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Are ectomycorrhizal fungi alleviating or aggravating nitrogen limitation of tree growth in boreal forests?

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Summary

- Symbioses between plant roots and mycorrhizal fungi are thought to enhance plant uptake of nutrients through a favourable exchange for photosynthates. Ectomycorrhizal fungi are considered to play this vital role for trees in nitrogen (N)-limited boreal forests.
- We followed symbiotic carbon (C)–N exchange in a large-scale boreal pine forest experiment by tracing $^{13}CO_2$ absorbed through tree photosynthesis and ^{15}N injected into a soil layer in which ectomycorrhizal fungi dominate the microbial community.
- We detected little ^{15}N in tree canopies, but high levels in soil microbes and in mycorrhizal root tips, illustrating effective soil N immobilization, especially in late summer, when tree belowground C allocation was high. Additions of N fertilizer to the soil before labelling shifted the incorporation of ^{15}N from soil microbes and root tips to tree foliage.
- These results were tested in a model for C–N exchange between trees and mycorrhizal fungi, suggesting that ectomycorrhizal fungi transfer small fractions of absorbed N to trees under N-limited conditions, but larger fractions if more N is available. We suggest that greater allocation of C from trees to ectomycorrhizal fungi increases N retention in soil mycelium, driving boreal forests towards more severe N limitation at low N supply.

Introduction

Many plants form mycorrhizal symbioses (Brundrett, 2009), which are thought to enhance their nutrient acquisition (Smith & Read, 2008). In the extensive boreal forests, where tree growth is strongly limited by low soil nitrogen (N) supply (Tamm, 1991), > 95% of the root tips of tree roots are covered by ectomycorrhizal fungal mantles (Taylor *et al.*, 2000). Hyphae extend from these, efficiently exploring the soil to acquire nutrients such as N, some of which is transferred to the host plant in return for carbon (C) substrates from photosynthesis (Smith & Read, 2008). Extramatrical hyphae may comprise a significant fraction of soil microbial biomass and represent a substantial fraction of soil microbial N (Wallander *et al.*, 2001; Högberg *et al.*, 2010). However, in spite of a significant input of C from tree photosynthesis to mycorrhizal fungi (Finlay & Söderström, 1992; Simard *et al.*, 2002), the trees remain N limited. The N limitation characteristic of boreal forests is associated with a high microbial N immobilization capacity of the surface organic mor layer (e.g. Melin *et al.*, 1983) and, intriguingly, this immobilization capacity is also positively correlated with the proportion of fungi relative to bacteria within the microbial community (Högberg *et al.*, 2006). Strong N immobilization in temperate and boreal forests has been shown in a number of studies, which has stimulated a discussion on the role of active roots and mycorrhizas in this context (e.g. Aber *et al.*, 1998; Schimel & Bennett, 2004). Furthermore, several pot studies have shown that mycorrhizal fungi may contribute to N immobilization, hence limiting rather than improving seedling N uptake (e.g. Colpaert *et al.*, 1996; Alberton *et al.*, 2007; Corrêa *et al.*, 2008). The potential role of ectomycorrhizal fungi in N immobilization in boreal soils has, however, not been tested in field settings.

In a recent meta-analysis, Corrêa *et al.* (2012) found evidence that C allocation from plants to mycorrhizal fungi is a result of excess C production by the plant, and thereby incurs no cost. The authors concluded that the effect of mycorrhizal symbiosis on plant growth was linked to its effect on plant N uptake, implying that the rates of N transfer from fungi to host plants, and not C–N exchange rates between the symbionts, are decisive for host plant performance. A wealth of studies have shown that plant allocation of C to belowground processes is stimulated at low N availability (cf. Hermans *et al.*, 2006), but it is not clear how mycorrhizal fungi balance the transfer of N critically needed by the host plants whilst, at the same time, providing N for their own N demand.

Based on the above, we speculate here that the large allocation of C from trees to ectomycorrhizal fungi in N-limited forests may drive a strong immobilization by stimulating the growth of these fungi. If this is the case, additions of N to the soil should override the N demand of mycorrhizal fungi, reducing the proportion of N they immobilize and thereby increasing the supply of N to tree canopies.

To test our hypothesis, we injected trace amounts of stable isotope-labelled nitrate, $^{15}NO_3^-$, into the ectomycorrhiza-dominated lower horizon (Lindahl *et al.*, 2007) of the mor layer of eight experimental plots in a young *Pinus sylvestris* L. stand in northern Sweden. Four plots had been fertilized with unlabelled NO_3^--N at a rate of 100 kg N ha^{-1} 2 wk before labeling, and four plots remained unfertilized as controls. We used nitrate because the high C requirements for the reduction and assimilation of this N source should lead to a lower efficiency of microbial immobilization of N than of organic N sources or ammonium, which are more easily immobilized by mycorrhizal fungi. Hence, using nitrate enabled a more critical test of our mycorrhiza immobilization hypothesis than using organic N sources or ammonium. Moreover, evidence from ^{15}N pool dilution studies suggests that nitrate turnover and immobilization may be significant in coniferous forest soils despite low concentrations of nitrate (Stark & Hart, 1997).

