Arbuscular mycorrhizal fungi do not enhance nitrogen acquisition and growth of old-field perennials under low nitrogen supply in glasshouse culture

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Summary

• Arbuscular mycorrhizal fungi (AMF) are known to promote plant growth when phosphorus is limiting, but the role of AMF in plant growth under nitrogen (N) limiting conditions is unclear.

• Here, we manipulated N (control vs inorganic and organic forms) and AMF species (control vs four AMF species) for five old-field perennials grown individually in a glasshouse under N-limiting conditions.

• We found that AMF were at best neutral and that some AMF species depressed growth for some plant species (significant plant–fungus interaction). Native plant species growth was strongly depressed by all but one AMF species; exotic plant species were less sensitive to AMF. We found no evidence of plant N preferences. Both natives and exotics were able to acquire more N with N addition, but only exotics grew more with added N.

• Our results suggest that AMF do not promote plant N acquisition at low N supply, and our results are consistent with other research showing that AMF can act as a parasitic carbon drain when phosphorus availability is relatively high.

Key words: arbuscular mycorrhizal fungi (AMF), niche partitioning, nitrogen (N), old-field perennials, plant nutrition.

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Introduction

Mycorrhizal fungi form symbiotic associations with plant roots, transferring soil nutrients to the plant in exchange for carbon (C). This mutually beneficial relationship is thought to have been critical to the colonization of land by vascular plants and is characteristic of the majority of plant families (Brundrett, 2002). Arbuscular mycorrhizal fungi (AMF), the most ancient and common type, have traditionally been associated with enhanced plant phosphorus nutrition. However, research on the role of AMF in plant nitrogen (N) nutrition has gradually been accumulating and suggests that AMF can also enhance plant N acquisition (e.g. Ames *et al.*, 1984; Azcón-Aquilar *et al.*, 1993).

The conventional wisdom is that plants are only able to acquire soil nutrients like N from the soil solution after microbial mineralization from organic (e.g. amino acids, proteins, chitins and urea) to mineral forms (ammonium and nitrate: NH_4^+, NO_3^-). More recently, this conventional view has been revised to include direct plant access to organic N pools, particularly in infertile ecosystems (Chapin, 1995). In tropical, boreal and temperate forests, as well as in heathlands and arctic tundra, plants may 'short-circuit' the N mineralization pathway by taking up dissolved amino acids or by accessing organic N in litter or protein–tannin complexes. Ecto- and ericoid mycorrhizae are capable of breaking down complex organic N and taking up dissolved organic N such as amino acids (Read, 1991; Turnbull *et al.*, 1995), and appear to be important in enabling certain plants to access organic N (Stribley & Read, 1980; Kielland, 1994; Northup *et al.*, 1995; Turnbull *et al.*, 1995, 1996; Schimel & Chapin, 1996), although some plants can take up amino acids independently (Chapin *et al.*, 1993; Näsholm *et al.*, 2001).

Although temperate grasslands have comparatively high N mineralization rates, N limitation is common (Tilman, 1987) and microbial competition might drive selection for more direct plant access to organic N even on relatively fertile sites (Kaye & Hart, 1997). Recent research has demonstrated *in situ* amino acid uptake by four agricultural grasses (Näsholm *et al.*, 2000). In temperate grassland and agricultural plants, AMF have been implicated in uptake of a variety of amino acids (Cliquet *et al.*, 1997; Hawkins *et al.*, 2000), in access to N from plant litter (Hodge *et al.*, 2001), and in uptake of NH_4^+ and NO_3^- (Ames *et al.*, 1983; Cliquet *et al.*, 1997; Hawkins *et al.*, 2000).

However, prior research has focused almost exclusively on a few AMF species in a single genus (*Glomus*), typically examining only one AMF species and one plant host per study. Substantial host-specificity of AMF with plant hosts has been demonstrated in a number of temperate grasslands (Bever *et al.*, 1996; van der Heijden *et al.*, 1998a). This host-specificity, in combination with AMF diversity, has been implicated in the maintenance of plant community diversity (van der Heijden *et al.*, 1998b; Bever *et al.*, 2001; van der Heijden, 2002), although the specific mechanism is not known. Access to different forms of mineral and organic N may be an important mechanism of niche diversification, by which a diversity of AMF promotes the coexistence of plant species (Reynolds *et al.*, 2003). N partitioning is expected to occur most strongly in low-fertility soils, where N is relatively more limiting than light and the benefits of partitioning N correspondingly higher (Reynolds *et al.*, 2003). To improve our understanding of the role of AMF in the N nutrition of old-field perennial grassland species, our research addressed whether, at low N supply:

1 AMF improve the ability of plants to grow not only on mineral forms of N, but also on organic forms; and

2 AMF enable different plant species to access *different* organic and mineral forms of N, as opposed to indiscriminate enhancement of plant N.

Materials and Methods

Study species

We studied five perennial plant and four AMF species common to temperate old-field grassland habitats throughout the midwestern and eastern USA. Plant species were chosen to span a range of life forms and included three herbaceous dicots (*Salvia lyrata* (L.), *Plantago lanceolata* (L.) and *Rumex acetosella* (L.)) and two cool-season grasses (*Panicum sphaerocarpon* (Elliott) and *Anthoxanthum odoratum* (L.)). *S. lyrata* and *P. sphaerocarpon* are native to North America; *A. odoratum*, *P. lanceolata*, and *R. acetosella* are Eurasian introductions (USDA, NRCS, 2004). Fungal species included representatives from three AMF genera: *Gigaspora gigantea* (Nicol. & Gerd.) Gerd. & Trappe, *Gigaspora decipiens* (Hall & Abbott), *Archaeospora trappei* (Ames & Linderman), and an unidentified *Glomus* called *Glomus* sp. D1 (Bever *et al.*, 1996). The plants and fungi were found co-occurring within a 1-ha abandoned agricultural field in North Carolina near Duke University, Durham, that was maintained by mowing for 60 yr and has been intensively studied (Bever *et al.*, 1996, 2001 and references therein). Although the individual fungal species are capable of infecting each of the plant species, specificity is known to exist in host plant and fungal response (Bever *et al.*, 1996; Bever, 2002).

