Global Change Biology (2007) **13,** 78–88, doi: 10.1111/j.1365-2486.2006.01279.x

Mycorrhizal responses to nitrogen fertilization in boreal ecosystems: potential consequences for soil carbon storage

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Abstract

Mycorrhizal fungi can contribute to soil carbon sequestration by immobilizing carbon in living fungal tissues and by producing recalcitrant compounds that remain in the soil following fungal senescence. We hypothesized that nitrogen (N) fertilization would decrease these carbon stocks, because plants should reduce investment of carbon in mycorrhizal fungi when N availability is high. We measured the abundance of two major groups of mycorrhizal fungi, arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) fungi, in the top 10 cm of soil in control and N-fertilized plots within three Alaskan boreal ecosystems that represented different recovery stages following severe fire. Pools of mycorrhizal carbon included root-associated AM and ECM structures; soil-associated AM hyphae; and glomalin, a glycoprotein produced by AM fungi. Total mycorrhizal carbon pools decreased by approximately 50 g C m^{-2} in the youngest site under N fertilization, and this reduction was driven mostly by glomalin. Total mycorrhizal carbon did not change significantly in the other sites. Root-associated AM structures were more abundant under N fertilization across all sites, and root-associated ECM structures increased marginally significantly. We found no significant N effects on AM hyphae. Carbon sequestered within living mycorrhizal structures (0.051–0.21 g m^{-2}) was modest compared with that of glomalin (33–203 g m^{-2}). We conclude that our hypothesis was only supported in relation to glomalin stocks within one of the three study sites. As N effects on glomalin were inconsistent among sites, an understanding of the mechanisms underlying this variation would improve our ability to predict ecosystem feedbacks to global change.

Keywords: global change, glomalin, hyphae, minirhizotron images, root length density, soil carbon sequestration

Received 25 April 2006 and accepted 23 July 2006

Introduction

In most ecosystems, N limits aboveground plant growth (Liebig, 1843; Vitousek & Howarth, 1991). N effects on the microbial community, however, are unclear (Fog, 1988), even though this issue is important in predicting responses of ecosystems to environmental changes. For example, field experiments have demonstrated that rates of N mineralization increase 46%, on average, when temperatures are raised 0.3–6 °C to simulate global warming (Rustad *et al*., 2001). If decomposers are limited by N, then this increase in N availability may stimulate the conversion of soil organic matter to CO_2 (Chapin, 1983; Mack *et al*., 2004).

Not all microbes consume soil organic matter, though. For example, arbuscular mycorrhizal (AM) fungi receive their carbon from host plants and do not break down soil organic matter. In addition, they produce glomalin, a glycoprotein that can remain in the soil for years to decades (Rillig *et al*., 2001). These long residence times allow glomalin to accumulate in the soil, where it can represent up to 3–8% of soil organic carbon (Rillig *et al*., 2001, 2003; Lovelock *et al*., 2004).

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AM fungi acquire inorganic nutrients from soils and provide a portion to their host plants in exchange for carbon (Smith & Read, 1997). Economic theory predicts that host plants should allocate less carbon to AM fungi as soil nutrients become less limiting (Bloom *et al*., 1985; Read, 1991b). As glomalin concentrations in the soil can be correlated with AM abundance (Treseder *et al*., 2004), it follows that an increase in N availability might reduce carbon storage in glomalin. Alternately, if AM fungi are directly limited by N, then AM growth and glomalin production could increase as N availability rises (Treseder & Allen, 2002).

Ectomycorrhizal (ECM) fungi also likely contribute to carbon storage in the soil. This group uses host plants, not soil organic matter, for 90% or more of their carbon intake (Abuzinadah & Read, 1989; Finlay *et al*., 1996; Treseder *et al*., 2006). Although ECM fungi release extracellular enzymes that target organic N compounds in the soil (Dighton, 1991; Read, 1991a), this activity contributes to mineralization of N, not necessarily the conversion of organic carbon to CO_2. ECM biomass can comprise a substantial carbon pool – up to 15% of soil organic matter in some ecosystems (Vogt *et al*., 1982). In addition, ECM fungi can competitively inhibit growth of other decomposer microbes (Gadgil & Gadgil, 1971; Leake *et al*., 2002). Each of these factors could contribute to carbon sequestration (Treseder & Allen, 2000). As with AM fungi, N may reduce ECM growth as plants shift allocation patterns (Treseder, 2004). As a result, N fertilization could reduce carbon stocks in ECM and AM pools alike.

Tests of N effects on soil microbes are rare in boreal ecosystems, even though these areas harbor large stocks of organic carbon (Batjes, 1996; Amundson, 2001) that could be converted to atmospheric CO_2 by decomposers. Global warming is also occurring faster in northern ecosystems than elsewhere; summer air temperatures have increased 0.5–2.0 °C over the past four decades in this region (Chapin *et al*., 2005). Microbes could play a key role in determining whether these ecosystems form a positive feedback on global warming. To better understand responses of boreal ecosystems to N fertilization, we documented changes in fungal hyphal lengths, mycorrhizal abundance, and glomalin. We focused on fungi, because they dominate microbial communities in northern temperate forests (Heal & Dighton, 1986; Dighton, 2003). We hypothesized that the abundance of AM and ECM fungi, as well as glomalin, should decline under N additions, because plants should allocate less carbon to their mycorrhizal fungi when N is more readily available (Lilleskov *et al*., 2001; Nilsson & Wallander, 2003).