Plots (i.e. trees and root-associated microbes) were also labelled with the stable C isotope ^{13}C by supplying the tree canopies with a short $^{13}CO_2$ pulse (Högberg *et al.*, 2010) that reached the roots and associated biota in the soil on the next day, that is, on the day on which the ^{15}N tracer was added to the soil. This enabled us to study interactions between the allocation of photosynthetic C and N taken up from the soil.

Materials and Methods

Field site and measurements of tree growth

The experiment was conducted in a *c.* 14-yr-old, 4-m-tall, boreal *Pinus sylvestris* L. forest at Rosinedalsheden (64°09′N, 19°05′E, 145 m above sea level) in northern Sweden (Högberg *et al.*, 2010; Figs S1, S2). The site has a sparse understorey of dwarf shrubs, *Calluna vulgaris* L. and *Vaccinium vitis-idaea* L., and a ground layer of *Cladonia* spp. lichens. The soil is podzolic, with a 2–3-cm-thick organic mor layer, with a C : N ratio of 33 and pH (in H_2O) of 4.5 (Högberg *et al.*, 2010).

Tree growth was measured on fertilized and control plots by coring trees 3 yr after the addition of fertilizer. Cores were taken at breast height from four trees per plot and from four fertilized and four nonfertilized plots. Measurements of annual tree ring width were taken for the periods 2004–2006 and 2008–2010.

Treatments and stable isotope labelling

Nitrogen was added to four 100-m^2 plots, designated N1–N4, 2 wk before labelling the soil with ^{15}N and the canopy with $^{13}CO_2$, by broadcasting pellets of $Ca(NO_3)_2$, at a dose of 100 kg N ha^{-1}, on 1 June 2007 (N1 and N2 plots; Table S1) or 6 August 2007 (N3 and N4 plots). Four plots, designated C1–C4 (each paired with an N plot), were used as unfertilized control plots. On the day after $^{13}CO_2$ labelling (11 and 19 June 2007 for plots C1–N1 and C2–N2, 21 and 23 August 2007 for plots C3–N3 and C4–N4, respectively), 57 mM ^{15}N-$NaNO_3$ (98 atom%) solution was injected into the lower half of the surface organic mor layer of the soil, using a 50-ml syringe fitted with a four-sideport needle (custom made at UNIMEG, Umeå University, Umeå, Sweden; http://www.service.umu.se/unimeg/), at 160 points (15 ml at each point) regularly distributed over the central 10 m^2 of the plots. This tracer addition corresponded to 2 kg N ha^{-1}. Products of canopy photosynthesis were labelled by enclosing 50-m^2 plots (centred around the 10 m^2 labelled with ^{15}N and within the 100-m^2 plots) in transparent 4-m-tall plastic chambers into which 25 l of 96 atom% $^{13}CO_2$ were released (Högberg *et al.*, 2010). This resulted in initial ^{13}C and [CO_2] concentrations of 22 atom% and 530 ppm, respectively, but, during the 1.5–3.5 h of exposure, the concentration of CO_2 decreased to *c.* 320 ppm (Högberg *et al.*, 2010). Untreated and unlabelled control plots, designated K1–K4, were sampled to determine the natural background abundances of ^{13}C and ^{15}N in the examined ecosystem components.

Sampling and analyses Current year needles were sampled from trees growing within the central 10 m^2 of the plots on three occasions (directly before labelling and 2 and 4 wk after labeling) and isotopic analyses were performed on dried and milled needles of four trees within each plot. For measurements of ^{13}C or ^{15}N contents of soil, root tips and microbial cytoplasm, and ^{13}C contents of PLFAs, samples of the organic mor layer were taken (0.15-m-diameter corer, $n = 4$ per plot) and roots were carefully extracted before the experiment and 2, 4, 6, 14 d and *c.* 28 d after labelling. Three additional samplings of root tips were made in October 2007 (*c.* 70 and 130 d after labelling) and in June and July the following year (*c.* 300–400 d after labelling; Högberg *et al.*, 2010; Keel *et al.*, 2012). Ectomycorrhizal root tips from pine trees were picked from the soil cores. Ectomycorrhizal roots of Scots pine are readily distinguished from nonmycorrhizal root tips on the basis of their swollen appearance, often associated with more or less conspicuously coloured fungal sheaths and extramatrical mycelium, and the frequent formation of dichotomous roots. The soil samples were fumigated with chloroform and microbial cytoplasm contents were extracted a few hours after sampling (Brookes *et al.*, 1985; Vance *et al.*, 1987). Nonassimilated $^{15}NO_3^-$ tracer or fertilizer was removed (Wyland *et al.*, 1994), and the resulting N fraction was hydrolysed through Kjeldahl digestion, and microdiffusion of N released as NH_3, which was captured on acidified cellulose paper which was subsequently analysed for N and ^{15}N (Stark & Hart, 1996). The N and ^{15}N in the microbial cytoplasm, as well as in root tips and needles, were analysed using a Delta+ isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The C and ^{13}C in the extracts were analysed by gas chromatography-isotope ratio mass spectrometry (GC-IRMS) after wet oxidation (Högberg *et al.*, 2010). PLFAs found in microbial membranes were extracted from freeze–dried soil and

used as microbial group-specific biomarkers (Frostegård & Bååth, 1996). The ^{13}C signatures of PLFAs were determined according to Paterson *et al.* (2007; cf. Högberg *et al.*, 2010). The chitin content of fine roots (< 1 mm) was analysed according to Ekblad & Näsholm (1996).