N treatments

We used two common mineral forms of N, nitrate (NO_3^-, as KNO_3^-; Sigma-Aldrich, St Louis, MO, USA) and ammonium (NH_4^+, as $(NH_4)_2SO_4$; EMD Chemicals, Gibbstown, NJ, USA); and three common organic forms, the amino acid glycine (Sigma-Aldrich), urea (Sigma-Aldrich) and chitin (purified; Sigma-Aldrich). Ammonium and nitrate are readily available to plants. Glycine can be a relatively poor substrate for microbial growth (Lipson *et al.*, 1999, but see Meyer *et al.*, 2004) and significant plant uptake has been observed in a number of studies (Chapin *et al.*, 1993; Kielland, 1994; Näsholm *et al.*, 2000; Näsholm & Persson, 2001; Näsholm *et al.*, 2001). Neither urea nor chitin can be taken up directly by plants, and were chosen to represent more and less easily degraded organic N sources, respectively. Urea (e.g. from animal excreta) is easily hydrolyzed to NH_4^+ and thus represents an important source of N in grassland communities (Day & Detling, 1990; Steinauer & Collins, 1995). Chitin, a major component of insect exoskeletons and fungal cell walls, is degraded by a more complex series of steps involving a series of enzymes (Paul & Clark, 1996).

Experimental design and setup

Seeds, soil and mycorrhizal fungal species were collected from the Duke University old-field site. Topsoil was sieved through a 13-mm screen and seeds and soil were stored in closed containers at room temperature. Mycorrhizal fungi were maintained in single AMF species culture on *Sorghum bicolor* host plants grown in the glasshouse in a 1 : 1 mixture of sterile Duke soil and sand and fertilized with 20-0-20 NPK fertilizer at 4 g N l^{-1} as needed to promote vigorous growth. Mature cultures with abundant sporulation were used as inocula in this experiment.

We used a modified randomized complete block factorial design of four blocks (four glasshouse benches in a 2×2 block matrix). Five levels of plant treatment (each of our five plant species) fully crossed with five levels of fungal treatment (each of our four AMF species and an autoclaved mixed-species control) were nested within six levels of N treatment (ammonia, nitrate, glycine, urea, chitin and tap water control) within each block, for a total of 600 units. We nested plant–fungal treatments within N treatments in order to avoid errors in applying N solutions.

Seeds of the five plant species were planted on July 12, 2002 in Petri dishes containing sterile medium–fine sand placed under supplemental lighting in a 12-h/12-h light/dark cycle at room temperature for approx. 3 wk, until cotyledons had fully emerged. On July 31 and August 1, 2002, single seedlings were transplanted into 6.4-cm-diameter, 25-cm-deep 'Deepot Cells' (Stuewe & Sons, Inc., Corvallis, OR, USA). The potting medium was a 3 : 1 mixture of medium–fine sand and Duke old-field topsoil prepared by steam sterilizing (2 h at 120°C for 2 consecutive days). We used a high sand content to promote the use of N from treatment sources and to facilitate root harvesting. After sterilization, the sand–soil mixture had a pH (H_2O) of 7.1 and contained 0.03% total N, 26 ppm NO_3^--N and 8 ppm NH_4^+-N (1 N KCl-extractable; Michigan State University, Soil and Plant Nutrient Laboratory, East Lansing, MI, USA). (In comparison, unsterilized Duke soil without sand contained 0.14% total N, 114 ppm NO_3^--N and 29 ppm NH_4^+-N.) Pots were filled with a bottom layer of 150 ml sterile sand–soil mix, followed by a middle layer of 178 ml sterile sand–soil mix combined with 22 ml AMF inoculum (chopped soil and *S. bicolor* root from the appropriate AMF culture), and capped with 200 ml of sterile sand–soil mix to inhibit cross-contamination between fungal treatments. After seedlings were planted, all pots were reinoculated with 35 ml of soil filtrate containing the indigenous soil microbial community minus AMF propagules (25 ml combined filtrate from the four *S. bicolor* AMF cultures and 10 ml filtrate from unsterilized Duke topsoil). Microbial filtrates were prepared by blending soil and water (or soil, roots and water, for *S. bicolor* cultures), sieving this mix through a 38-μm mesh sieve and then filtering through Whatman #1 filter paper (Whatman International Ltd, Maidstone, UK).

After transplanting, seedlings were transferred to a temperature-controlled glasshouse (average 21°C, range 18–35°C) and arranged in the modified randomized complete block design described above. We supplemented the natural light to maintain 12-h days in the fall. We used a ceptometer that measured over 80 cm (Decagon Model PAR80, Pullman, WA, USA) to determine that ambient photosynthetically active radiation (PAR) at plant level averaged 245 μmol m^{-2} s^{-1} (range: 129–640 μmol m^{-2} s^{-1}, readings taken at 11 : 00, 13 : 00 and 15 : 00 h on a clear day at two locations within each block) and that our supplemental lights increased the ambient PAR by an average of 97 μmol m^{-2} s^{-1}.

N treatments were applied in solution form twice a week during the experiment at 10 mg N l^{-1}, a low concentration designed to ensure limiting N conditions. A total of 8.7 mg N was applied per pot over the course of the experiment. Plants were watered with tap water between nutrient applications as necessary to prevent drought stress. The pH of the N solutions ranged from 7.4 to 7.8 and was not adjusted; the pH of tap water was 7.7. Dilute (≈ 5%), N-free Hoagland's solution (pH 7.0) was applied twice a week to provide all other nutrients in proportion; however, we did not correct for the extra sulphate or potassium given in the NO_3^- and NH_4^+ treatments, and we increased phosphorus to 5.7 mg l^{-1} to ensure N-limiting conditions. Because tap water contains minerals, all of our plants received some level of additional nutrients from waterings.

Inoculation potential

We assayed the inoculum potential of each of our fungal cultures by growing *S. bicolor* host plants with 1, 5 and 10% dilutions of each fungal culture in a 1 : 1 mixture of sterile soil and sand. Plants were grown from seed in 3.8-cm-diameter, 21-cm-deep 'Cone-tainer Super Cells' (Stuewe & Sons, Inc., Corvallis, OR, USA) under the same glasshouse conditions as the main experiment, and fertilized as described above for the fungal cultures. After 3 wk of growth, roots were harvested and prepared and scored for percentage fungal colonization as described in the next section.