Materials and methods

Sites

Our study sites consisted of three ecosystems that represented different stages of recovery following severe forest fire. Fire is a common form of disturbance in boreal forests (Kasischke & Stocks, 2000), and a chronosequence was used to represent the range of ecosystem types present in fire-prone areas of the boreal region. The sites are described in detail by Treseder *et al*. (2004). Briefly, the sites were located in upland boreal forests within a 100 km^2 area (63°55′N, 145°44′W) near Delta Junction, Alaska. One site had burned in 1999, and another in 1987. A third site contained a mature forest of *Picea mariana* (black spruce), and was approximately 80 years old. We will refer to these sites as the 'youngest,' 'middle-aged,' and 'oldest' sites, respectively. The two youngest sites contain herbaceous perennials, deciduous trees and shrubs, and evergreen shrubs. The youngest site harbors a relatively equitable distribution of these plant groups; the middle-aged site is dominated by *Populus tremuloides* and *Salix* spp. The youngest site is occupied predominantly by AM host plants and the oldest by ECM host plants (Treseder *et al*., 2004). The middle-aged site is co-dominated by both AM and ECM plants. The soils are gelisols. Permafrost is discontinuous in this area and is not present in any of the sites. N mineralization rates are highest in the middle-aged sites, and soil organic matter accumulates with site age (Treseder *et al*., 2004). The local climate is cold and dry, with a mean annual temperature of −2 °C and a precipitation rate of 303 mm yr^{-1} (http://weather.noaa.gov/).

N fertilizer was applied as ammonium nitrate at a rate of 200 kg N ha^{-1} yr^{-1} in one dose starting June 2002, and continued at an annual rate of 100 kg N ha^{-1} yr^{-1}. We established four blocks in each site; each block contained an N-fertilized and control plot. Treatments were assigned randomly within each block. Plots were 10×10 m^2 and were separated from one another by at least 10 m.

Sample collection

Sampling occurred during the growing season, which is May through September. We collected minirhizotron images during 2003 and 2004 and soil samples in 2004. These images and samples were used to estimate the abundance of fungal hyphae, mycorrhizal structures, and glomalin.

AM, ECM, and free-living nonmycorrhizal fungi grow hyphae through the soil, and we used the standing lengths of these hyphae as one proxy for fungal biomass. We employed a minirhizotron microscope to

view intact fungal hyphae within the soil. Because image collection is nondestructive, we were able to conduct several repeat samplings in the same locations. One limitation of the minirhizotron approach, however, was the inability to discern among nonmycorrhizal, AM, and ECM hyphae in the images. AM hyphae are morphologically distinct from nonmycorrhizal and ECM hyphae (Bonfante-Fasolo, 1986), but phase-contrast microscopy is required to identify these features. Minirhizotron microscopes do not possess this capability. As such, we considered hyphal lengths obtained by minirhizotron analysis to represent the fungal community as a whole.

Soil cores were collected to measure glomalin concentrations and the standing length of AM hyphae in the soil. By extracting fungal hyphae from the soil and examining them with phase contrast microscopy in the laboratory we could identify and quantify AM hyphal lengths. Nonmycorrhizal hyphae could not be distinguished from ECM hyphae by this method, as these two groups are interrelated and possess similar morphologies. Thus, we did not specifically report ECM hyphal lengths.

Mycorrhizal fungi also form structures within and around fine roots of host plants. Specifically, AM fungi produce arbuscules, vesicles, and intraradical hyphae within root cells. ECM fungi form a fungal sheath around root tips. We assessed the abundance of these structures by extracting roots from the soil cores. Specifically, we calculated the percent root length colonized (%RLC) by AM and ECM fungi. Higher values of %RLC indicate a greater proportion of resources allocated by plants to their mycorrhizal symbionts (Allen, 2001; Treseder & Cross, 2006). Furthermore, to obtain an index of the abundance of root-associated AM and ECM structures, we calculated the total standing root length colonized by AM and ECM fungi. To do this, we took the product of %RLC and the standing length of fine roots, which was derived from the minirhizotron images. We will refer to this index as 'AM (or ECM) root length.'

Soil cores were collected in May, July, and August 2004. We used a 10 cm diameter corer to sample soils to 10 cm depth. Within each plot, cores were obtained from two random locations and then compiled. Soils were frozen at $-20\,^{\circ}C$ within 2 h of sampling, and were stored at this temperature until analyses could be conducted at the University of California, Irvine.

Fungal hyphae and root lengths

In June 2002, we used a custom-fit borer (BTC Reamer, Bartz Technology, Santa Barbara, CA, USA) to install three minirhizotron observation tubes at random locations in each plot. Tubes were 5 cm diameter and 1 m long, and were composed of cellulose acetate butyrate (Bartz Technology). They were installed at an average angle of 29°. We inserted each tube as far as possible into the soil, although layers of rocks and gravel often limited insertion depth. The average vertical depth reached by the tubes was 13 cm. Tubes were capped on both ends, and any length of tube exposed above the soil was covered in duct tape to prevent light penetration. We allowed 1 year for roots to recolonize the soil surrounding the tubes before sampling. Images were collected in May, June, July, and September 2003; and June and August 2004.

