, ECM sporocarps around defoliated and control Scots pine seedlings (n = 30) among young Scots pine trees were monitored during 2002–2003 from August to October. However, due to the small size of the seedlings and the proximity of other seedlings, sporocarps only at a distance of 1 m from the trunk were counted. Also non-ECM sporocarps were counted in both experiments to control to the overall treatment effect on fungal fruiting.

For ECM sporocarps and morphotypes species richness, Shannon diversity index (I, IV) and Simpson's dominance index (IV) were applied in assessing the above- and belowground community structure in the natural stands. Sporocarp numbers around defoliated and non-defoliated Scots pine seedlings were compared with t-tests performed separately for both defoliation years (2002–2003). All fungal data were analysed as plant parameter data (cf. chapter 2.3).

Table 2. Summary of the examined fungal parameters in the experiments. ECM diversity refers to the Shannon diversity index calculated from sporocarp (I) or morphotype (IV) data. ECM dominance refers to Simpson's dominance index calculated from morphotype data.

Paper	I	II	III	IV
Host plant	*Pinus sylvestris*	*Pinus sylvestris*	*Betula pubescens*	*Salix repens*
ECM colonisation	x	x	x	x
ECM morphotypes	x	x	x	x
ECM diversity	x			x
ECM dominance				x
ECM sporocarps	x			
Root fungal biomass	x	x	x	
Soil fungal biomass	x			x
AM structures				x

2.5 Response of fungal symbiont in relation to host response

To assess, whether defoliation or clipping treatment resulted in a response of similar magnitude in the host plant and ECM fungal symbionts, relative effects were calculated from data presented here (I–IV) as well as from previously

published data (Gehring & Whitham 1995, Markkola 1996, Kolb *et al.* 1999, Mueller *et al.* 2005, Stark & Kytöviita 2005). Relative effects of simulated or insect herbivory were calculated by taking into account the most severely effected plant and ECM fungal parameters in damaged plants in comparison to intact plants. Plant parameters used in Figure 3 are stem or total plant biomass (II, III, Markkola 1996, Kolb *et al.* 1999, Stark & Kytöviita 2005), current shoot growth (I), shoot mortality (Gehring & Whitham 1995, Mueller *et al.* 2005) and host flowering shoot proportion (IV). Respective ECM fungal parameters are total colonisation (Gehring & Whitham 1995, Kolb *et al.* 1999, Mueller *et al.* 2005), the number of ECM roots (Markkola 1996), fungal biomass in soil or in fine roots (IV, Stark & Kytöviita 2005), colonisation of ECM morphotypes (II, III) and number of ECM sporocarps (I). Other details are presented in Table 5.

3 Results

3.1 Mycorrhizal response to defoliation

Defoliation or shoot clipping had a generally negative effect on different fungal parameters, although many neutral responses were also detected (Table 3). In none of the experiments did the treatments decrease the total ECM colonisation. Instead, a shift in ECM morphotype community was often found. In the experimental field, both 50 and 100% defoliation in two consecutive years reduced colonisation by high-biomass ECM morphotypes in white birch roots, while neither of the defoliation intensities applied during only one growing season had a significant effect (III). Likewise, in Scots pine repeated defoliation in two or three consecutive years increased colonisation by low-biomass ECM with a simultaneous though non-significant decline in high-biomass ECM after two years of defoliation (II). A similar declining trend in high-biomass morphotypes, molecularly identified as *Suillus* and *Rhizopogon* spp., was found in a nutrient poor, natural Scots pine stand (I). Despite the observed reduction of high-biomass morphotypes, fine root fungal biomass (μg g^{-1}; indicated as ergosterol) was found to decrease by defoliation only in white birch (III), while in Scots pine fungal biomass did not show a significant response (I, II). Effects of defoliation on soil fungal biomass were studied in natural field experiments (I, IV), where soil fungal biomass only in clipped male *Salix repens* showed a decreasing trend.

The number of ECM sporocarps around defoliated Scots pine trees decreased to about one third compared to controls in the first defoliation year (I). The same pattern was seen in the fungal biomass allocation to sexual reproduction and mycelial growth in roots and soil: in the first defoliation year the proportion of sporocarp biomass of the estimated total fungal biomass was 21% in controls and only 6.3% in defoliated trees, and in the second defoliation year 11.5% in controls and 3.9% in defoliated trees. Defoliation only affected the fungal biomass allocation pattern, while the total fungal biomass in ECM symbionts remained unchanged (I). However, a similar defoliation treatment conducted in two successive years (2002–2003) on Scots pine seedlings among the young Scots pine trees in the same natural stand did not result in a similar reduction in ECM sporocarps ($t_{28} = 0.641$, $p = 0.572$ in 2002; $t_{28} = –0.483$, $p = 0.633$ in 2003; Fig 2). Defoliation did not affect the number of non-ECM sporocarps around the studied

trees or seedlings. Further, in young Scots pine trees defoliation also mediated qualitative changes in the aboveground ECM community by reducing diversity of ECM sporocarps measured as species richness and Shannon diversity index (I). The strongest decline was detected in absolute number of *Suillus bovinus* sporocarps, but in none of the ECM species did the relative sporocarp number show a significant reduction due to defoliation. In *Salix repens* a decreasing trend in morphotype diversity (Shannon index) and an increase in morphotype dominance (Simpson's index) were detected (IV).