Plant performance and fungal colonization

After four months of growth, we harvested above- and below-ground biomass. Plant shoots were clipped at stem bases and roots were sieved from soil and washed in tap water. Fresh roots were cut into 1–2 cm sections and weighed to the nearest mg. A representative subsample of approximately 0.4 g of fresh roots was taken from all plants with a sufficiently large root mass (50% or more roots remaining after subsampling), placed in tissue cassettes (HistoPrep OmniSette, Fisher Scientific Healthcare, Pittsburgh, PA, USA), and dried and stained for analysis of percentage fungal colonization using protocols modified from McGonigle *et al.* (1990). Shoots and remaining roots were dried at 60°C for 48 h and weighed to the nearest mg. Dry weights of root subsamples were estimated using a wet weight to dry weight conversion factor obtained from the main root mass and used to calculate total root dry biomass. Shoots and roots were finely clipped with scissors. The fragments were placed in borosilicate glass vials containing 2–4 stainless steel rods, ground to a fine powder on a roller mill, and analyzed for percentage C and N on a PerkinElmer Series II CHNS/O analyzer (Wellesley, MA, USA). We calculated the total amount of N acquired by plants from the percentage N content and biomass of shoots and roots:

$$\begin{aligned}\text{mg N/plant} &= \text{mg N/shoot} + \text{mg N/root}\\ &= (\text{percentage N shoot}/100) \times \text{mg shoot}\\ &\quad + (\text{percentage N root}/100) \times \text{mg root.}\end{aligned}$$

Root morphology

We measured total root length and surface area and average root diameter of five replicates of each of our study species, grown under similar conditions and the same nutrient regime as the main experiment, except that plants were not inoculated with background microbes or AMF. After growing for 11 wk,

roots were washed from soil and root systems were measured using the WINRHIZO root image analysis system (Regent Instruments, Quebec).

Statistical analysis

Data were analyzed using SYSTAT's general linear models procedure (Version 10.2, 2002; SYSTAT Software Inc., Richmond, CA, USA). We tested for differences in inoculation potential among our fungal cultures using a two-way ANOVA with fungal species and dilution as categorical variables. Treatment effects on percentage colonization, plant biomass, total N, and percentage N and C of shoots and roots were tested using a general linear model tailored to our main experimental design (Table 1). We tested assumptions of homogeneity of variance and normality by inspecting residual plots (normal probability plots, histograms and residual-estimate scatter plots). Biomass and total N data were ln-transformed to meet assumptions of ANOVA (normalized, homoscedastic residuals) and to remove any artifacts of scale from interaction terms, but untransformed data are presented in figures (mean ± 1 SE). Significant two-way interaction terms were interpreted by performing one-way ANOVAs for each main effect. For these ANOVAs, F was calculated using the ANOVA mean square (MS) as the error numerator. The error denominator was obtained by summing the appropriate MS errors from the full model. Where F-tests indicated significant main effects, we used the Tukey–Kramer honestly significant difference (HSD) (Bonferroni-protected) test to conduct pairwise mean comparisons [using the within subject mean square error (MSE) and degrees of freedom (d.f.) from the F-test]. Species differences in total root length, total surface area and average root diameter were tested with a one-way ANOVA followed by Tukey-Kramer HSD pairwise comparisons. ANOVA assumptions were tested as above and data were ln-transformed if necessary. We also calculated the mycorrhizal dependency and mycorrhizal species sensitivity (MSS) of our five plant species using the methods suggested in van der Heijden (2002). If $\sum_{1}^{n} a_n > bn$, then mycorrhizal dependency (%) $= \left(1 - \left(\frac{bn}{\sum_{1}^{n} a_n}\right)\right) \times 100$. If $\sum_{1}^{n} a_n < bn$, then mycorrhizal dependency (%) $= \left(-1 + \left(\frac{\sum_{1}^{n} a_n}{bn}\right)\right) \times 100$, where a is the mean total plant dry mass in a given AMF treatment, n is the number of AMF treatments and b is the mean plant dry mass of the non-AMF treatment. For MSS, we calculated the coefficient of variation on total plant dry mass among treatments with our four different AMF species. For both dependency and MSS, our calculations were made across N treatments, because we did not detect AMF–N interactions within plant species. Pearson correlations of mean plant species values of mycorrhizal dependency or MSS and total biomass, percentage N and C of shoots and roots, root length, root surface area and root diameter were used to examine possible mechanisms of plant responses to AMF. Given the low power available with only five species points, we considered these analyses exploratory and chose not to apply Bonferroni-corrected P-values.

Table 1 The ANOVA model used to test for main effects and interactions: Y = constant + Block + Plant + AMF (AMF) + Nitrogen (N) + Plant × AMF + Plant × N + AMF × N + Plant × AMF × N + Block × Plant + Block × AMF + Block × N + Block × Plant × AMF + Block × Plant × N + Block × AMF × N

Source	d.f.	Error term
Block	3	Block × N
Plant	4	Block × Plant
AMF (AMF)	4	Block × AMF
Nitrogen (N)	5	Block × N
Plant × AMF	16	Block × Plant × AMF
Plant × N	20	Block × Plant × N
AMF × N	20	Block × AMF × N
Plant × AMF × N	80	MS Error (ANOVA Table)
Block × Plant	12	MS Error (ANOVA Table)
Block × AMF	12	MS Error (ANOVA Table)
Block × N	15	MS Error (ANOVA Table)
Block × Plant × AMF	48	MS Error (ANOVA Table)
Block × Plant × N	60	MS Error (ANOVA Table)
Block × AMF × N	60	MS Error (ANOVA Table)

Results

Inoculation and percentage colonization

Although highest in *A. trappei*, inoculation potential, as gauged by percentage colonization on *S. bicolor* (number of hyphae, arbuscules, vesicles or coils found at 20–30 intersections per root subsample) was relatively similar (31–44%) at the dilution level (5%) closest to that used in our experimental pots (Table 2). Percentage colonization of our experimental plant

Table 2 Inoculation potential of cultures of the four arbuscular mycorrhizal fungi (AMF) study species, expressed as percentage (mean ± SE) of *Sorghum bicolor* host plant roots colonized

	Dilution		
AMF	1%[a]	5%[b]	10%[b]
A. trappei[a]	41 ± 6	44 ± 5	54 ± 5
G. decipiens[b]	13 ± 6	34 ± 4	38 ± 6
G. gigantea[b]	14 ± 3	31 ± 4	29 ± 5
Glomus D1[b]	28 ± 4	41 ± 1	34 ± 7

Lower-case letters indicate significant differences between AMF species or dilutions ($P \leq 0.05$).