We collected images of fungal hyphae and roots by using a minirhizotron microscope (BTC-10 with I-CAP software, Bartz Technology). The minirhizotron microscope magnifies images up to $\times 100$. At highest magnification, individual fungal hyphae could be seen. At lowest magnification ($\times 3$) and above, fine roots were visible. We collected one set of images at $\times 100$ magnification for assessments of fungal hyphal length, and another at $\times 3$ magnification for measurements of fine root length. The soil area represented in each image was $1.9 \times 1.3\,cm^2$ at low magnification and $0.27 \times 0.20\,cm^2$ at high magnification. To collect the images, we inserted the microscope into the tube until it reached the bottom, and then moved the lens upward in increments of 1.3 cm. One vertical column of images was collected per tube. A guide hole at the top of each tube ensured that we returned to the same location within the tube at each subsequent sampling date. On average, 21 images were obtained per tube and stored as JPEG files.

We used WinRHIZO Tron MF software (Regen Instruments Inc., Quebec, Canada) to manually calculate the standing length of all fungal hyphae or roots present in at least one randomly selected tube per plot per sampling date. In addition, we counted the number of fungal hyphae and roots in each tube. We found that the length of fungal hyphae (or roots) in each tube could be predicted based on the number of fungal hyphae (or roots), according to the linear regression model $y = bx$, where y is length in centimeters, b is a constant, and x is the number of hyphae or roots. Separate linear regression models were developed for fungal hyphae and roots. In each case, the regression was derived from all dates and all tubes in which fungal hyphal or root length was measured directly. For fungal hyphae, $b = 0.039$ ($r^2 = 0.90$, $P < 0.001$); for roots, $b = 0.47$ ($r^2 = 0.92$, $P < 0.001$). The y-intercept was fixed at 0. We used these linear regression models to estimate fungal hyphal and root length in the remaining tubes, by counting numbers of fungal hyphae and roots. This approach was suggested by Crocker *et al*. (2003) as a means of facilitating analyses of minirhizotron images,

as manual measurements of lengths can be time consuming. Fungal hyphal and root lengths were reported per unit area of minirhizotron tube (e.g. cm hyphae cm^{-2} tube).

AM hyphal length

We used a modified procedure from the work of Sylvia (1992) to determine lengths of AM hyphae in the soil for May, July, and August 2004. For each sample, 200 mL sodium metaphosphate solution (39.5 g L^{-1}) was added to 10 g of air-dried soil. The mixture was shaken for 1 min and sonicated for 20 s. After being allowed to settle for 1 h, the mixture was passed through a 425 μm sieve. Any material captured by the sieve was discarded. We diluted the remaining solution with 400 mL deionized water, and then agitated the solution for 1 min. Hyphae were collected on a 45 μm sieve. We transferred the hyphae to a graduated cylinder by rinsing the sieve with deionized water. The volume of the hyphae and water was brought to 50 mL. The solution was poured into a beaker and stirred continuously to suspend the hyphae. A 10 mL subsample of the solution was collected in a syringe, and then pushed through a 0.22 μm polycarbonate filter. We used polyvinyl-lacto-glycerol (PVLG) mounting medium to mount the filter on a glass slide (Koske & Tessier, 1983), which was then dried overnight at 60 °C.

We examined the filter for the presence of hyphae by using a phase contrast microscope (Nikon Eclipse E400, A. G. Heinze, Lake Forest, CA, USA) at ×200 magnification. Hyphal lengths were estimated with the point-intersect method (Paul & Clark, 1996); 100 points were scanned per sample. Hyphae from AM fungi could be distinguished from those of non-AM fungi based on morphological features. Specifically, AM hyphae have irregular walls, lack septa, and tend to branch angularly (Bonfante-Fasolo, 1986).

AM colonization of roots

We stained roots with Trypan Blue to assess AM colonization (Koske & Gemma, 1989) in the July and August 2004 samples. We restricted our analyses to fine (<2 mm diameter) roots. Roots were isolated by hand sieving and were cut into 1 cm lengths. Approximately five root pieces were then selected at random. Roots were incubated in 2.5% potassium hydroxide for 20 min at 90 °C, rinsed with deionized water three times, soaked for 20 min in 0.525% sodium hypochlorite, and then rinsed three times. Roots were acidified in 1% hydrochloric acid overnight, and then heated at 90 °C for 20 min in acidic glycerol mixed with 0.05% Trypan blue. The samples were destained overnight in acidic glycerol (50% glycerol, 5% hydrochloric acid, 45% deionized water), and then mounted with PVLG medium onto glass slides. To quantify %RLC, we used the magnified intersections method (McGonigle *et al.*, 1990) and a Nikon phase-contrast microscope to quantify the presence of arbuscules, vesicles, and intraradical hyphae. One hundred intersections or more were assessed for each sample.

