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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

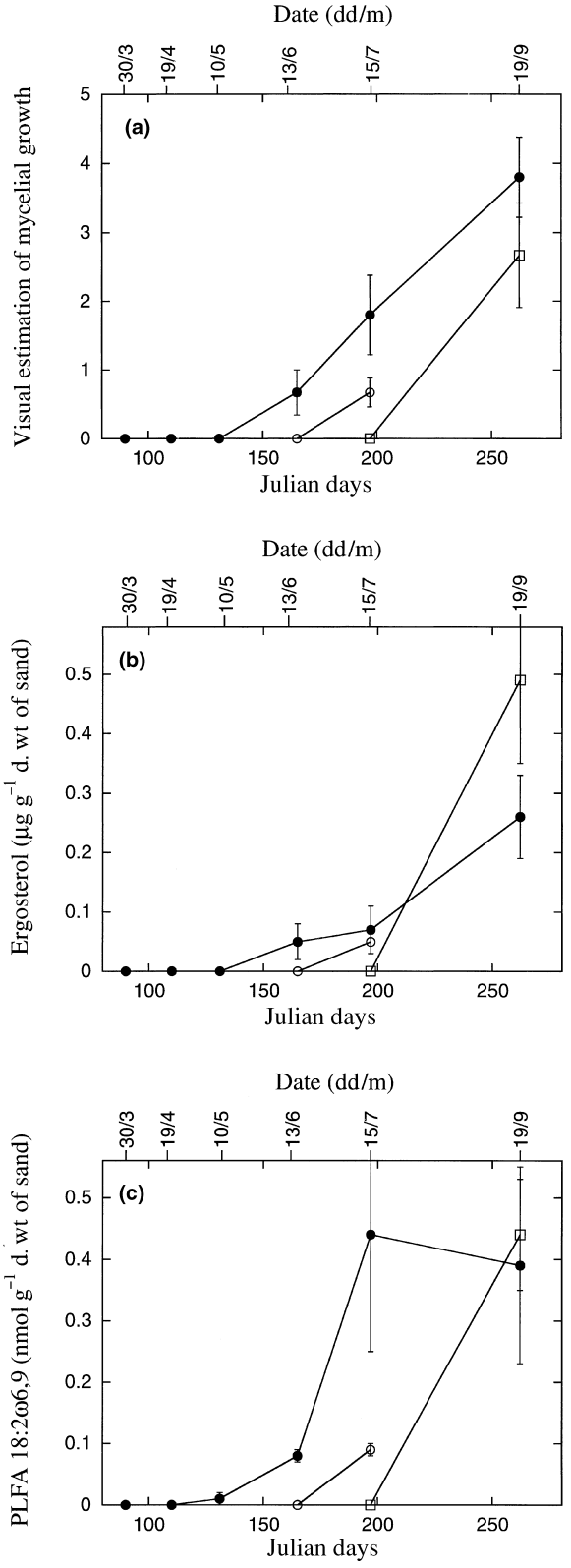

Fig. 1 Colonization of sand in mesh bags at Silvåkra, south-west Sweden estimated by: (a) visual estimation (b) ergosterol analysis and (c) phospholipid fatty acid 18 : 2ω6,9 analysis. Different symbols indicate mesh bags buried at different dates during the year. Bars indicate ± SE, n = 3–6.

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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