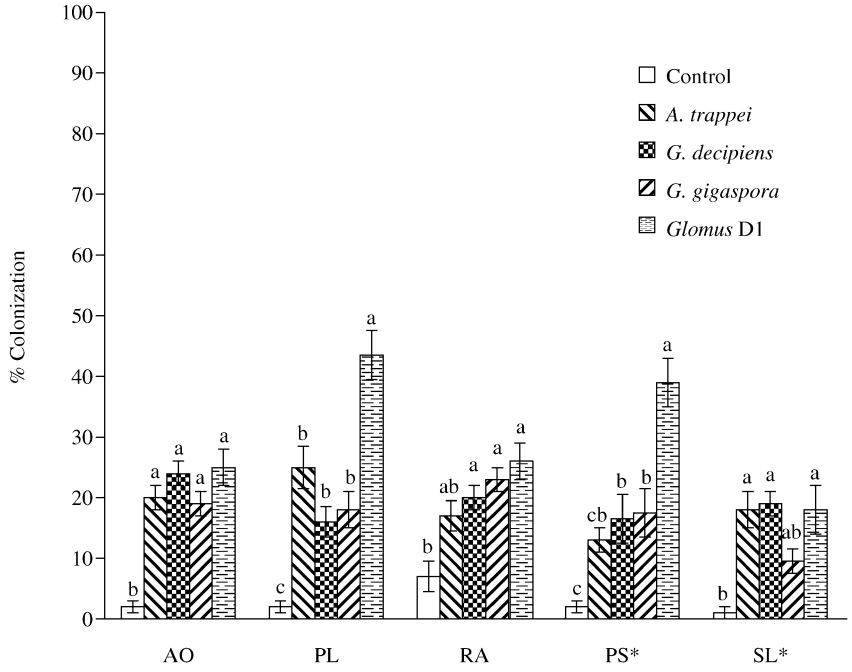

Fig. 1 Arbuscular mycorrhizal fungal (AMF) colonization levels of plant species at harvest (averaged across levels of N; AO, *Anthoxanthum odoratum*; PL, *Plantago lanceolata*; RA, *Rumex acetosella*; PS*, *Pancium sphaerocarpon*; SL*, *Salvia lyrata*; *indicates native species). Significance ($P \leq 0.05$) of AMF treatment differences is indicated by differing lower-case letters (comparisons apply within, not across, plant species).

species at harvest shows that AMF levels in AMF controls were generally near zero and significantly lower than AMF levels in AMF treatments (Fig. 1). Percentage colonization in AMF treatments was at fairly consistently low (10–25%) levels across plant species (Fig. 1), with the exception of *Glomus* D1 with *P. lanceolata* and *P. sphaerocarpon*, which averaged *c.* 40% colonization (significant plant–AMF interaction, Table 3, Fig. 1). Arbuscules were detected in AMF treatments but not in any AMF controls.

Biomass, mycorrhizal dependency, MSS, total plant N, and percentage N and C

After 4 months of growth on a range of N sources at low supply rates, we found that plant biomass and N content were typically either unaffected or depressed by AMF inoculation, depending on plant species (significant plant–AMF interactions, Table 3, Fig. 2). In particular, the two native species *P. sphaerocarpon* and *S. lyrata* showed a distinct drop in biomass with *A. trappei*, *G. decipiens* and *G. gigantea*, but were unaffected by *Glomus* D1, whereas the exotic grass *A. odoratum* was unresponsive to AMF treatment (Fig. 2a). These biomass responses translate into mycorrhizal dependency and MSS values of −55% and 106% and −61% and 97% for *P. sphaerocarpon* and *S. lyrata*, respectively (with 3% and 31%, although not significant, for *A. odoratum*). The exotic forbs *P. lanceolata* and *R. acetosella* responded to AMF much like the native species, although growth declines were generally less dramatic, and even *Glomus* D1 depressed growth of *P. lanceolata* (Fig. 2a; mycorrhizal dependency and MSS of −40% and 45% and −26% and 48% for *P. lanceolata* and *R. acetosella*, respectively). Total plant N exhibited similar patterns except that *P. lanceolata* showed little response to AMF in total N acquired (Table 3, Fig. 2b). We also detected a significant AMF–N interaction for total plant biomass and total plant N (averaged across all plant species, Table 3), apparently simply due to overall lower growth and less variable response to AMF in N control and chitin pots (data not shown).

Percentage N in shoots tended to exhibit an opposite pattern of response to AMF treatments across plant species than that of biomass and total plant N, increasing in AMF treatments for which biomass and total plant N exhibited decreases (significant plant–AMF interaction, Table 3, Fig. 2c). Percentage N in shoots was unresponsive to AMF in *A. odoratum* and unresponsive to inoculation with *Glomus* D1 in the other four plant species (Fig. 2c). Percentage N was lower in roots compared with shoots (overall means: 0.96 ± 0.02 for roots and 1.76 ± 0.03 for shoots) and responses to AMF did not depend on plant species. Root percentage N showed small (≈ 10–20%) increases with inoculation by *A. trappei* or either of the two *Gigaspora* species compared with inoculation with *Glomus* D1 or with uninoculated controls (significant main effect of AMF, Table 3, Tukey post hoc comparisons $P \leq 0.05$).

Percentage C in shoots and roots was much less variable than percentage N; however, both plant and AMF had significant main effects on percentage C in shoots, and percentage C in roots showed a significant plant–AMF interaction (Table 3). Differences among plant species in percentage C in shoots were in the order *S. lyrata* < *R. acetosella* = *P. lanceolata* = *P. sphaerocarpon* = *A. odoratum* (Tukey post hoc comparisons, $P \leq 0.05$). For AMF, *Gigaspora decipiens*, *G. gigaspora*, and especially *A. trappei* tended to reduce percentage C in shoots

Table 3 Summary of ANOVA results for percentage colonization, total biomass, total plant N, and percentage N and C in shoot and root

	Percentage colonization				Total biomass				Total N			
Source	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Block	3	0.003	0.18	0.91	3	0.10	7.94	< 0.01	3	0.150	1.39	0.28
Plant	4	0.10	10.81	**< 0.01**	4	6.68	433.02	**< 0.01**	4	17.36	102.31	**< 0.01**
AMF	4	0.77	73.84	**< 0.01**	4	1.09	39.63	**< 0.01**	4	4.38	38.84	**< 0.01**
N	5	0.01	0.90	0.51	5	0.57	44.04	**< 0.01**	5	5.13	47.43	**< 0.01**
Plant × AMF	16	0.07	3.75	**< 0.01**	16	0.10	8.55	**< 0.01**	16	1.16	13.47	**< 0.01**
Plant × N	20	0.01	0.74	0.77	20	0.08	5.26	**< 0.01**	20	0.25	2.75	**< 0.01**
AMF × N	20	0.02	1.71	0.06	20	0.02	1.90	**0.03**	20	0.13	2.07	**0.02**
Plant × AMF × N	80	0.02	1.13	0.26	80	0.02	1.20	0.15	80	0.07	1.14	0.23
Block × Plant	12	0.01	0.65	0.79	12	0.02	1.07	0.39	12	0.17	2.61	< 0.01
Block × AMF	12	0.01	0.76	0.69	12	0.03	1.90	0.04	12	0.11	1.74	0.06
Block × N	15	0.02	1.12	0.35	15	0.01	0.90	0.57	15	0.11	1.66	0.06
Block × Plant × AMF	48	0.02	1.28	0.13	48	0.01	0.82	0.80	48	0.09	1.32	0.10
Block × Plant × N	60	0.02	1.14	0.26	60	0.02	1.03	0.43	60	0.09	1.39	0.05
Block × AMF × N	60	0.01	0.91	0.66	60	0.01	0.88	0.72	60	0.06	0.96	0.56
Error	161	0.01			231	0.01			198	0.07		