To calculate AM root length, we took the product of %RLC by AM fungi and standing fine root length. For both variables, we used average values for all 2004 sampling times. We estimated AM root length on a ground area basis (e.g. km m^{-2}) for the top 10 cm of soil. To convert our estimates of fine root length from a tube area basis (see 'Minirhizotron images') to a ground area basis, we assumed that the viewing depth of our minirhizotron microscope was 2 mm (Taylor *et al.*, 1970; Merrill & Upchurch, 1994). We calculated root lengths for the top 10 cm of soil (vs. the entire tube).

ECM colonization of roots

We measured %RLC of ECM fungi on fine roots collected in July and August 2004. About 25 cm of fine roots were selected randomly, rinsed in deionized water three times, and then examined for ECM tissues by using a stereo microscope (Olympus SZ40, Olympus Inc., Melville, NY, USA) at ×40 magnification. We employed a point intersection method to estimate the percentage of root length covered by ECM sheaths (Brundett *et al.*, 1996). Hyphae of ECM fungi were not quantified, as they are morphologically indistinguishable from other hyphae. Fungal hyphal lengths from the minirhizotron images should represent an upper bound for ECM hyphal length. ECM root length was calculated as for AM root length.

Glomalin

An enzyme-linked immunosorbent assay (ELISA) was used to characterize concentrations of glomalin in our May 2004 samples (Wright & Upadhyaya, 1996; Wright, 2000). Glomalin was isolated from 1 g of soil by adding 8 mL of 50 mM sodium citrate (pH 8.0), and then autoclaving the mixture at 121 °C for 1 h. Samples were centrifuged for 15 min at 5000 ***g***. The supernatant was reserved and stored at 4 °C. This process was repeated until the supernatant became transparent; all supernatants were combined within each sample. Glomalin concentrations of the extracts were assayed with an ELISA procedure detailed by Wright (2000). Briefly, the extract was dried on the bottom of a microtiter plate

well and then incubated with a glomalin-specific monoclonal antibody (MAb32B11). We determined antibody concentrations by using a microplate reader equipped with a 405 nm filter (EL800, Bio-tek instruments, Winooski, VT, USA), and then comparing values with those of a glomalin standard derived from fresh AM hyphae. Our glomalin concentrations represent immunoreactive glomalin only, and they are equivalent to the 'IRSP' fraction named in Rosier *et al*. (2006). We used values for soil bulk density (72, 94, and 21 kg m^{-2}; King *et al*., 2002; Neff *et al*., 2005) to adjust results to a ground area basis (e.g. g glomalin m^{-2}) for the top 10 cm of soil.

Mycorrhizal carbon stocks

To assess the extent to which N could influence mycorrhizal carbon stocks, we estimated the amount of carbon sequestered within glomalin and within tissues of AM and ECM fungi. Glomalin carbon pools were calculated by assuming that glomalin contained 41% carbon by weight (Lovelock *et al*., 2004). To calculate carbon pools in other mycorrhizal structures, we calculated biovolumes of each structure type and then assumed a fresh tissue density of 1.1 g cm^{-3}, a solids content of 40%, and a carbon content of 40% (Paul & Clark, 1996). The biovolume of AM hyphae (V_h) was estimated by applying the standard equation for a cylinder: $V_h = \pi \cdot r_h^2 L_h$, where r_h is radius and L_h is standing hyphal length. The radius used for AM hyphae was 1.1 μm (Friese & Allen, 1991). The biovolume of AM structures within roots (V_{AM}) and of ECM structures surrounding roots (V_{ECM}) was approximated as $V_{AM/ECM} = \pi \cdot r_r^2 L_{AM/ECM} K_{AM/ECM}$, where r_r is fine root radius, $L_{AM/ECM}$, AM or ECM root length, and $K_{AM/ECM}$, the fraction of colonized root volume that is fungal (Toth *et al*., 1991). We assumed a value of 0.06 for K_{AM} (Toth *et al*., 1991) and 0.3 for K_{ECM} (Harley, 1971).

To determine the radius of fine roots in the study plots, we extracted all fine roots from soil cores collected September 2003. Two 10 cm diameter cores were collected and compiled from the top 10 cm of soil in each plot. Roots were extracted by hand sieving, were washed three times in deionized water, and were digitally scanned. The radius of each root was measured manually with WinRHIZO Tron MF software (Reagent Instruments Inc.). Values averaged 0.02 mm across plots. We calculated biovolume (and carbon pools) separately for each plot by using the average radius and root length colonized for each plot. To determine net changes in each mycorrhizal carbon pool, we calculated the difference in pool size between N-fertilized and control plots within each block.

Statistics

We used analyses of variance (ANOVAs) to test our hypotheses that N fertilization would reduce the prevalence of mycorrhizal fungi and glomalin (Sokal & Rohlf, 1995; SPSS 2002, Chicago, IL, USA). Specifically, fully factorial ANOVAs were conducted with site and N fertilization as independent variables. For measurements with more than one sampling time, a repeated measures model was applied. Root lengths and glomalin were square-root transformed to achieve normal distribution (Sokal & Rohlf, 1995). The remaining results could not be transformed to meet assumptions of ANOVA, so tests were performed on ranked data. To determine whether N fertilization altered net carbon storage within mycorrhizal pools, we conducted one-sample *t*-tests in which we compared net changes with a fixed mean of zero. A Pearson correlation was conducted between glomalin and AM hyphal lengths from the May 2004 sampling. For all statistical tests, the unit of replication was the plot. Differences were considered significant when $P \leq 0.05$.