Calculations

Data on N and C contents, and atom% ^{15}N and ^{13}C in excess of the natural abundances of ^{15}N and ^{13}C in the K1–K4 natural abundance control plots, were used to calculate atom% ^{15}N excess and ^{13}C excess for each sample from the $^{13}CO_2$- and $Na^{15}NO_3^-$-labelled N1–N4 and C1–C4 plots. Concentrations of excess ^{15}N and ^{13}C in microbial cytoplasm and in foliage were calculated from the atom% excess values and corresponding values for total N concentrations.

Measured values of the amounts of ^{15}N per gram biomass in needles and ^{15}N per gram organic matter were converted to units of mg $^{15}N\,m^{-2}$ ground area based on an estimate of total needle dry biomass per m^2 common to all plots (150 g m^{-2}), and measurements of organic matter per ground area for each plot (1.1 kg m^{-2} in all treatments).

Statistics

Effects of N fertilization on excess ^{13}C or ^{15}N in microbial cytoplasm (Figs 1, 2) were tested using paired *t*-test using treatment means from five samplings up to 1 month after labelling ($N = 5$, $n = 2$ plots per treatment, alpha = 0.05). Treatment effects on excess ^{15}N of ectomycorrhizal root tips (Fig. 3) were tested using paired *t*-test using treatment means from eight samplings up to *c.* 1 yr after labelling ($N = 8$, $n = 2$ plots, alpha = 0.05).

Mathematical modelling of mycorrhizal N–C exchange

Fungal biomass production (db/dt_{gross}, g $m^{-2}\,d^{-1}$) is limited by C allocation from the plant (C_{myc}, g $m^{-2}\,d^{-1}$) and is in equilibrium with turnover, that is, net growth (db/dt_{net}) is zero (Eqn 1). These assumptions result in a fungal biomass (b, g m^{-2}, Eqn 2) and fungal N demand (N immobilization, N_{im}, g m^{-2}, Eqn 3) proportional to C_{myc}.

$$\frac{db}{dt_{net}} = \frac{db}{dt_{gross}} - \text{turnover} = y\,C_{myc} - \frac{b}{t_m} = 0 \qquad \text{Eqn 1}$$

$$b = y\,C_{myc}\,t_m \qquad \text{Eqn 2}$$

$$N_{im} = y\,C_{myc}\,r_{N:C} \qquad \text{Eqn 3}$$

In Eqns 1–3, y is a yield coefficient ($y = 1 -$ respiration costs per biomass constructed), $r_{N:C}$ is the N : C ratio of fungal biomass and t_m is the mean lifespan of the fungi.

Without going into the underlying mechanisms (e.g. direct uptake and enzymatic N mobilization) and their spatial heterogeneity, we assume that overall net fungal N uptake (N_{up}, Eqn 4) increases with soil N availability (N_{av}, g $m^{-2}\,d^{-1}$) and fungal biomass b. The effect of increasing b gradually declines as a result of a limited soil volume and increasing competition among hyphae.

$$N_{up} = N_{av}\,\frac{b\,u_b}{1 + b\,u_b} \qquad \text{Eqn 4}$$

In Eqn 4, the constant u_b determines the maximal N uptake rate per b, that is, uptake efficiency at small b.

Nitrogen transfer to the trees (N_{exp}, Eqn 5) is the difference between N uptake and N_{im}.

$$N_{exp} = N_{up} - N_{im} \qquad \text{Eqn 5}$$

The results from this model are illustrated in Fig. 6 and further elaborated in the Discussion section.

Results

The average annual tree ring widths for the trees (mean ± SE, $n = 4$) were 2.5 ± 0.16 and 2.4 ± 0.18 mm yr^{-1} for control and fertilized trees, respectively, during the period 3 yr before fertilization (2004–2006), and the corresponding ring widths for the period after fertilization (2008–2010) were 1.7 ± 0.06 and 3.1 ± 0.11 mm yr^{-1}, respectively. This corresponds to an 80% increase in tree annual ring growth during the 3 yr after fertilization (*t*-test, $P < 0.001$).