	Shoot %N				Root %N				Shoot %C				Root %C			
Source	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Block	3	1.30	6.85	< 0.01	3	0.07	0.79	0.52	3	31.65	2.95	0.07	3	22.52	0.80	0.51
Plant	4	31.16	134.63	**< 0.01**	4	13.86	244.24	**< 0.01**	4	572.01	25.24	**< 0.01**	4	568.11	21.06	**< 0.01**
AMF	4	4.28	30.61	**< 0.01**	4	0.62	13.26	**< 0.01**	4	118.65	13.59	**< 0.01**	4	138.55	15.78	**< 0.01**
N	5	2.83	14.85	**< 0.01**	5	0.31	3.48	**0.03**	5	46.59	4.34	**0.01**	5	42.81	1.52	0.24
Plant × AMF	16	0.37	2.50	**0.01**	16	0.07	1.68	0.08	16	14.58	1.10	0.38	16	39.30	2.53	**0.01**
Plant × N	20	0.26	1.23	0.27	20	0.12	1.92	**0.03**	20	16.60	0.76	0.75	20	15.2	0.50	0.95
AMF × N	20	0.20	1.54	0.10	20	0.05	0.90	0.59	20	16.55	0.83	0.67	20	20.3	1.30	0.22
Plant × AMF × N	80	0.14	0.82	0.86	80	0.05	0.79	0.88	80	17.54	1.05	0.38	80	14.68	0.67	0.98
Block × Plant	12	0.23	1.35	0.19	12	0.06	0.83	0.62	12	22.66	1.36	0.19	12	26.97	1.24	0.26
Block × AMF	12	0.14	0.82	0.63	12	0.05	0.69	0.76	12	8.73	0.52	0.90	12	8.78	0.40	0.96
Block × N	15	0.19	1.11	0.35	15	0.09	1.32	0.19	15	10.74	0.64	0.84	15	28.16	1.29	0.21
Block × Plant × AMF	48	0.15	0.87	0.71	48	0.04	0.60	0.98	48	13.27	0.80	0.83	48	15.55	0.71	0.92
Block × Plant × N	60	0.22	1.25	0.13	60	0.06	0.92	0.64	60	21.99	1.32	0.08	60	30.56	1.40	0.04
Block × AMF × N	60	0.13	0.75	0.90	60	0.06	0.86	0.76	60	19.97	1.20	0.18	60	15.65	0.72	0.93
Error	212	0.17			213	0.07			212	16.70			213	21.79		

Significant ($P \leq 0.05$) *P*-values are in bold except for terms involving Block, in which we were not interested. (Note: we present results for total plant biomass, but analysis of shoot biomass gave identical results.)

relative to uninoculated controls while *Glomus* D1 had no effect (Tukey post hoc comparisons, $P \leq 0.05$). Percentage C in roots declined for the two natives, *P. sphaerocarpon* and *S. lyrata*, when grown with *A. trappei*, *G. decipiens* and *G. gigantea*, whereas percentage C in roots was unaffected by AMF in the three exotic plant species (Fig. 2d).

We found no evidence for AMF-facilitated N niche partitioning (nonsignificant plant–AMF–N interactions, Table 3, Fig. 3), although plant species responded differently to N treatments in growth and N acquisition (significant plant–N interactions, Table 3, Fig. 3). The native plant species exhibited overall less growth than exotics and were unresponsive to amount or form of N in terms of growth (Fig. 3a), despite generally acquiring more N in N treatments compared with unfertilized controls (Fig. 3b). In contrast, both N acquisition and growth of exotics were promoted by all forms of N, although significantly less so on the most recalcitrant N source, chitin (Fig. 3a,b). Percentage N in shoots increased with N addition, regardless of form of N, plant species, or AMF treatment (significant main effect of N, Table 3, Tukey post hoc comparisons, $P > 0.05$). However, plant species differed overall in percentage N in shoots, in an order roughly opposite to that for percentage C in shoots: *A. odoratum* < *P. lanceolata* < *R. acetosella* = *P. sphaerocarpon* < *S. lyrata* (significant main effect of plant, Table 3, Tukey post hoc comparisons, $P \leq 0.05$). Percentage N in roots increased with added N in only one native (*S. lyrata*), with other species constant or nearly so in percentage N in roots across N treatments (significant plant–N interaction, Table 3, Fig. 3c). Percentage C in shoots increased with N addition, regardless of form of