Results

Fungal hyphal length

N did not significantly influence the standing length of hyphae visible in minirhizotron images (Fig. 1). These hyphae should include AM and ECM hyphae as well as nonmycorrhizal hyphae. Fungal hyphal lengths varied significantly by date, and did not mirror root dynamics (Fig. 2). Sites differed significantly in temporal patterns, especially during the first growing season. During that period, fungal hyphal lengths peaked earliest in the oldest site and latest in the youngest site.

Root length

Overall, N significantly increased standing root length almost twofold (control: 4.7 cm cm^{-2}, N: 8.8 cm cm^{-2}; Fig. 2). In addition, root lengths varied among sites depending on sampling date. Specifically, root lengths remained relatively constant over time in the oldest site. In the younger two sites, root lengths increased consistently over the first 3 months of sampling, and then tended to level off.

AM hyphal length

Standing hyphal lengths of AM fungi were not significantly altered by N fertilization (Table 1). Sites differed significantly from one another, however, in that AM hyphal lengths were lowest in the oldest site.

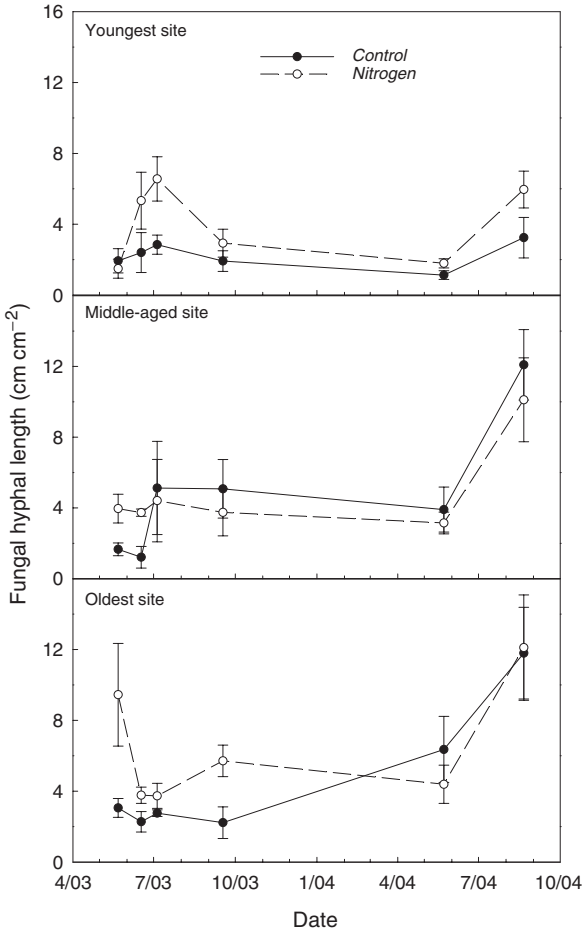

Fig. 1 Fungal hyphal lengths visible per unit area of minirhizotron tube. Tubes extended 13 cm deep into the soil, on average. Images were collected at ×100 magnification. Hyphae were not differentiated among nonmycorrhizal, AM, and ECM fungi, and thus represent the fungal community. Neither site, N fertilization, nor their interaction significantly influenced fungal hyphal length (Table 1). However, there were significant effects of sampling date ($P < 0.001$) and date × site interactions ($P = 0.002$). Symbols indicate means ± 1 SE for two to four plots. Measurements were repeated in the same plots for each sampling date.

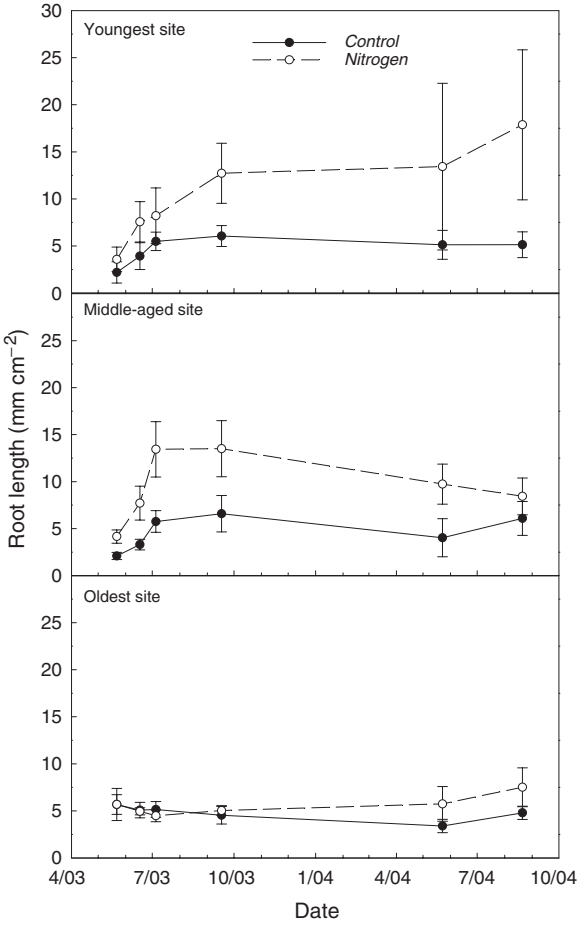

Fig. 2 Fine root lengths visible per unit area of minirhizotron tube. Images were collected at ×3 magnification from the top 13 cm of soil, on average. Nitrogen fertilization significantly increased standing root length ($P = 0.044$), but there were no significant differences among sites (Table 1). There was no significant site by N fertilization interaction. Effects of sampling date ($P < 0.001$) and date × site interactions ($P = 0.039$) were significant. Symbols represent means ± 1 SE for two to four plots. Measurements were repeated in the same plots for each sampling date.