Within weeks after N addition, there was no sign of altered C allocation below ground; neither the ^{13}C labelling of the cytoplasm of soil microorganisms, nor that of the fungal biomarker PLFA 18:2ω6,9 differed between fertilized and control plots (Figs 1; S3). However, ^{13}C labelling of the cytoplasm and of PLFA 18:2ω6,9 was more than three times higher in August than in June. Moreover, the amount of ^{15}N in the cytoplasm of soil microorganisms was lower in fertilized than in nonfertilized control plots, an effect that was statistically significant in August ($P < 0.001$). The much lower incorporation of ^{15}N into microbial cytoplasm in N-fertilized plots was associated with a decline in the C : N ratio from 7 to 5 (paired *t*-test, $P < 0.009$, $n = 10$), but was not the result of a change in microbial cytoplasm C ($P = 0.194$ in June and $P = 0.241$ in August, $n = 5$), that is, there was no difference in microbial cytoplasm C between N-treated plots and control plots in either June or August. In addition, no difference in microbial community structure was detected ($P = 0.421$, Fig. S3). This enabled us to study the effects of N availability *per se* on N immobilization by ectomycorrhizal fungi and other soil microbes. Fine roots (< 1 mm) of control trees and fertilized trees sampled 1 and 2 wk following labelling exhibited similar chitin concentrations (10.7 ± 0.5 mg g^{-1} DW, average ± SE, $n = 12$ and 11.1 ± 0.1 mg g^{-1} DW, average ± SE, $n = 14$, respectively).

The amount of ^{13}C derived from the allocation of recently fixed photosynthates and found in microbial cytoplasm was significantly and positively correlated with the ^{15}N content of microbial cytoplasm in control plots (Fig. 2), but no such correlation was found for N-amended plots. Thus, microbial N immobilization was significantly linked to the amount of C acquired

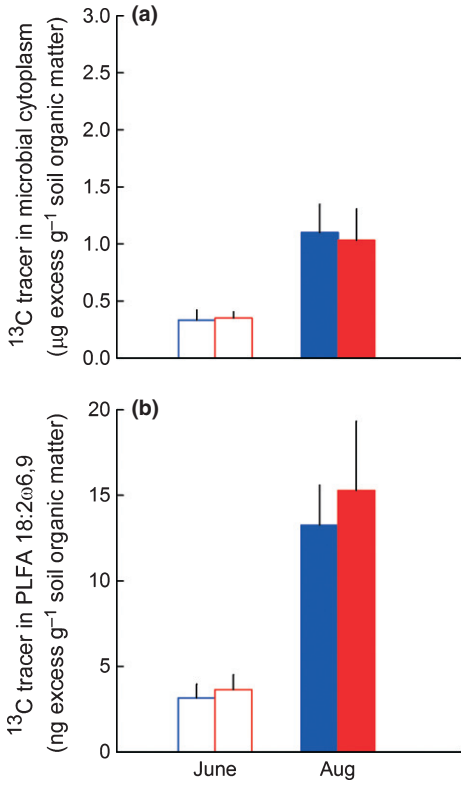

Fig. 1 Excess ^{13}C (μg g^{-1} soil organic matter) in the cytoplasm of soil microbes (a) and excess ^{13}C (ng g^{-1} soil organic matter) in the fungal biomarker phospholipid fatty acid 18:2ω6,9 (b). Bars show means ± 1SE ($N = 5$, $n = 2$ plots) of the temporal average of ^{15}N and ^{13}C enrichment for N-fertilized (red bars) and control (blue bars) plots. The N fertilizer was applied 14 d before tracer injection, which was performed in early (12–20 June) and late (22–24 August) season.

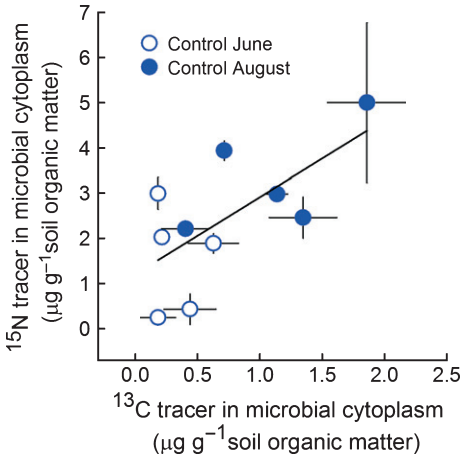

Fig. 2 Relationship between excess ^{15}N and excess ^{13}C in the cytoplasm of soil microbes (μg g^{-1} soil organic matter) in control plots ($R^2_{adj} = 0.38$, $P = 0.034$, $n = 10$) in June (open circles) and August (closed circles). Samples were collected up to 4 wk after labelling of tree canopies (^{13}C) and soil (^{15}N). Data are means ± 1SE ($n = 2$ plots).

by soil organisms. Apparently, N fertilization decreased significantly the capacity of soil microbes, including ectomycorrhizal fungi, to incorporate the soil-injected labelled N (Figs 3, 4).

The temporal dynamics of ^{15}N in the cytoplasm of soil microbes showed a strong increase and peaked 1 and 2 wk following tracer application in June and August, respectively (Fig. 4a,c). ^{15}N of tree foliage (current year needles) showed a very moderate increase for nonfertilized plots, but a steep increase in fertilized plots, especially in August (Fig. 4b,d). These temporal patterns reveal how added tracer N is efficiently trapped by soil microbes under low N and, in particular, when belowground C allocation is high (in August; Fig. 1a). Under high N, however, soil microbes are less efficient at immobilizing tracer N, resulting in higher rates of transfer of ^{15}N to tree foliage (Fig. 3b). To further investigate the interaction between tracer N in microbes and in tree foliage, we calculated the amounts of ^{15}N in microbial cytoplasm and tree foliage per unit area, and found a significant negative correlation between the two (Fig. 5).