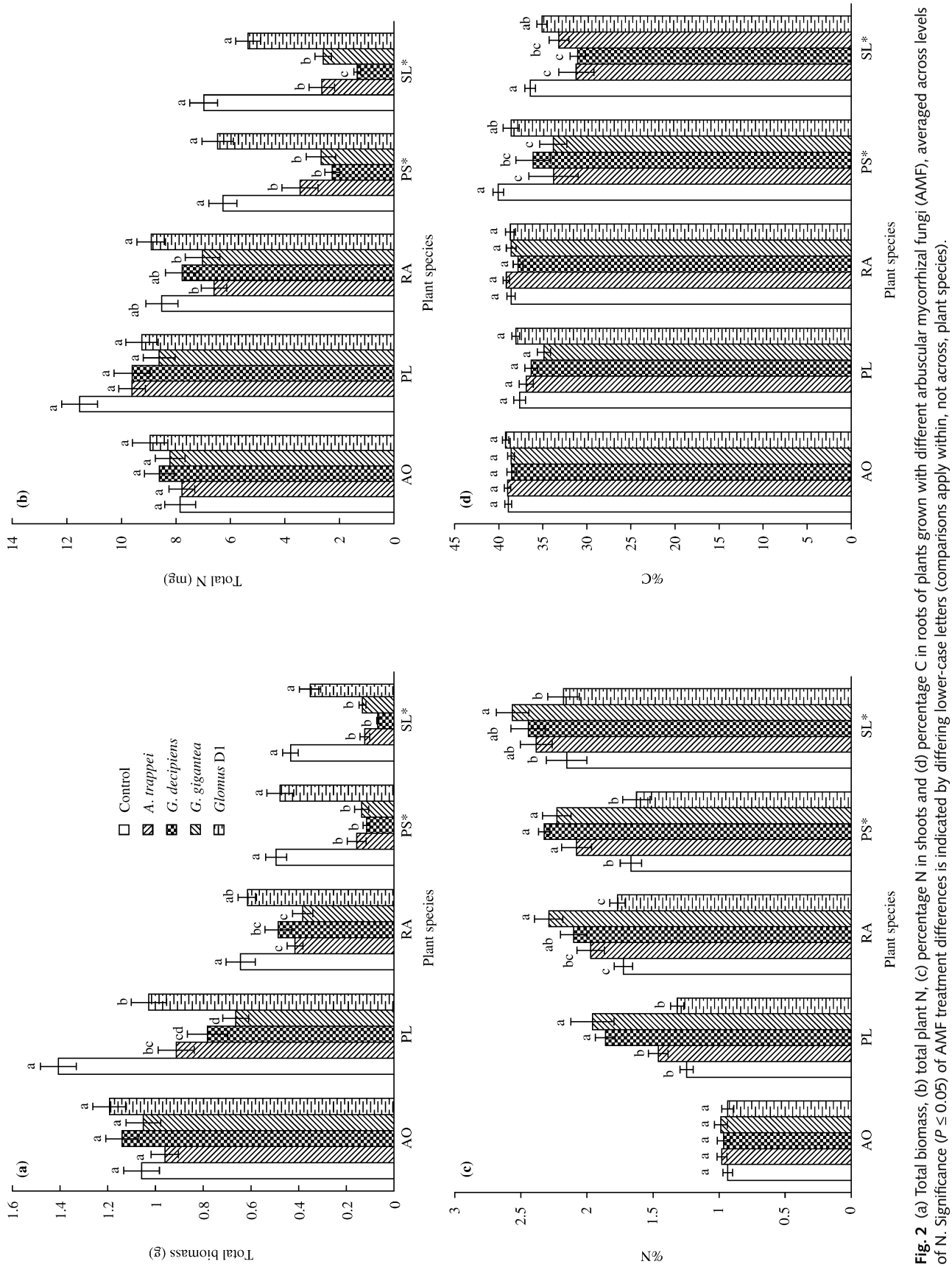

Fig. 2 (a) Total biomass, (b) total plant N, (c) percentage N in shoots and (d) percentage C in roots of plants grown with different arbuscular mycorrhizal fungi (AMF), averaged across levels of N. Significance ($P \leq 0.05$) of AMF treatment differences is indicated by differing lower-case letters (comparisons apply within, not across, plant species).

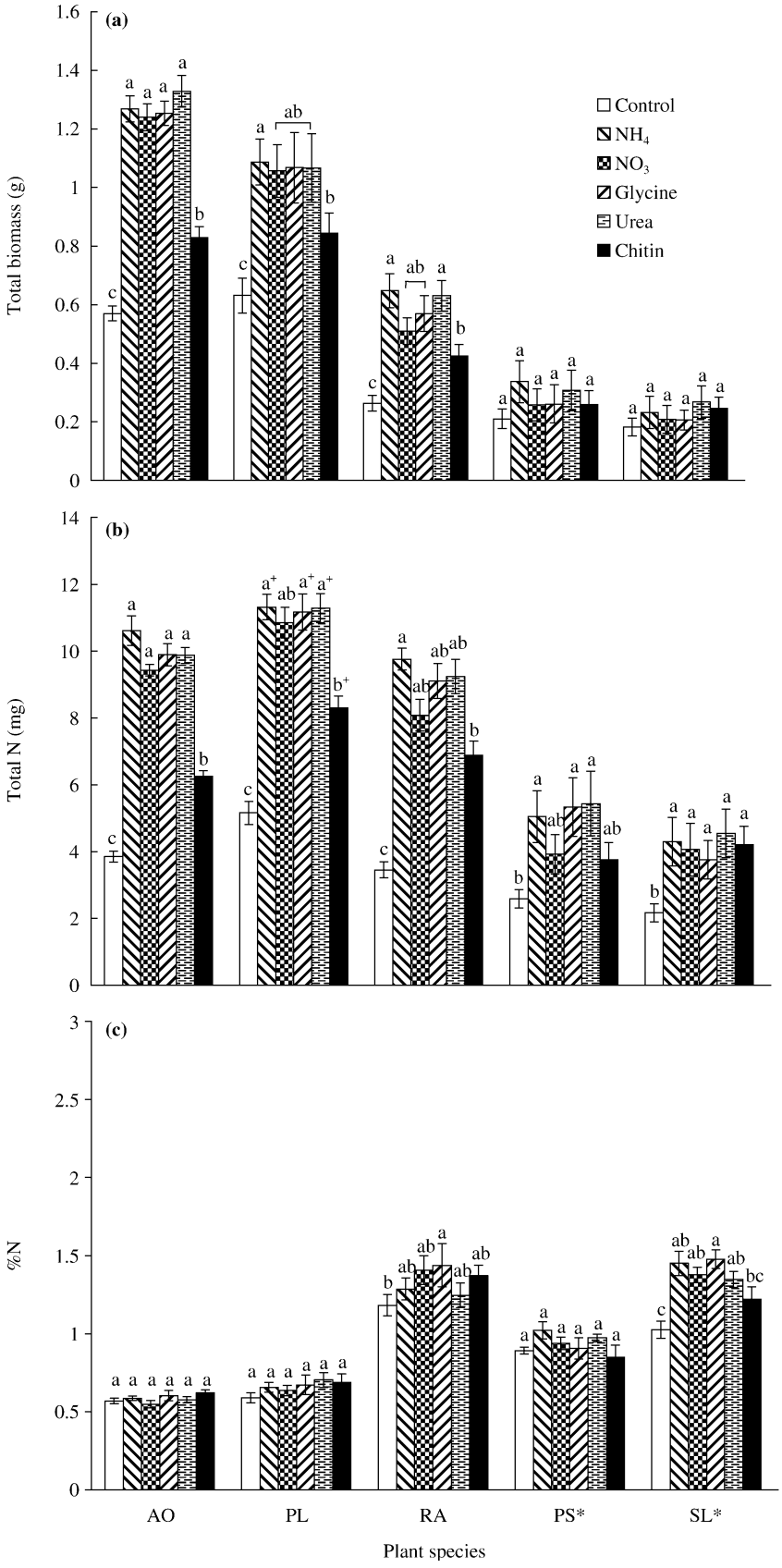

Fig. 3 (a) Total biomass, (b) total plant N and (c) percentage N in roots of plants grown with different N treatments, averaged across levels of arbuscular mycorrhizal fungi (AMF). Significance ($P \leq 0.05$) of N treatment differences is indicated by differing lower-case letters (comparisons apply within, not across, plant species; $^{+}$indicates significance at $P \leq 0.10$).