The negative effect of defoliation on ECM parameters was related to how early in the growing season the treatment was applied. In Scots pine roots colonisation of low-biomass ECM increased in the early- and middle-season-defoliated seedlings. High-biomass ECM colonised slightly, but non-significantly, less root tips in defoliated seedlings, while in early-defoliated seedlings the proportion of non-mycorrhizal root tips was decreased, possibly indicating reduced growth potential in this treatment (II). Root fungal biomass did not express any clear response pattern to seasonal defoliation treatments.

Table 3. Summary of the impact of different defoliation treatments or shoot clipping on studied fungal parameters. Neutral response is indicated as '0'; statistically significant ($p < 0.05$) negative or positive responses as '–' and '+' , and nearly significant ($p < 0.1$) negative response as '(–)'. ECM diversity refers to Shannon diversity index calculated from sporocarp (I) or morphotype (IV) data. ECM dominance refers to Simpson's dominance index calculated from morphotype data.

Paper	I	II	III	IV
Host plant	*Pinus sylvestris*	*Pinus sylvestris*	*Betula pubescens*	*Salix repens*
ECM colonisation	0	0	0	0
ECM morphotypes				
High biomass ECM	(–)	(–) / –	–	
Low biomass ECM		+		
ECM diversity	–			(–)
ECM dominance				+
ECM sporocarps	–			
Root fungal biomass	(–)	0	–	
Soil fungal biomass	0			(–)
AM structures				0

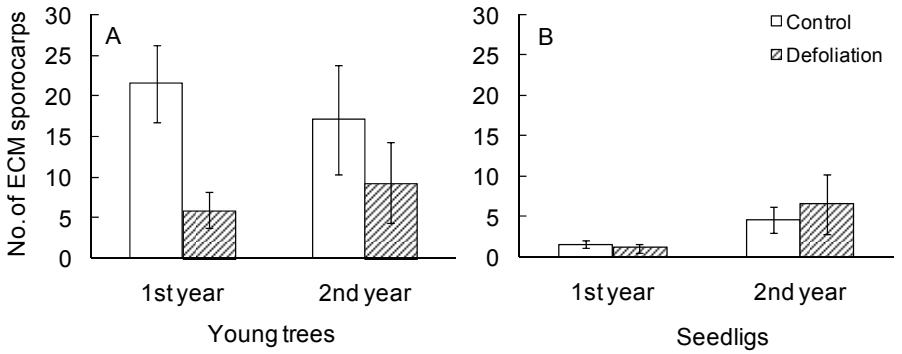

Fig. 2. ECM sporocarps (mean ± SE) produced (A) around young trees (I) and (B) seedlings of Scots pine in a primary seashore succession stand. 1st and 2nd year refer to studied years, when 100% consecutive removal of mature needles was applied (1998–1999 for young trees and 2002–2003 for seedlings).

3.2 Host responses to defoliation

Host plants typically showed a strong negative response to defoliation or shoot clipping (Table 4). One of the most striking effects of defoliation in Scots pine was reduction in shoot growth (I, II). In white birch seedlings full defoliation on two consecutive years reduced shoot biomass as much as 86% (III). A reduction of the same magnitude was also detected in Scots pine shoot biomass in the most severe treatment (early season defoliation of pine seedlings in three consecutive years; II). Further, in Scots pine current main shoot length was especially reduced by previous year defoliation in both young trees in the natural stand and in seedlings in the experimental field (I, II). In young Scots pine trees (I) also a significant increase in carbon-based secondary metabolites in needles accompanied by a decrease in foliar nitrogen was observed (Roitto *et al.* 2003). In belowground parts, coarse roots were strongly reduced by defoliation treatments in the experimental field. Defoliation decreased root biomass relatively more than shoot biomass, which in Scots pine and white birch seedlings was also seen as a reduction in root to shoot ratio (II, III). No significant reduction in root biomass estimated from soil cores was detected in the natural Scots pine stand.

Defoliation and shoot clipping also negatively affected host reproduction. After two years of repeated defoliation, female cone production in young Scots pine trees was almost completely inhibited (I). Similarly, in *Salix repens* shoot

clipping performed during the previous year reduced the proportion of shoots producing either female or male inflorescences.

Seasonal timing of defoliation significantly affected the response of Scots pine seedlings to foliar damage (II). Similar to the fungal symbionts, defoliation induced a more negative effect in relation to the earlier in the growing season it was applied. Current main shoot length and root to shoot ratio were significantly decreased by early and middle season defoliation. Total shoot and coarse root biomasses showed a similar decreasing pattern, except that defoliation always had a significant negative effect on both parameters irrespective of season when defoliation was conducted (II).