AM colonization of roots

The %RLC by AM fungi increased significantly from an average of 18% in the control treatment to 30% in the N-fertilized treatment (Table 1). This value also differed significantly among sites and was lowest in the middle-aged site. N fertilization significantly augmented AM root length (Table 1), but there were no significant site effects or interactions between site and N.

ECM colonization of roots

We found no evidence of significant N effects on %RLC by ECM fungi (Table 1). Sites varied significantly in %RLC, though, with the youngest site having the lowest values. ECM root length increased marginally significantly under N fertilization (Table 1, $P = 0.054$), and differed significantly among sites, peaking in the intermediate-aged site. There was no significant interaction between site and N for ECM root length.

Glomalin

Glomalin concentrations (as IRSP) responded differently to N fertilization depending on site (Table 1). Specifically, glomalin tended to increase under N fertilization in the middle-aged site, decrease in the young-

Table 1 Indices of mycorrhizal abundance under N fertilization for the top 10 cm of soil, reported as means ± SE*

Mycorrhizal index	Youngest site		Middle-aged site		Oldest site		Significant effects (*P*-values)
	Control	N fertilized	Control	N fertilized	Control	N fertilized	
AM hyphal length (km m^{-2})							
May 2004	116 ± 66	84 ± 33	134 ± 53	207 ± 51	16 ± 4	39 ± 16	Site (0.001)
July 2004	36 ± 25	81 ± 27	59 ± 23	47 ± 19	8 ± 5	11 ± 0	Date (0.007)
August 2004	30 ± 10	17 ± 5	24 ± 12	37 ± 12	11 ± 3	13 ± 6	
AM colonization (% root length)							Site (0.036)
July 2004	27.9 ± 2.3	42.3 ± 7.0	6.6 ± 4.0	14.0 ± 3.3 (n = 3)	27.2 ± 10.7	33.7 ± 3.6	N fert (0.029)
August 2004	24.8 ± 1.7	27.0 ± 8.9	14.1 ± 3.2	29.4 ± 7.1	9.1 ± 4.5	30.2 ± 14.7 (n = 3)	Date × site (0.035)
AM root length (km m^{-2})	0.69 ± 0.19	2.19 ± 0.87	0.21 ± 0.06	1.17 ± 0.40	0.31 ± 0.09	1.24 ± 0.43	N fert (0.001)
ECM colonization (% root length)							Site (<0.001)
July 2004	1.7 ± 0.7	0.4 ± 0.4	19.1 ± 1.8	15.8 ± 2.8 (n = 3)	19.5 ± 7.1	17.8 ± 5.6	Date (0.023)
August 2004	15.4 ± 2.9	10.7 ± 1.3	25.8 ± 4.2	20.4 ± 3.5	13.4 ± 3.1	15.6 ± 1.8	Date × site (0.011)
ECM root length (km m^{-2})	0.20 ± 0.07	0.42 ± 0.22	0.51 ± 0.17	0.87 ± 0.24	0.30 ± 0.03	0.49 ± 0.12	Site (0.032)
IRSP glomalin concentration (g m^{-2})	248 ± 46	124 ± 42	310 ± 53	509 ± 120	83 ± 7	90 ± 34	Site (<0.001)
							Site × N (0.050)

*n = 4 unless otherwise noted.
AM, arbuscular mycorrhizae; ECM, ectomycorrhizae.

Fig. 3 Correlation between IRSP glomalin concentrations and AM hyphal lengths for May 2004. The correlation was significantly positive (r = 0.578, n = 47, P < 0.01). All plots within all sites are included, and each symbol represents one plot.

est site, and remain unchanged in the oldest site. Glomalin concentrations also varied significantly among sites, peaking in the intermediate site. AM hyphal lengths from May 2004 were significantly correlated with glomalin concentrations across sites (Fig. 3; df = 45, r = 0.578, P < 0.01), and also within the youngest site (df = 6, r = 0.720, P < 0.05).

Mycorrhizal carbon stocks

Total mycorrhizal carbon stocks decreased significantly under N fertilization in the youngest site, owing to a significant decrease in glomalin stocks there (Table 2). The remaining sites displayed no change. Glomalin carbon was considerably larger than the other carbon stocks, with average values of 34–149 g C m^{-2} (depending on the site) in control plots. Carbon stocks in ECM root tips were second largest (0.012–0.037 g C m^{-2}), followed by AM structures within roots (0.001–0.012 g C m^{-2}) and AM hyphae in soil (0.001–0.008 g C m^{-2}).