Table 4 Summary of ANOVA results for root morphological variables

Species	Total length (cm)	Total surface area (cm^2)	Average root diameter (mm)
Anthoxanthum	916.1 ± 147.0[b]	219.2 ± 43.3[b]	0.7 ± 0.1[ab]
Plantago	1919.9 ± 121.5[a]	371.1 ± 35.4[a]	0.6 ± 0.0[bc]
Rumex	507.2 ± 41.1[b]	109.8 ± 8.3[b]	0.7 ± 0.1[ab]
*Panicum**	697.4 ± 62.2[b]	190.5 ± 16.1[b]	0.9 ± 0.0[a]
*Salvia**	769.1 ± 42.6[b]	114.2 ± 3.9[b]	0.5 ± 0.0[c]

Lower-case letters indicate significant difference among plant species ($P \leq 0.05$). Total length: d.f. = 4, MS = 1.23, $F = 15.62$, error d.f. = 19, error MS = 0.08, $P \leq 0.001$; Total surface area: d.f. = 4, MS = 1.16, $F = 10.10$, error d.f. = 19, error MS = 0.11, $P \leq 0.001$; Average root diameter: d.f. = 4, MS = 0.04, $F = 7.83$, error d.f. = 19, error MS = 0.01, $P \leq 0.001$.

N (significant main effect of N, Table 3, Tukey post hoc comparisons, $P > 0.05$); percentage C in roots did not respond to N addition.

Root morphology

Total root length and surface area were ≈ 2–4 and ≈ 1.5–3 times higher, respectively, in *P. lanceolata* compared with the other plant species, which did not significantly differ in these variables (Table 4). Average root diameter was highest in *P. sphaerocarpon* and lowest in *S. lyrata*, with the three exotic species showing intermediate values (Table 4).

Correlations

Of the traits examined, only percentage N in shoots was significantly correlated with mycorrhizal dependency and only total plant biomass was significantly correlated with MSS. The greater the percentage N in shoots, the more negative the mycorrhizal dependency ($R = -0.87$, $P = 0.05$) and the larger the mean plant size, the lower the MSS ($R = -0.90$, $P = 0.04$).

Discussion

Our study was unique in examining a large number of combinations of plant species, AMF species and N sources, but provides little evidence for N partitioning or a role for AMF in N acquisition of old-field plant species. Rather, AMF were at best neutral and most often negative in their effect on plant growth and N acquisition under conditions of low N supply. Although AMF did not promote plant growth in this study, we detected specificity in whether plants experienced AMF as neutral or detrimental to growth as well as in plant species responses to N sources.

Inoculation potential and percentage colonization

Our inoculation potential assay demonstrated that all AMF cultures were viable and roughly equivalent in ability to colonize a common plant host. Levels of AMF colonization at the end of the experiment were generally low, but confirmed that our AMF treatments were effective. The relatively higher colonization of *P. lanceolata* and *P. sphaerocarpon* roots by *Glomus* D1 did not appear to be associated with greater mycorrhizal benefit in these species. It should be noted that we chose to score a lower number of root intersections (20–30) per root subsample than is normally recommended for estimates of percentage mycorrhizal colonization (at least 100, McGonigle *et al.*, 1990). This represented a trade-off in volume of samples scored vs accuracy of scoring. Tests show that variability among subsamples is inflated with lower numbers of intersections scored (McGonigle *et al.*, 1990). However, levels of variation were relatively low in our percentage colonization data (Table 2, Fig. 1), and we were able to confirm that our AMF treatments were effective (AMF controls near zero and significantly less colonized than AMF treatments).

Plant species responses to AMF

Mycorrhizal fungi are known to act as facultative parasites at relatively high phosphorus availability (Buwalda & Goh, 1982), especially when photosynthate is limiting, such as may occur when young plants are establishing (Smith, 1980; Hayman, 1983; Brundrett, 1991) or when light levels are low (Smith, 1980; Smith *et al.*, 1986; Brundrett, 1991). We initiated our plants as newly germinated seedlings, so it is possible that AMF drains on limited photosynthate during early establishment contributed to the growth depressions observed at final harvest. However, early establishment drains typically disappear after 1–2 wk (Hayman, 1983). Because our plants grew for four months, it is likely that some other factor contributed to the observed growth depressions. Light availability is a possible factor, although the day length and light intensity provided in our experiment were sufficient to promote vigorous growth of our *S. bicolor* cultures and were above those found to depress or reverse positive growth responses to mycorrhizal colonization in other studies (onion, Hayman, 1974; maize, Daft & El-Giahmi, 1978).

Photosynthate production is also strongly limited by N availability; plants require substantial amounts of N (2–5% N content by dry weight, compared with 0.3–0.5% phosphorus

content, Marschner, 2002), and well over 50% of leaf N is devoted to photosynthesis (Field & Mooney, 1986). In order to benefit plant growth under N-limiting conditions, AMF must enhance plant access to N to an extent sufficient to allow photosynthate production in excess of AMF demand for photosynthate. Given the relatively high N demands of plants, the expectation that net benefits of AMF exceed net costs in unfertilized soil and fall below net costs in fertilized soil (Johnson *et al.*, 1997) may not apply when N is the more limiting nutrient. In our study, percentage N in shoots increased for those plant–AMF combinations that typically resulted in lower overall plant growth and total N acquisition. Also, percentage N in roots tended to increase, and percentage C in shoots tended to decrease, with inoculation by any of the three AMF species (*A. trappei*, *G. decipiens*, *G. gigaspora*) that caused growth declines in most plant species. When inoculated with these same AMF species, percentage C in roots declined for the two plant species that showed the strongest growth depressions. These results suggest that AMF often imposed a C drain on plants, preventing plants from using acquired N for increased growth.