Table 4. Summary of the impact of different defoliation treatments or shoot clipping on plant parameters. Symbols as in Table 3.

Paper	I	II	III	IV
Target plant	*Pinus sylvestris*	*Pinus sylvestris*	*Betula pubescens*	*Salix repens*
Current shoot length	–	–		
Total shoot biomass		–	–	
Lateral shoot length				0
Host reproduction	–			–
Root carbohydrates	0		(–)	
Fine root biomass		0	–	
Coarse root biomass		–	–	
Total root biomass	0			
Root to shoot ratio		–	–	

3.3 Interactions between fungal and plant partners

In all studies it was possible to relate root fungal biomass, colonisation by high-biomass ECM or morphotype richness to host tree growth. In young Scots pine trees (I) current shoot growth was positively correlated with root fungal biomass ($r = 0.526$, $p = 0.003$) and number of sporocarps ($r = 0.384$, $p = 0.036$). In Scots pine seedlings (II) current shoot growth had a negative relation to colonisation by low-biomass morphotypes ($r = –0.229$, $p = 0.027$), while root fungal biomass showed no trend. In fully (100%) defoliated white birch there was a strong positive relation between high-biomass ECM and leaf biomass (proportionated to non-photosynthetic plant biomass; III). This relation was less evident in 50% defoliation and was absent in controls. In *Salix repens* ECM morphotype richness and host lateral shoot growth was positively correlated (IV).

4 Discussion

4.1 Mycorrhizal responses to defoliation

Reduced aboveground biomass of the host was also reflected in belowground mycorrhizal fungal symbionts in all present studies. Host defoliation or shoot clipping induced a negative response in different ECM fungal compartments (sporocarps, fungal biomass in roots and to a lesser extent in soil) or caused a shift in ECM morphotypes, though no reduction in the intensity of ECM colonisation was found in any of the host species studied. Results from the present experiments are well in line with previous studies (Table 5), although some dissimilarities, which are discussed below, also emerged.

4.1.1 ECM colonisation

Total ECM colonisation is a conservative, although important measure, since it describes the degree of individual nutrient transport connections between the host and fungal symbiont, and is thus closely related to the functioning of the symbiosis. In all the present experiments total ECM colonisation remained unaffected despite severe defoliation treatments, while in previous studies both negative and neutral effects have been reported (Table 5). Several different factors may account for the discrepancy in the responses of ECM symbionts. Firstly, ECM colonisation was mainly decreased in studies where trees were defoliated repeatedly by natural long-term (≥ 4 years) herbivory on pinyon pine (*Pinus edulis*; Gehring & Whitham 1991, 1995, Del Vecchio *et al.* 1993, Gehring *et al.* 1997) or on willows and balsam poplar (Rossow *et al.* 1997). In addition, in pinyon pine studies, insects were allowed to choose the trees they were feeding on resulting in heavily attacked susceptible trees and resistant trees with only minor herbivore defoliation. Natural food plant selection implies that herbivore-susceptible and herbivore-resistant pinyon pines may differ, e.g. genetically, which may have contributed to the reduced ECM colonisation in the susceptible trees (Gehring & Whitham 1991). However, defaunation of herbivore insects from susceptible pinyons recovered the ECM colonisation to the same level as in resistant trees, which implies that severity and duration of herbivore damage, rather than inherent differences in hosts, drive the degree of ECM colonisation in pinyon pine (Del Vecchio *et al.* 1993).

Table 5. Summary of the reported effects of simulated or natural herbivory on ECM fungi. Modified from Kuikka *et al*. 2007.

Treatment	Host plant	Colonisation	High biomass ECM	Community	Root fungal biomass	Sporocarps	Reference
Insect defoliation	*Pinus edulis*	Reduced					Gehring & Whitham 1991, 1995
Insect defoliation	*Pinus edulis*	Reduced					Del Vecchio *et al.* 1993
Insect defoliation	*Pinus edulis*	Reduced					Gehring *et al.* 1997
Insect defoliation	*Pinus edulis*			Changed			Gehring & Whitham 2002
Manual defoliation	*Pinus sylvestris*	No change	Reduced	Changed	No change		Saikkonen *et al.* 1999
Manual defoliation	*Pinus sylvestris*	No change			No change		Markkola 1996
Manual defoliation	*Pinus sylvestris*	No change	(Reduced)	Changed [1]	No change	Reduced	Kuikka *et al.* 2003 (I)
Manual defoliation	*Pinus sylvestris*	No change	Reduced		No change		Saravesi *et al.* 2008 (II)
Manual defoliation	*Pinus contorta*	No change		Changed			Cullings *et al.* 2001
Manual defoliation	*Pinus contorta*		Reduced [2]	No change			Cullings *et al.* 2005
Insect defoliation	*Pseudotsuga menziesii*	Reduced					Kolb *et al.* 1999
Manual defoliation	*Betula pubescens*	No change	Reduced		Reduced		Markkola *et al.* 2004 (III)
Manual shoot clip	*Betula pubescens*				Reduced		Stark & Kytöviita 2005
Manual defoliation	*Betula pendula*, *B. pubescens*					Reduced	Last *et al.* 1979
Manual shoot clip	*Salix repens*	No change		Changed	Reduced		Saravesi *et al.* unpubl. (IV)
Mammal browsing	*Salix* spp., *Populus balsamifera*	Reduced					Rossow *et al.* 1997
Insect defoliation	*Populus* × *canadensis*	No change		No change			Kosola *et al.* 2004
Insect stem galling	*Quercus turbinella*	Reduced					Mueller *et al.* 2005