Discussion

We asked whether, in N-limited boreal forests, the C flow from tree canopies to ectomycorrhizal fungi promotes or constrains the transfer of N from the soil to tree canopies. Although it is well known that ectomycorrhizal fungi can mobilize more stable soil organic N (cf. Smith & Read, 2008) and thereby promote N cycling in ecosystems, our data suggest that the ectomycorrhizal fungi of this boreal pine forest used a major fraction of absorbed ^{15}N for their own growth and transferred only minute amounts of ^{15}N to their hosts under conditions of high C and low N availability, but this transfer was increased following N amendment. Such a critical role of ectomycorrhizal symbiosis in N immobilization is further supported by the significant decrease in ^{15}N abundance in ectomycorrhizal root tips, which, to a significant degree consists of fungal N (Finlay & Söderström, 1992), in response to N fertilization (Fig. 3). Thus, the lower N tracer levels in root tips of N-amended plots in August, but not in June, support our hypothesis of efficient N immobilization in ectomycorrhizal fungi at high C but low N supply. We observed these patterns in both early (Fig. 4a,b) and late (August; Fig. 4c,d) season, but more so in August. The larger difference in N tracer of root tips and of microbial cytoplasm between N treatments in August coincides with the time for high C allocation to roots and ectomycorrhizas (Högberg *et al.*, 2010), suggesting that high rates of C allocation to mycorrhizal fungi lead to N immobilization and not to high rates of N transfer to hosts. This interpretation of our results is in line with those of Alberton *et al.* (2007), demonstrating progressive N limitation as a consequence of N immobilization by ectomycorrhizal fungi under elevated atmospheric CO_2 conditions. Here, ^{15}N labelling of soil organisms and trees was studied during 4 wk after labelling, and it could be argued that the rate of transfer through ectomycorrhizal fungal mycelia to the trees is very slow, but would ultimately benefit the trees. Plots labelled in June had a weak tendency for a gradual increase in needle ^{15}N over the time of sampling (Fig. 4b), potentially corroborating this view. However, for plots labelled in August, we observed higher ^{15}N tracer levels in the ectomycorrhizal root tips in nonfertilized control plots relative to N-fertilized plots, even 1 yr after labelling (Fig. 3). Moreover, longer term studies in similar boreal N-limited forests also speak strongly against the

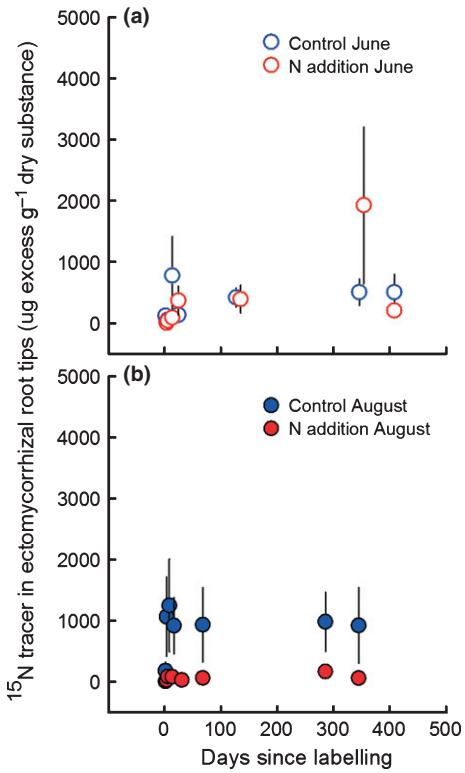

Fig. 3 ^{15}N of ectomycorrhizal root tips of *Pinus sylvestris* trees from N-fertilized plots (red symbols) and control plots (blue symbols). Ectomycorrhizal root tips were sampled at 2 d to 1 yr after ^{15}N labelling. Data are means ± 1SE ($n = 2$ plots) of ^{15}N enrichment of *P. sylvestris* root tips (μg ^{15}N excess per gram dry weight). Paired *t*-test showed a significant difference between control plots and N-amended plots in August ($P = 0.001$, $N = 8$ samplings) but not in June.