Discussion

We had hypothesized that the abundance of mycorrhizal fungi and glomalin should decrease under N fertilization in boreal ecosystems, if plants allocate fewer carbohydrates to mycorrhizal fungi upon alleviation of N-limitation. This hypothesis was only supported for glomalin concentrations, which decreased with N fertilization in the youngest site. Glomalin tended to increase in the middle-aged site, however, and did not change in the oldest site. Contrary to our hypothesis, living tissues of mycorrhizal fungi did not become less

Table 2 Changes in mycorrhizal carbon pools under N fertilization (N-fertilized minus control, in $g\,C\,m^{-2}$ for the top 10 cm of soil, reported as means ± SE)

Pool	Youngest site	Middle-aged site	Oldest site
AM structures within roots	+ 0.050 ± 0.049	+ 0.017 ± 0.009	+ 0.036 ± 0.015
AM hyphae in soil	+ 0.000 ± 0.007	+ 0.018 ± 0.008	+ 0.005 ± 0.003
ECM root tips	+ 0.047 ± 0.062	+ 0.052 ± 0.024	+ 0.050 ± 0.022
IRSP glomalin	−50 ± 5*	+ 79 ± 34	+ 3 ± 14
Total	−50 ± 5*	+ 79 ± 34	+ 3 ± 14

*Significant change ($P = 0.002$).
AM, arbuscular mycorrhizae; ECM, ectomycorrhizae.

prevalent under N fertilization. Instead, N additions led to an increase in AM structures within plant roots, as indicated by AM root length. We found no changes in AM hyphal length, %RLC by ECM fungi, and total fungal hyphal length. Moreover, there was only a marginally significant increase in ECM root length under N fertilization. Altogether, we documented little evidence that plants reduced investment in mycorrhizal fungi under higher levels of N availability in these sites.

The increase in AM root length under N fertilization was driven by an increase in %RLC coupled with an augmentation of standing fine root length. This rise in %RLC is not standard. In a meta-analysis of 31 field-based studies, Treseder (2004) found that AM abundance decreases 24%, on average, under N fertilization. Most of these studies had quantified %RLC as an index of abundance, but measurements of spore count and hyphal length were also included. Our results are not unprecedented, however. Studies vary significantly in N responses, as indicated by the meta-analysis, and AM abundance tended to increase in 30% of the studies. This study was one of the first to examine N effects on AM fungi in boreal ecosystems, so it is not clear if our findings were typical of this biome.

A number of possibilities might underlie the increase we observed in %RLC by AM fungi. AM fungi may have been N limited, and could have proliferated following N additions even if plants did not alter investments in AM fungi (Treseder & Allen, 2002). This response may occur if C : N ratios of fungal material shift under fertilization. Alternately, the availability of AM host plants could have influenced %RLC (Treseder & Cross, 2006). In our sites, grass and herbaceous species that hosted AM fungi seemed to constitute a larger portion of the plant community in the N-fertilized plots compared with controls (personal observation). Such a response might have elicited greater allocation of carbon to AM fungi at the community scale. In addition, AM fungi themselves may have altered their allocation patterns to favor intraradical structures instead of external hyphae. We found that AM hyphal lengths in the soil did not change significantly under N fertilization, while AM structures within plant roots (i.e. AM root length) became more abundant. AM fungi might have invested a greater proportion of their resources in intraradical structures like arbuscules, vesicles, and hyphal coils in order to improve uptake and storage of carbon from plants, especially if the fungi became carbon-limited following N fertilization (Johnson *et al*., 2003; Treseder, 2005). Another possibility includes a shift in the AM community toward *Glomus* species, which tend to produce more intraradical structures and less external hyphae than do other AM species (Klironomos *et al*., 1998; Dodd *et al*., 2000; van Aarle *et al*., 2002; Treseder, 2005). We did not assess the AM community structure in our sites, but proliferation of *Glomus* has often been observed in other N fertilization studies (Johnson, 1993; Klironomos *et al*., 1997; Eom *et al*., 1999; Egerton-Warburton & Allen, 2000; Treseder & Allen, 2002). Any of these mechanisms, singly or in combination, could have been responsible for the increase in %RLC.

Fine root length is a strong predictor of AM root length among ecosystems (Treseder & Cross, 2006). The increase in AM root length under N fertilization in our study is consistent with this pattern, as standing fine root length also increased. Several field studies have reported positive effects of N on root length density (Ahlstrom *et al*., 1988; Weber & Day, 1996; Rahman *et al*., 2000; Vamerali *et al*., 2003), though others have documented no effect (Durieux *et al*., 1994; Ostertag, 2001; Sharifi *et al*., 2005). Aboveground plant growth appears to increase under N fertilization in the youngest and middle-aged sites (personal observation), which could also influence the availability of photosynthate for allocation to AM fungi. Another possibility is that in our field sites, phosphorus limitation could have been induced as N availability increased. Phosphorus and N increase nonmycorrhizal hyphal lengths independently in the middle-aged site and in combination in the oldest site (T. Gartner, unpublished data). Phosphate is relatively immobile in the soil (Walker & Syers, 1976), so

augmentations of fine root length should be an effective means of improving phosphorus acquisition (Chapin, 1980; Bloom *et al.*, 1985). Phosphorus uptake could occur via the fine roots themselves, the AM fungi, or both (Johnson *et al.*, 2003). Regardless of the mechanism, the increase in fine root length could have provided more opportunities for colonization by AM fungi.