Notes for Table 5:

[1] Based on ECM sporocarps

[2] Based on ECM soil mycelium of *Cenococcum geophilum*

However, in the present studies even three years of repeated, severe defoliation did not reduce ECM colonisation in Scots pine seedlings in the moderately nutrient rich experimental field (II), suggesting that response of mycorrhizal colonisation may be system specific. Studies reporting neutral response of ECM colonisation to defoliation have mostly been conducted in boreal trees (Table 5). Boreal trees are adapted to grow in nutrient-poor conditions in rather moist soils with a high organic nitrogen content and low pH, which in turn provides an excellent breeding ground especially for ECM fungi and results in very high ECM colonisation in roots. A dramatic decrease in fungal colonisation potential in boreal soils would require large-scale deterioration of host trees or the soil ecosystem, and hence, defoliation treatments conducted on individual trees, may not reduce fungal inoculum enough to have an impact on total colonisation. Studies showing reduced ECM colonisation in pinyon pine roots (Gehring & Whitham 1991, 1995, Del Vecchio *et al.* 1993, Gehring *et al.* 1997) were conducted on less favourable semi-arid sites, where ECM colonisation has been shown to be strongly dependent on soil moisture conditions (Swaty *et al.* 1998). In these environments ECM colonisation of pinyon roots is highly variable and distinctly lower (~ 30–80%, Del Vecchio *et al.* 1993, Gehring *et al.* 1995) compared to nearly 100% constant ECM colonisation typical for boreal tees (Taylor *et al.* 2000). Defoliation in less favourable environments for fungal growth could result in a shortage of fungal inoculum, and thus lead to reduced colonisation. This idea is supported by Gehring *et al.* (1995), who report that shoot clipping decreased ECM colonisation in a water and nutrient-stressed site whereas colonisation remained unchanged in a less stressful site. Further, in the sandy seashore site shoot clipping did not reduce ECM colonisation in dual mycorrhizal *Salix repens* (IV). In another dual mycorrhizal woody host, *Quercus turbinella*, herbivory has been reported to decrease ECM colonisation, accompanied by a simultaneous increase in AM colonisation (Mueller *et al.* 2005). The present results agree with Kosola *et al.* (2004) who reported similar ECM and AM colonisation in defoliated and undefoliated hybrid poplars.

High investment in ECM fungal colonisation seems to be a common feature in boreal trees adapted to low-nutrient conditions. In laboratory studies, where nutrient addition is adjusted so that the host is not able to derive any nutritional benefits from ECM symbiosis, fungal colonisation has been shown to decrease host growth (Colpaert *et al.* 1992, Colpaert *et al.* 1996, Goriseen & Kuyper 2000), which can be regarded as a direct carbon cost of the symbiont (Hobbie 2006). In these studies the host plant exhibited almost full ECM colonisation, which

suggests that trees have a limited potential to regulate ECM colonisation in laboratory conditions even if the symbiosis would be detrimental to host growth. Therefore, the mechanism behind the possible reduction in ECM colonisation in herbivore-damaged trees in the field would most likely be reduced inoculum potential of the fungi. This hypothesis was recently tested by Lewis *et al.* (2008), who reported a reduced ECM colonisation and morphotype richness in oak seedlings planted in herbivore-damaged *Tsuga canadensis* forest compared to transplants in intact oak forest. However, since no herbivore-intact *Tsuga* forest was available to control the effect of host tree species on fungal inoculum (Lewis *et al.* 2008), the decline in oak seedlings' ECM colonisation could be due to incompatibility with the *Tsuga* ECM community or due to an actual reduction in fungal inoculum in the herbivore-damaged forest.