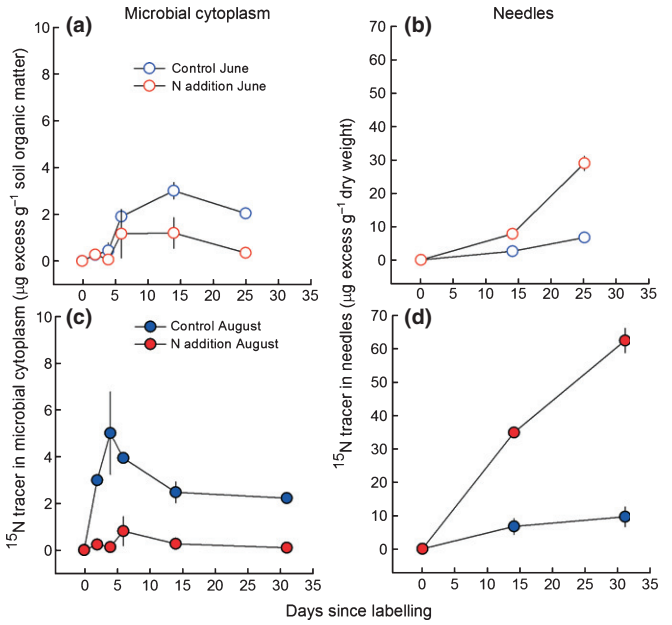

Fig. 4 ^{15}N of the cytoplasm of soil microorganisms (μg ^{15}N excess g^{-1} soil organic matter) (a, c) and of *Pinus sylvestris* foliage (μg ^{15}N excess g^{-1} dry weight needles) (b, d). Samples of soil and tree foliage were taken from two replicate plots per treatment over 4 wk after injecting the ^{15}N label in plots fertilized with N (red symbols and lines) or in control plots (blue symbols and lines). N treatments were applied 14 d before tracer injection and the experiment was performed in both June (a, b) and August (c, d). Data are means ± 1SE ($n = 2$).

argument of slow transfer, because the ^{15}N tracer remains far higher in the soil than in the trees even a long time after labelling (Melin *et al.*, 1983).

We propose that, in the specific system of our study, the PLFA 18:2ω6,9 would, to a significant degree, represent ectomycorrhizal fungi, because we found a significant relationship between the ^{13}C labelling of this compound and the ^{13}C labelling of ectomycorrhizal fungal sporocarps (regression analysis, $R^2_{adj} = 0.89$, $P = 0.038$, $n = 4$). Strong correlations ($R^2_{adj} = 0.7$–0.9) between this PLFA and DNA sequences of ectomycorrhizal fungi also support its use as a biomarker for these fungi in other boreal *P. sylvestris* forests in this area (Yarwood *et al.*, 2009; Högberg *et al.*, 2011). The fact that only four of the 35 PLFAs analysed became labelled, and that 18:2ω6,9 was the most highly ^{13}C-labelled biomarker in this experiment (Högberg *et al.*, 2010), provide further support for its usefulness as an indicator of ectomycorrhizal fungi in this particular forest type. This biomarker is also present in saprotrophic fungi (Frostegård & Bååth, 1996). However, ectomycorrhizal fungal mantles cover the finest tree roots and, especially, the root tips, and are thus the first soil microorganisms to receive tree photosynthate allocated below ground, whereas saprotrophic fungi live on C substrates of several years of age in this type of boreal pine forest (Lindahl *et al.*, 2007), and are hence not expected to become significantly ^{13}C labelled through recent photosynthate. Furthermore, we injected the ^{15}N tracer into the so-called F and H layers of the mor layer, in which ectomycorrhizal fungi dominate, and in which most N uptake is assumed to occur (Lindahl *et al.*, 2007); saprotrophic fungi predominate higher up in the mor layer.

We are aware of the problems inherent in the interpretation of results from field studies, where levels of control of various parameters cannot be compared with those of studies in glasshouse or climate chambers. The results of the latter cannot, however, safely be extrapolated to explain phenomena in the field. Our interpretation of the results as showing that ectomycorrhizal fungi are active in immobilizing available N is not the only possible explanation; at least two additional hypotheses to interpret the observed patterns in ^{15}N are possible. First, added ^{15}N may have been diluted to a greater extent in fertilized than in nonfertilized plots, hence giving rise to a lower abundance of ^{15}N in microbes of the fertilized plots. However, if this was the case, we would expect to see lower ^{15}N abundance in all sampled compartments. This was not the case, as we recorded a strong increase in ^{15}N in tree foliage of fertilized plots (Fig. 4). Moreover, if differential dilution in fertilized and nonfertilized plots had a major impact on the results, we would expect this impact to be equally large in June and in August, which was not the case (Fig. 4). Second, saprophytic fungi are active in N immobilization in forest soils and may hence restrict N acquisition by trees (Koide & Kabir, 2001) and cause the low ^{15}N transfer to trees in N-limited plots. However, these fungi preferentially inhabit the uppermost layer of the mor of boreal forest, whereas we injected ^{15}N below this level,

in the layer in which ectomycorrhizal fungi dominate and where tree N uptake occurs (Lindahl *et al.*, 2007). In addition, the correlation between ^{13}C and ^{15}N in microbial cytoplasm in control plots (Fig. 2) suggests that the mycorrhizal fungi that have direct access to the tree-derived ^{13}C in our experiment are responsible for the uptake of ^{15}N, rather than the saprophytic fungi.