A recent study involving a greater range of old-field plants and AMF, grown in low-nutrient soil with supplemental phosphorus, found growth-depressing effects of AMF in half of the plant–AMF combinations (Klironomos, 2003). Yet, other studies, including a number conducted under low N conditions, have used ^{15}N labeling to show that AMF can transport N to the plant host, increasing host acquisition of $^{15}NH_4^+$ (celery, Ames *et al.*, 1983; cucumber, Johansen *et al.*, 1992), $^{15}NO_3^-$ (ryegrass, Cliquet *et al.*, 1997; lettuce, Azcón *et al.*, 2001), ^{15}N-labelled amino acids (ryegrass, Cliquet *et al.*, 1997; wheat, transformed Queen Ann's Lace roots, Hawkins *et al.*, 2000, experiments i, iv), or ^{15}N derived from plant litter (plantain, Hodge *et al.*, 2001). However, ^{15}N-labelling studies must be interpreted with care because of artifacts related to differences in size of nonmycorrhizal vs mycorrhizal plants (Högberg *et al.*, 1994). Furthermore, the amount of ^{15}N transferred in tracer studies is small compared with total plant needs, and in four of the six studies no increases in plant growth or total N were detected in mycorrhizal vs nonmycorrhizal treatments, despite the evidence for N transfer. Cliquet *et al.* (1997) and Azcón *et al.* (2001) did find significantly enhanced plant growth and N content for mycorrhizal treatments, although in the latter study plants were fertilized with a phosphorus-free fertilizer, so mycorrhizal benefit might best be explained by phosphorus-limiting conditions.

Mechanisms of AMF-mediated N acquisition include increased surface area for uptake or increased mineralization of N from organic forms. The dominant perspective has been that AMF are not saprotrophic (Brundrett, 1991), although recent work has shown that AMF may stimulate mineralization of organic patches in soil (Hodge, 2001; Hodge *et al.*, 2001; but see Hodge *et al.*, 2000). Our results do not support a significant role for either mechanism of AMF-mediated N acquisition, at least not in individual pot culture with homogeneous soil (but see Cliquet *et al.*, 1997). Perhaps AMF-mediated N acquisition manifests itself only under more realistic field conditions, where plants are competing for limited N in spatially unbounded and heterogenous soil. Then, one plant's gain in N is another's loss, and AMF may enhance N acquisition by virtue of access to a larger soil volume than roots and/or via exploitation of nutrient-rich hot spots (Hawkins *et al.*, 2000). Few such studies yet exist, although Šmilauerová & Šmilauer (2002) found that foraging responses to N and phosphorus patches were unrelated to AM mycorrhizal dependency under competitive field conditions. Similarly, Hodge (2003) found no effect of AM mycorrhizal inoculum on plant N capture from a dispersed vs patchy organic N source, although competing plant species did obtain more N from either source with AMF inoculation. More generally, competitive environment might also affect whether AMF act as parasites or mutualists, altering our understanding of the parasitism to mutualism continuum emerging from single plant pot culture studies (e.g. Klironomos, 2003).

Although both native and exotic plant species experienced growth-depressing effects of AMF in this study, the two native species were affected most. Yet the natives were also the smallest species on average, and we found that mean plant size was inversely correlated with MSS. We found no relationship between other measured plant traits (percentage N and C and root length, surface area or diameter) and MSS, although percentage N in shoots was negatively correlated with mycorrhizal dependency. The latter relationship was due to the fact that *A. odoratum* had the lowest percentage N in shoots (highest N use efficiency; Chapin & Van Cleve, 1989) of all our plant species, and was the one species whose growth and N acquisition was unaffected by colonization with any AMF species – all other species had negative values of mycorrhizal dependency. Other work has suggested that root morphology (Hetrick *et al.*, 1990; Brundrett, 2002), perhaps in combination with phenology (Hetrick *et al.*, 1988) is important in determining plant responses to AMF. Also, although not addressed here, greater allocation to nonstructural carbohydrates, especially in roots, has been associated with greater mycorrhizal dependency (Graham, 2000), and could thus also influence vulnerability to parasitic effects of AMF. Traits of AMF species may be of equal importance in understanding why AMF are beneficial or parasitic under any given resource conditions or for any particular plant species. Aggressiveness in rate of root colonization, for example, can predict nonbeneficial AMF effects on plants at high phosphorus supply (Graham, 2000; Graham & Abbott, 2000). C and N stoichiometry (especially relative to that of plant species), size and growth rate, and nativity and phenology of AMF are also logical traits to explore. Such information may have helped to understand why *Glomus* D1 was most frequently neutral and the other AMF species most frequently nonbeneficial in effect on the plant species in our experiment.

Plant species responses to N

Even if AMF play such a little role in N partitioning, plant species might partition forms or sources of N by virtue of root characteristics or associations with other microbes (Reynolds *et al.*, 2003). Our plants were exposed to other indigenous soil microbes in addition to the AMF treatments, yet still we found little evidence that different plant species grew better on different forms of N. Ability to use multiple forms and sources of N may be a generally advantageous strategy for a sessile organism in a temporally and spatially heterogenous world. It is also possible that partitioning manifests itself only under competitive conditions. We grew our plants singly in pots, and so perhaps we saw only the 'fundamental N niche' of these species, and the 'realized N niches' when grown in mixture would have been different. Recent work has shown, for example, that alpine plant species exhibit preferences for different forms of N in the presence vs absence of neighbors (A. Miller *et al.*, National Park Service, unpublished data).

Although we did not detect N partitioning, we found that natives and exotics again behaved differently in response to treatments. The exotic species experienced some release from N limitation under all N treatments (although less so with chitin, the most recalcitrant N source), but the two native species did not. Yet tissue N data show that the natives were able to acquire more N from N treatments. These results are consistent with the greater growth reductions due to AMF for natives. That is, in *S. lyrata* and *P. sphaerocarpon*, gains in photosynthate from added N may have more often gone to AMF rather than to growth.

Conclusions and future directions

This study suggests that AMF do not promote inorganic or organic plant N acquisition in a constrained rooting volume at low N supply, and that such conditions promote growth-depressing effects of AMF in responsive plant species. The study also suggests that plants do not exhibit N preferences under such conditions. Further study is needed to determine whether these results also hold when plants are grown in larger rooting volumes under competitive conditions/with heterogeneous soil nutrients, and to uncover the basis of plant responses to AMF in terms of plant and/or AMF species traits.

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