4.1.2 Belowground ECM community

Nitrogen availability in the environment has been recognised as an important factor in shaping belowground ECM fungal communities (Taylor *et al.* 2000, Peter *et al.* 2001, Lilleskov *et al.* 2002). There is an increasing body of evidence indicating that also host carbon resources affect ECM community composition by causing shifts in morphotype or species abundances (reviewed by Kuikka *et al.* 2007). As discussed in the previous chapter, in boreal forest soil stacked with fungal mycelium, the host tree may have only a limited control over being fully colonised. However, the host may reduce carbon flow to the individual ECM fungal symbiont by competing for carbohydrates in the apoplast (Grunze *et al.* 2004) or possibly by aborting such ECM short roots in which the carbon costs outweigh the nutritional benefits (Hoeksema & Kummel 2003). Fungi with rapid growth rates are presumably the first ones to colonise newly-emerged root tips, but after that succession of fungal colonisers will take place (Shaw *et al.* 1995, Wu *et al.* 1999). In a situation where host photosynthesis is limited, an ECM fungus which is able to maintain its growth and metabolism at low carbon levels would have a competitive advantage (Saikkonen *et al.* 1999). A superior fungal competitor may reduce colonisation by the inferior ECM species (Kennedy *et al.* 2007, Hortal *et al.* 2008) or in an extreme case, take over already colonised root tips (Wu *et al.* 1999). Thus, competition between fungi with differing carbon demands would be a plausible mechanism for ECM community change when energy supply is low.

Saikkonen *et al.* (1999) were first to report a shift in ECM morphotype assemblage in defoliated Scots pine, where a thick-mantled and rhizomorphous type was decreased and smooth types with less external mycelium increased compared to non-defoliated controls. Similarly, a lower colonisation of thick-mantled ECM morphotypes was found with severely defoliated *Betula pubescens* (III), and a decreasing trend in rhizomorphous and thick-mantled *Suillus*/*Rhizopogon* type was observed in defoliated Scots pine trees in the field (I). Respectively, in Scots pine seedlings (II) defoliation increased the abundance of low-biomass morphotypes. Changes in morphotype assemblages may originate from different carbon demands of the morphotypes, fungi with high biomass in mantle and rhizomorphs having a higher carbon requirement (Colpaert *et al.* 1992, 1996, Godbold & Berntson 1997, Saikkonen *et al.* 1999, Gorissen & Kuyper 2000). At the species level, defoliation of *Pinus contorta* altered ECM community composition detected by molecular methods, while overall ECM species richness remained unchanged (Cullings *et al.* 2001). Species from the genera *Inocybe* and *Cortinarius* dominated the undefoliated control plots, while defoliation reduced or inhibited their occurrence (Cullings *et al.* 2001). Especially species in the large fungal genus *Cortinarius* often form an abundant mycelial network with rhizomorphs in the soil, and are known to be sensitive to disturbance, such as nitrogen deposition (Lilleskov *et al.* 2002, Tarvainen *et al.* 2003) and forestry practices (Lazaruk *et al.* 2005). The change in ECM fungal species composition in roots of defoliated *Pinus contorta* was not detected in soil mycelium by molecular methods, but reductions in individual species were attributable to mycelial production (Cullings *et al.* 2005). In pinyon pine ECM communities in herbivore-resistant trees were dominated by Basidiomycetes, while in susceptible trees Ascomycetes were more abundant (Brown *et al.* 2001, Gehring & Whitham 2002). Ascomycetous ECM fungi typically produce a low amount of fungal mycelium in the soil (Agerer 2001, Tedersoo *et al.* 2006). In addition, Ascomycetous ECM have been reported to be more abundant in slowly rather than fast-growing host clones, possibly due to lower biomass allocation below ground and inability to maintain ECM symbionts with high mycelial production in slow-growth hosts (Korkama *et al.* 2006, 2007).

Due to differing phenologies in host trees and ECM fungi, the growth of the latter mainly occurring later in the season than that of the host (Lippu 1998, Wallander *et al.* 2001), a contrasting response of host and fungal symbionts to different seasonal defoliation treatments could have been expected. However, despite the differences in the seasonal growth dynamics, ECM fungi followed the

response patterns of the host (II). Seasonal defoliation treatments had a stronger negative effect on Scots pine growth and biomass allocation in relation to the earlier in the growing season they were applied. Similarly, early and middle season defoliation increased root colonisation of low-biomass morphotypes by over 20% compared to controls, with a simultaneous, though non-significant reduction in high-biomass morphotypes in all seasonal defoliation treatments (II).

Although clipping did not reduce lateral shoot growth in *Salix repens*, the lateral shoot length correlated positively with the number of ECM morphotypes. Jonsson *et al.* (2001) have shown that host productivity is positively related to ECM fungal species richness, although this may depend on host plant species and soil fertility. Different ECM fungal species have specialised nutrient uptake capacities (Landeweert *et al.* 2001, Read & Perez-Moreno 2003), and thus a more diverse ECM community may lead to better nutrition and consequently, to enhanced growth of the host. On the other hand, the driving force for this kind of positive relation might come from the host: a more species rich ECM community in better growing hosts may be due to higher belowground carbon allocation in hosts that have high productivity (Korkama *et al.* 2006).