Studies over more than a century have provided numerous examples of the benefits plants can receive from mutualistic relationships with mycorrhizal fungi (Smith & Read, 2008). The transfer of N from the soil by fungal hyphae to host plants is well documented, as is the reverse flow of photosynthetically fixed C to mycorrhizal fungi (Smith & Read, 2008). More generally, the superior capacity of mycorrhizal fungi to acquire nutrients from the soil has been thought to confer the greatest benefits to their host plants under nutrient-limiting conditions (Read & Perez-Moreno, 2003). However, mycorrhizal interactions represent a continuum from mutualism to parasitism (Leake, 1994; Johnson *et al.*, 1997; Jones & Smith, 2004; Corrêa *et al.*, 2008; Plett & Martin, 2011), and our results suggest that boreal mycorrhizal fungi immobilize a significant fraction of added tracer N, indicating that they may transfer little N absorbed from the soil under conditions of low N, but high C, availability (Fig. 4d).

Using a mathematical model (equations in Materials and Methods - Mathematical modelling of mycorrhizal N–C exchange), we sought the most parsimonious mechanisms explaining the observed dynamics of N and C exchange between C-limited mycorrhizal fungi and N-limited host trees. Previously, it has been assumed that mycorrhizal fungi transfer N to the plant in exchange for C at a fixed exchange rate favourable for the plant (Cowden & Peterson, 2009). However, supported by experimental evidence (e.g. Alberton & Kuyper, 2009), we suggest that, under N-limiting conditions, mycorrhizal fungi down-regulate their exchange rate (N exported per C received) to cover their own demand for growth. The consequence of this interaction is that mycorrhizal fungi transfer to trees only N in surplus to their own demand. The transfer of more N would make the fungus progressively more N limited and unable to use any further gains in plant C allocation, whilst transferring less N would give it less C in return. Although this may be an unconventional assumption for mycorrhizal fungi, in terms of N release it corresponds to how other soil fungi (and bacteria) act as regulators of nutrient cycling, that is, nutrients in excess of the demand (nonlimiting nutrients) are released (mineralized) to the environment (Jennings, 1995). In response to a decline in soil N availability, this fungal behaviour, in combination with the expected plant behaviour, may result in a feedback loop reducing plant N uptake. Although increased C export reduces the fungal N–C exchange rate, the plant, whilst aiming to maximize its N acquisition, increases its C export in proportion to the declining fungal N–C exchange rates (Alberton & Kuyper, 2009; Corrêa *et al.*, 2012), which further reduces the fungal N–C exchange rate.

In accordance with our observations (Figs 1–5), this model suggests that the proportion of N absorbed by fungi that is exported to trees is correlated with soil N availability (Model testing in Supporting Information, Notes S1). Moreover, we tested the implications of increased C allocation from trees to mycorrhizal fungi on N export from fungi to the host trees using our model (Modelled effects of elevated $[CO_2]$ in Notes S1). Increases in $[CO_2]$ have been linked to increases in plant C allocation to mycorrhizal fungi (Alberton *et al.*, 2007; Cairney, 2012). In line with the results of glasshouse studies (e.g. Treseder, 2004; Alberton *et al.*, 2007), the model suggests that increased C fluxes to mycorrhizal fungi can increase N uptake and transfer to trees (Fig. 6a), but reduces the N per C transfer efficiency (Fig. 6b). The C-fuelled increase in fungal growth and N immobilization may reduce soil N availability, which further increases C allocation to roots and associated mycorrhizal fungi, inducing a feedback mechanism suppressing soil N availability (Fig. 6c).

Corrêa *et al.* (2012) suggested that plants control the resource allocation to fungi through a variable production of surplus C. Here, we propose the hypothesis that, in N-poor ecosystems, such as the boreal forest, allocation of C from trees to mycorrhizal fungi results in a gradual decrease in N allocation from fungi to trees. This negative interaction (more C to fungi leading to less N for trees, less N for trees leading to more C to fungi) drives N-poor systems towards stronger N limitation.

A positive feedback between reduced soil N availability and increased mycorrhizal N immobilization through increased belowground C allocation may exacerbate differences in plant N limitation between N-rich and N-poor soils (Högberg *et al.*, 2006). Such a feedback mechanism could largely account for the strong N limitation in N-poor ecosystems with ectomycorrhizal trees (Fig. 6c). The mechanism suggests that mycorrhizal symbiosis is reinforced at the ecosystem level by creating an ecosystem from which plants, of the same or other species, needing more N

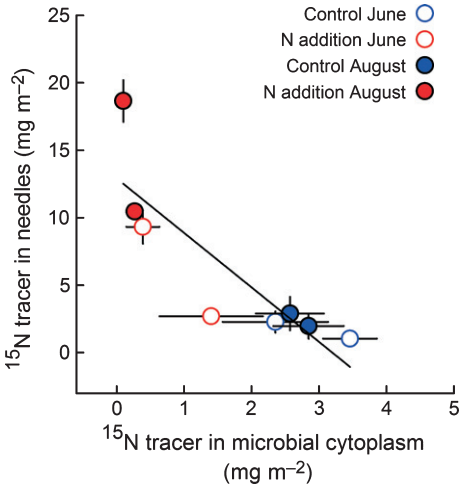

Fig. 5 The effect of N additions on the relationship between tracer ^{15}N in needles and microbial cytoplasm. The figure shows the measured (symbols) and modelled (line) relationship between ^{15}N in needles and in microbial cytoplasm. The symbols and error bars show means ± 1SD ($n = 2$ plots) among plots for control plots (blue symbols) and N-treated plots (red symbols) measured in June (open symbols) and August (closed symbols), 2 and 4 wk after ^{15}N labelling. The model (Eqns S1 and S2, Notes S1) was fitted to the data, yielding $R^2 = 0.73$ and a significantly negative slope (linear regression, $P = 0.002$). ^{15}N in needles was significantly higher ($P = 0.000$) and ^{15}N in microbial cytoplasm was significantly lower ($P = 0.0067$) in N-treated plots than in control plots (factorial ANOVA).