Our estimates indicated that N fertilization reduced carbon storage in mycorrhizal pools, but only for the youngest site. A decline in glomalin carbon stocks in that site was the major factor contributing to this change. The decrease in glomalin, in turn, may have resulted from a decrease in AM hyphal lengths, as the two were correlated. Glomalin is a component of hyphal walls (Driver *et al.*, 2005), so a causal relationship likely exists between AM hyphal lengths and glomalin concentrations. N fertilization might also have accelerated turnover of glomalin (Treseder & Allen, 2000). The physiological or ecological functions of glomalin – if any – for AM fungi are unknown. In addition, few experiments have examined N effects on glomalin stocks, although Wuest *et al.* (2005) observed no changes in glomalin concentrations across N fertilization regimes in a winter wheat field. Lovelock *et al.* (2004) noted no significant relationship between total soil N and glomalin content along a natural fertility gradient in Costa Rica. In our study, the inconsistency in N response among sites limited our ability to predict how increases in N availability might alter glomalin dynamics in boreal ecosystems.

Carbon stocks were much larger in glomalin than in living mycorrhizal tissues. The differences in mycorrhizal pool sizes could have been related to turnover times of these tissues (Steinberg & Rillig, 2003). For example, the residence time of glomalin is approximately 6 years in Hawaiian tropical forests (Rillig *et al.*, 2001) and 12 years in our field sites (K. K. Treseder, unpublished data). In contrast, AM hyphae display turnover times on the order of days to months (Staddon *et al.*, 2003; Zhu & Miller, 2003; Olsson & Johnson, 2005). The relatively rapid turnover times of AM hyphae could produce a comparatively large flux of carbon through the AM hyphal pool, even if the size of the pool is small at any given time (Driver *et al.*, 2005). A portion of this carbon should be allocated to glomalin production, and glomalin turnover is slow. Together, these conditions could yield relatively large glomalin pools in the soil.

Even though glomalin pools were large compared with other mycorrhizal pools, they represented only 1–2% (depending on the site) of soil organic carbon (Treseder *et al.*, 2004) in these boreal ecosystems. These percentages are low compared with other studies, which report that glomalin constitutes 4–5% of soil carbon in a Hawaiian rainforest (Rillig *et al.*, 2001), 3% in a Costa Rican rainforest (Lovelock *et al.*, 2004), and 4–8% in Ohioan forests, and agricultural land (Rillig *et al.*, 2003). AM fungi are not very abundant in our study sites. Specifically, AM root lengths averaged $0.4\,km\,m^{-2}$ in control plots and $1.5\,km\,m^{-2}$ in N-fertilized plots. For comparison, the global average is approximately $3\,km\,m^{-2}$ in the top 10 cm of soil (Treseder & Cross, 2006). In addition, organic carbon pools are relatively large in our sites, with $4–12\,kg\,C\,m^{-2}$ in the top 10 cm of soil (Treseder *et al.*, 2004). Such values are typical for mesic boreal forests (Amundson, 2001). Together, these two conditions may account for the comparatively small proportion of soil carbon contained within the glomalin pool. The loss of glomalin carbon following N fertilization, at $50\,g\,C\,m^{-2}$ over 2 years, was 10 times less than N-induced losses of soil carbon – on the order of $5\,000\,g\,C\,m^{-2}$ over 20 years recently reported for an arctic ecosystem (Mack *et al.*, 2004). Moreover, as the reduction in glomalin carbon was observed only within our youngest site, its significance at a regional scale may be constrained by the prevalence of recent fire scars. Our results do not indicate that increases in N availability will notably reduce carbon sequestration in mycorrhizal pools within the boreal region.

In conclusion, plants in our sites did not seem to reduce allocation of resources to mycorrhizal fungi under N fertilization, given that we found no significant decreases in abundance of AM or ECM fungi. Instead, AM fungi appeared to become more prevalent, as demonstrated by an increase in AM root length. We speculate that plants could be secondarily limited by phosphorus, so they invest more in AM fungi under N fertilization to improve phosphorus uptake. The increase in AM abundance under N is not typical of field studies in other biomes, and it remains to be seen if these effects are common for AM fungi in boreal forests. Likewise, other studies have noted decreases in ECM abundance under N fertilization in boreal ecosystems (Lilleskov *et al.*, 2002; Nilsson & Wallander, 2003). The discrepancy between our results and these previous studies could be related to differences in N or phosphorus limitation, dominant plant or ECM taxa, or soil types. Glomalin, alone, decreased under N fertilization, but in one site only. The variation in glomalin responses among sites highlights the need for caution in generalizing results from one ecosystem to the biome as a whole. As little is known about glomalin, it is difficult to pinpoint potential controls over its abundance in the soil. N availability is one factor that might merit further study.

Acknowledgements

We thank Fort Greely Army Base for access to field sites; B. Bergner, B. Ji, D. Kam, J. L. Lansing, L. Marzec, K. Son, B. Van

Sickle, S. E. Wright, and D. Yoo for technical or field assistance; and J. W. Harden, E. S. Kasischke, and J. T. Randerson for help with establishment of field sites. This work was supported by grants from the NSF (DEB-0445458, DEB-0430111, and EAR-0433918).

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