Results form the present studies strongly support the hypothesis, that biomass produced by an ECM symbiont is in relation to its carbon cost, since defoliation shifted ECM communities from high-biomass towards low-biomass morphotypes. However, a possibility of morphological changes taking place in ECM in a carbon-limited environment cannot be excluded. Therefore, defoliation may have caused an originally high-biomass ECM fungi to produce a low amount of external fungal biomass and reduced fungal mantle, especially in Scots pine seedlings (II) in which the dominating ECM type was very poor. Nevertheless, ECM fungal species show substantial variance in biomass production, which can be attributed to their carbon requirement (Colpaert *et al.* 1992, 1996, Gorissen & Kuyper 2000). Carbon demand of ECM fungal species could be one important factor in determining their niche partitioning (Bruns 1995), according to which fungal species could be specified similarly to nitrophilic and nitrophobic ECM species (see Taylor *et al.* 2000). Moreover, ECM fungal species have been distinguished in relation to their external mycelial production by Agerer (2001), who classified, e.g., smooth-mantled *Russula* and *Lactarius* ECM to the short-distance contact exploration type, whereas highly rhizomorphous *Suillus* and *Rhizopogon* are regarded as functioning in long-distance nutrient exploration.

4.1.3 ECM external mycelium and sporocarps

Although the unquestionable importance of ECM mycelium in nutrient exploration from soil and symbiosis functioning, production of external mycelium especially by individual ECM taxa has been less studied due to methodological difficulties (see Wallander 2006). Here in natural seashore succession stands soil fungal biomass, measured as ergosterol concentration, was not decreased around defoliated Scots pine trees (I), but showed a decreasing trend in clipped male *Salix repens*. This latter negative trend in soil fungal biomass is likely to be mediated via *Salix repens*, since in young coastal dune ecosystem soil fungal biomass mainly consists of mycorrhizal fungal mycelium due to low organic matter in the soil.

Sporocarp production of the ECM symbionts constitutes a considerable carbon sink for the host tree. Although sporocarp biomass forms only a minor proportion of the total ECM biomass, it may vary between sites, consisting of up to 2% of total ECM fungal biomass in mature Scots pine and Norway spruce forest stands (Markkola *et al.* 1995, Wallander *et al.* 2001) compared to estimates of 10–20% in the young Scots pine stand in the seashore succession area (I). Moreover, ECM fruiting occurs during a rather short period in the late summer and fall, which puts a heavy short-time carbon requirement on the hosts. ECM sporocarp production seems to be dependent on recent photosynthesis and carbohydrate storage, e.g., in fungal mycelium cannot be used to maintain the fruiting (Högberg *et al.* 2001). Tight coupling of host photosynthesis and ECM sporocarp growth was also shown by Lamhamedi *et al.* (1994), who reported a decline in sporocarp growth shortly after the host was transferred to a low-light environment. Similarly to Last *et al.* (1979), who reported a decline in ECM sporocarps associated with defoliated *Betula* spp., current year defoliation reduced the number of ECM sporocarps produced and the estimated proportion of sporocarp biomass to total fungal biomass in young Scots pine trees (I). Differences in non-ECM fungi were not detected, which confirms that the effect on ECM sporocarps was mediated through host defoliation. ECM sporocarp production recovered gradually, and reached equal numbers as in the control trees two years after the last defoliation treatment (see Fig. 3 in paper I). However, similar defoliation did not reduce ECM sporocarps around Scots pine seedlings in the same stand. The lack of responses in defoliated seedlings may be explained by the dominant position of larger trees in the community sharing the ECM mycelial network in the area. Previously it has been show that naturally regenerating Scots

pine seedlings and mature trees maintain similar ECM communities, implying a shared fungal network between them (Jonsson *et al.* 1999). Young Scots pine trees with much more photosynthesising foliage than small seedlings, apparently dominate in the carbon flow to the ECM community resulting in reduced sporocarp production in defoliated young trees but not in seedlings.

The most common ECM species fruiting in the young Scots pine stand (I) was *Suillus bovinus*. The number of *S. bovinus* sporocarps declined due to defoliation in the first treatment year, but this decline was not seen in the relative abundance or in any other species. Further, diversity and richness in fruiting ECM species decreased in defoliated trees in the second treatment year (I). Similar to belowground ECM community, changes in fruiting species composition due to defoliation may favour less carbon-costly symbionts that produce smaller or fewer sporocarps. This may partially explain the reduction in *Suillus bovinus*, which produces large sporocarps and abundant external mycelia in soil and in high-biomass mycorrhizas. The number of *Suillus* and *Cortinarius* sporocarps declined also at forest sites with high nitrogen deposition (Tarvainen *et al.* 2003) possibly due to decreased carbon flow to belowground nutrient foraging in a high nitrogen environment and consequent suffering of costly ECM symbionts. Further, results from the present experiment (I) imply, that ECM fungal symbionts may be able to change their allocation pattern by distributing proportionally less biomass to reproductive structures when carbon flow from the host is limited.