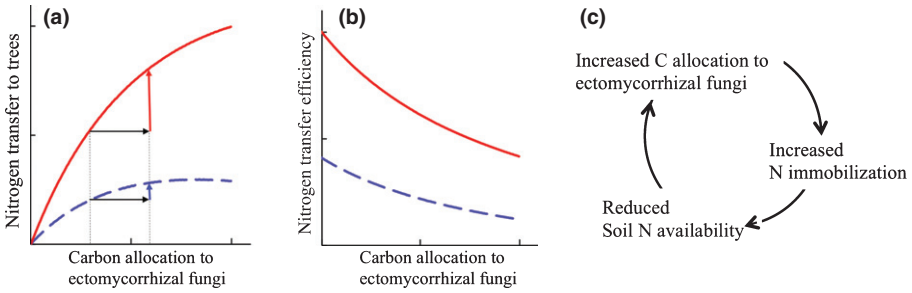

Fig. 6 Model output showing how increasing atmospheric [CO_2] increases C allocation to mycorrhiza (horizontal arrows), which increases N transfer to trees (vertical arrows) more strongly at high soil N availability (solid red lines) than at low soil N availability (dashed blue lines) (a). N transfer to host trees per C allocated to mycorrhizal fungi (transfer efficiency) increases with soil N availability (red vs blue line in Fig. 4b,d), but decreases with increasing C allocation to mycorrhizal fungi (b). Increasing C allocation to mycorrhizal fungi increases N immobilization, which reduces soil N availability for plants, which further increases C allocation to mycorrhizal fungi by the host trees, that is, a positive feedback loop (c). The results in (a) and (b) (relative values) are derived by modelling (see Materials and Methods section). High and low soil N availabilities differ by a factor of 2.

to close their life cycles (Chapin, 1980), are effectively excluded. The dominance of the ectomycorrhizal symbionts in boreal forest ecosystems diminishes when disturbances, such as wildfire or forest clear felling, weaken or break the link between the symbionts. This allows the temporary development of a richer flora of higher plants with more N-demanding life cycles (Bartels & Chen, 2010), a change that can also be driven by N additions. However, as shown in long-term studies in boreal *P. sylvestris* forests, the original N-limited ecosystem will gradually re-establish if N additions cease, allowing the role of ectomycorrhizal symbiosis to be restored (Högberg *et al.*, 2011). Moreover, experiments with elevated [CO_2] commonly show that plant belowground C allocation to mycorrhizal fungi increases (Treseder, 2004; Alberton *et al.*, 2007), and several studies have also reported aggravated N limitation of trees after extended exposure to elevated [CO_2], also known as progressive N limitation (PNL, Luo *et al.*, 2004). The mechanism suggested here should further contribute to PNL caused by elevated [CO_2].

The competition for N between soil microorganisms and plants has long been a major concern of agronomists and, more recently, ecologists (Kaye & Hart, 1997; Hodge *et al.*, 2000). However, it has been theoretically and methodologically difficult to identify the role of mycorrhizal fungi in this context. Our double (^{13}C,^{15}N) labelling approach raises the question of whether the large investment of plant C in fungal symbionts under N-limiting conditions may contribute to immobilization of soil N by the mycorrhizal fungi, rather than the expected mobilization. Clearly, a positive role for ectomycorrhizal symbiosis in tree N uptake in boreal forests has been taken for granted, to the extent that it has not previously been critically tested under relevant field conditions. We hypothesize that the large C investments of trees in the ectomycorrhizal symbiosis results in a strong N limitation of the ecosystem. This N limitation is a crucial condition for coniferous trees to be competitive against more fast-growing, but also more N-demanding, species. Thus, according to this hypothesis, the dominance and resilience of the extensive boreal forests may rest on a strong N limitation driven by the vast C investments of the trees in the ectomycorrhizal symbiosis. Evidently, additional field studies targeting the interdependence of C and N fluxes between trees and their symbiotic fungi are needed if we are to fully appreciate the physiology and ecology of the ectomycorrhizal symbiosis, and to better understand their ramifications on the biogeochemistry of forest ecosystems.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Map of experimental plot layout.

Fig. S2 Photograph showing $^{13}CO_2$ labelling of plots C1 and N1 in June 2007.

Fig. S3 Ordination of data on microbial community structure and ^{13}C enrichment based on data of 35 phospholipid fatty acids (PLFAs).

Table S1 Experimental treatments

Notes S1 Model testing.

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