4.2 Host response to defoliation

Generally, host growth declines due to simulated or natural leaf herbivory (Krause & Raffa 1992, Reich *et al.* 1993, Kolb *et al.* 1999), with defoliation intensity and timing affecting the responses (May & Carlyle 2003, Lyytikäinen-Saarenmaa 1999). In all the present experiments defoliation affected host growth negatively, except in *Salix repens*, in which shoot clip tended to increase total shoot number. In the experimental field studies biomass of Scots pine and white birch seedlings was reduced with a greater decline in root than in shoot biomass, resulting in a reduced root to shoot ratio. Reduced belowground allocation was also seen in a decreasing trend in carbohydrate concentration in birch roots (III). In Scots pine current main shoot length responded negatively to previous year defoliation (I, II). An increase in carbon-based secondary metabolites and a decrease in foliar nitrogen (Roitto *et al.* 2003) probably also contributed to growth loss in young Scots pine trees (I). Also, as reported previously (e.g. May & Carlyle 2003),

reductions in host growth were proportional to lost foliage, the highest defoliation intensity or number of repeated defoliation years resulting in the highest growth losses (II, III). Simulated herbivory also affected plant reproduction, since it nearly inhibited female cone production in young Scots pine trees (I) and reduced the proportion of flowering shoots of especially female *Salix repens* (IV).

Timing of foliar damage within the growing season is likely to differentially affect biomass accumulation due to periodically changing the growth phase of above- and belowground parts of the tree (Lippu 1998, Iivonen *et al.* 2001). This was confirmed with Scots pine seedlings (III), where defoliation treatments had a stronger negative effect on host growth and biomass allocation the earlier in the growing season they were applied. Loss of previous years' needles early in the growing season has been shown to be more detrimental to height growth of Scots pine than defoliation later in the season (Lyytikäinen-Saarenmaa 1999). On the other hand, severe loss of effectively photosynthesising current needles late in the season may also be harmful for tree growth (Ericsson *et al.* 1980). This implies that the effect of seasonal defoliation depends on the relative importance of different needle age classes during the growing season.

Evergreen plants have substantial carbon reserves in their needles, while in deciduous plants carbon is mainly stored in bark and roots. Foliar herbivory is thus predicted to affect evergreens more negatively than deciduous plants (Bryant *et al.* 1983). Further, due to a predetermined growth pattern, in which needle and internode primordia are already formed in the previous season, conifers have a limited capacity to compensate for herbivore damage (Kozlowski *et al.* 1991). Although conifers are not able to replace lost foliage or shoots during the same growing season, enhanced photosynthesis in remaining needles of defoliated balsam fir has been reported (Little *et al.* 2003). Two years after the last defoliation treatment, current main shoot length in young Scots pine trees was still reduced to about one-third that of controls (I), indicating a very slow recovery. Biomass of Scots pine seedlings in the experimental field showed a dramatic decrease (> 80%) due to the most detrimental treatment of early-season defoliation on three consecutive years (II). However, in deciduous white birch defoliation, treatments were equally effective. Defoliation of white birch conducted in the previous year more severely reduced plant biomass than current year defoliation, indicating that previous year storages are also important for the growth of boreal deciduous trees (III). Contrary to results from Scots pine and white birch, shoot clipping seemed not to affect lateral shoot growth of *Salix repens*. Moreover, clipping increased density in *Salix repens* patches by

increasing the number of vegetative shoots. *Salix* spp. typically show a high potential for compensatory growth (e.g. Johnston *et al.* 2007).

4.3 Defoliation effects on the host in relation to ECM fungi

Growth loss in host trees was proportional to the severity of the defoliation treatments (e.g. degree of removed foliage or repeated years of defoliation), and the ECM fungal symbionts seemed to follow the host response patterns (II, III). A similar trend emerged when studying the relative effects of defoliation on host and ECM fungal symbionts using present and previously published data (Fig. 3).

Firstly, the observed relation between host and fungal response suggests a threshold level (approx. 10%), below which defoliation effects on the host are not expressed in ECM symbionts. Secondly, the impact on ECM symbionts appears weaker than on host trees. Equal response in both partners would have resulted in a linear relation (dashed line in Fig. 3), where ECM symbiont response exactly mirrors the host response. A stronger effect on ECM symbionts in relation to hosts would shift data points above the linear relation, implying a lower dependence of the host on fungi in a facultative symbiosis.

The observed pattern, however, shows a weaker response in ECM fungi compared to the host (seen as data points below the linear relation), suggesting that it is beneficial for the host to continue carbon allocation to fungal symbionts, i.e., to nutrient acquisition, despite the reduced carbon availability.

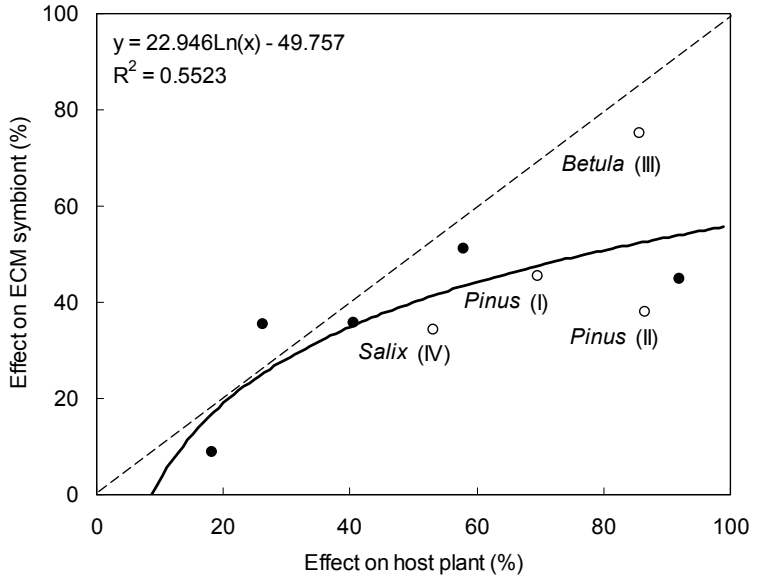

Fig. 3. Relative effect (%) of defoliation or shoot clipping on host plant and related ECM symbionts. Studies in the present thesis are marked with open symbols. Dashed line describes hypothetical equal response in both partners.

Alternatively, a lower effect on ECM fungi than on the host plant may imply a parasitic behaviour, where carbon costs exceed the nutritional benefits provided by the symbiont. However, especially conifers are highly dependent on ECM fungi in their nutrient acquisition, and thus even very high carbon costs for maintaining the symbiosis seem to be acceptable (Johnson *et al.* 1997). Further, by facilitating a host's nutrient status and enhancing photosynthesis by providing a strong belowground sink (Dosskey *et al.* 1990, Rousseau & Reid 1990, Conjeaud *et al.* 1996), ECM fungi may act in recovery from foliar damage. This is also supported by the present results, since sporocarp production around defoliated trees recovered to similar level as in controls within two years, while current host shoot growth showed no recovery yet (I). Overall, the above results imply a strong interdependence between host trees and ECM fungi.

5 Conclusions

Present results confirm that aboveground herbivory does not only change host plant growth and biomass allocation, but may also affect belowground ECM fungal symbionts. Host defoliation induced changes in different ECM fungal parts including sporocarps, fine root fungal biomass and both the above- and belowground ECM community. Partially diverging from previous results, defoliation and shoot clipping treatments did not affect the intensity of ECM colonisation. Instead of decreasing colonisation in roots, ECM fungi seemed to respond to defoliation through changes in fungal morphotype composition.

Both the plant and ECM fungal partner depend on resources provided by each other, resulting in a highly obligatory symbiosis for both the host and fungal symbionts. Especially boreal conifers growing in nutrient-poor conditions rely on ECM fungi in their nutrient acquisition. In such conditions, it would not be beneficial, or even possible to reduce the degree of nutrient-transferring ECM connections, although at the same time ECM fungi comprise a considerable carbon sink. Besides affecting host carbohydrate pools, defoliation may result in nitrogen deficiency especially in conifers due to loss of foliar nitrogen reserves (Roitto *et al.* 2003). Maintenance of ECM association in most root tips creates a positive feedback for host nutrient status, and may thus improve the ability of the host to recover from herbivore damage.

The present results strongly support the hypothesis suggesting that in a carbon-limited environment biomass production of ECM fungi, which largely determines the carbon consumption of an individual fungal symbiont, would also define the morphotype composition. However, in all cases it was not possible to separate whether shift in morphotypes was due to true morphotype community change or deterioration of originally high-biomass types into low-biomass types. Change in morphotypes was accompanied by a reduction in fungal biomass in sporocarps or fine roots and to a lesser extent in soil, indicating further reduction in ECM structures.

In addition, defoliation induced a parallel response pattern in the host and in ECM fungi with a stronger response in relation to increasing severity of treatment (e.g. intensity or consecutive years of defoliation). Further, seasonal defoliation treatment affected host and ECM symbionts similarly, the early season defoliation being most detrimental to host growth but also resulting in the highest colonisation of low-biomass morphotypes. This suggests that herbivore species might have different effects on the composition or condition of ECM fungal

communities depending on their seasonal feeding regime. These observations were confirmed, when the magnitude of host and ECM symbiont response was compared by calculating relative effects of defoliation from the present and previous data available. A similar pattern emerged, where the ECM symbiont's response was positively related to the host response. However, defoliation seemed to inflict a weaker impact on ECM fungi than on hosts, which further emphasises the importance of the symbiosis for trees.

In a broader context, the present results confirm that belowground adaptation of boreal trees to the changing environment does not occur through altering the intensity of root colonisation but rather via changing the fungal community (Taylor *et al.* 2000, Lazaruk *et al.* 2005) or modifying production of external mycelium or sporocarps (Nilsson & Wallander 2003, Gange *et al.* 2007). Thus, reduced colonisation in highly ECM boreal trees would require large-scale or long-lasting disturbance. Changes in the ECM fungal community may also be reflected onto the whole belowground ecosystem, since mycorrhizal fungal mycelium is suggested to be a major pathway by which carbon enters the soil organic matter pool. Thus, an altered ECM community may eventually contribute to possible cascading effects of herbivory on the biological properties of soil (Ayres *et al.* 2004).

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