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NITROGEN ENRICHMENT ALTERS MYCORRHIZAL ALLOCATION AT FIVE MESIC TO SEMIARID GRASSLANDS

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Abstract. Arbuscular mycorrhizal (AM) fungi are integral components of grasslands because most plants are associated with interconnected networks of AM hyphae. Mycorrhizae generally facilitate plant uptake of nutrients from the soil. However, mycorrhizal associations are known to vary in their mutualistic function, and there is currently no metric that links AM functioning with fungal colonization of roots. Mycorrhizal structures differ in their physiological and ecological functioning, so changes in AM allocation to intraradical (inside roots) and extraradical (in soil) structures may signal shifts in mycorrhizal function. We hypothesize that the functional equilibrium model applies to AM fungi and that fertilization should reduce allocation to arbuscules, coils, and extraradical hyphae, the fungal structures that are directly involved in nutrient acquisition and transfer to plants. This study compared AM responses to experimental N enrichment at five grasslands distributed across North America. Samples were collected from replicated N-enriched (and some P-enriched) and control plots throughout the growing season for three years. Intraradical AM structures were measured in over 1400 root samples, extraradical hyphal density was measured in over 590 soil samples, and spore biovolume was analyzed in over 400 soil samples. There were significant site × N interactions for spore biovolume, extraradical hyphae, intraradical hyphae, and vesicles. Nitrogen enrichment strongly decreased AM structures at Cedar Creek, the site with the lowest soil N:P, and it increased AM structures at Konza Prairie, the site with the highest soil N:P. As predicted by the functional equilibrium model, in soils with sufficient P, relative allocation to arbuscules, coils, and extraradical hyphae was generally reduced by N enrichment. Allocation to spores and hyphae was most sensitive to fertilization. At the mesic sites, this response was associated with a shift in the relative abundance of Gigasporaceae within AM fungal communities. This study demonstrates that N enrichment impacts mycorrhizal allocation across a wide range of grassland ecosystems. Such changes are important because they suggest an alteration in mycorrhizal functioning that, in turn, may impact plant community composition and ecosystem function.

Key words: arbuscular mycorrhizae; functional equilibrium; grasslands; intersite comparison; LTER; nitrogen eutrophication; N:P ratio.

INTRODUCTION

Arbuscular mycorrhizal (AM) fungi are among the most abundant fungi in grassland and agricultural soils. Mycorrhizal symbioses facilitate plant uptake of soil resources (including P, N, and water), affect plant–pathogen interactions, and mediate the outcome of plant competition (Allen 1991, George et al. 1995, Newsham et al. 1995, Zobel and Moora 1997). Moreover, extensive networks of these ubiquitous fungi are pivotal to the formation of soil aggregates and the development of soil structure (Miller and Jastrow 2000). Humans inadvertently impact AM fungi through management (Schenck and Kinloch 1980, Bentivenga and Hetrick 1992, An et al. 1993, Eom et al. 1999) and pollution (Egerton-Warburton and Allen 2000, Egerton-Warburton et al. 2001). It is important to understand the nature of these impacts because AM fungal communities can directly influence the diversity and species composition of plant communities (Francis and Read 1994, Van der Heijden et al. 1998*a*, Hartnett and Wilson 1999, Klironomos et al. 2000, O'Connor et al. 2002).

Wet and dry deposition of nitrogenous compounds emitted from agricultural operations and internal combustion engines have quickly become a dominant source of N in many natural ecosystems (Vitousek et al. 1997). Nitrogen enrichment of grasslands often dramatically increases aboveground productivity, alters plant species composition, and reduces species diversity (Tilman 1988, Berendse et al. 1993, Gough et al. 2000). As plants become enriched with mineral nutrients, they tend to allocate more photosynthate to shoots and leaves and less to roots and AM fungi (Marschner et al. 1996). The impact of this perturbation on grass-

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land AM fungi and their feedbacks on plant communities is not well understood, but it is likely to be substantial because AM fungi acquire all of their carbon from living plants (Jakobsen and Rosendahl 1990). Experimental N enrichment has been shown to decrease, increase, or have no effect on AM colonization of roots (e.g., Hayman 1982, Sylvia and Neal 1990, Bentivenga and Hetrick 1992). Such inconsistencies among studies could stem from differences in initial soil fertility (primarily soil N:P), climate, disturbance regime, and host community. Also, it is likely that N enrichment alters both plant and AM fungal allocation patterns so that measuring only total intraradical (inside roots) colonization may not adequately reveal these responses (Klironomos et al. 1996).

Total intraradical AM colonization is a poor predictor of mutualistic functioning (McGonigle 1988). Nevertheless, more detailed measurements of intraradical and extraradical (in soil) structures might be useful for linking mycorrhizal structure and function (Graham et al. 1982, Johnson 1993). Intraradical structures include vesicles, arbuscules, coils, and hyphae, and extraradical structures include extraradical hyphae and spores (Fig. 1). From a plant nutrition perspective, high allocation to arbuscules, coils, and extraradical hyphae relative to other AM structures could indicate greater mutualistic functioning, because these structures are directly involved with supplying host plants with nutrients and water (Smith and Gianinazzi-Pearson 1988, Sylvia 1990, Jakobsen et al. 1992). Extraradical hyphae form extensive mycelial networks in grassland soils, with up to 111 m of hyphae per cubic centimeter of soil (Miller et al. 1995). In addition to absorbing and translocating minerals and water, these hyphae initiate new colonization sites and connect root systems of unrelated plants. AM fungi also spread and reproduce with asexual spores that form either as individual spores or clusters of spores at the end of hyphae in the soil or inside plant roots.

The functional equilibrium model predicts that the availability of above- and belowground resources control plant allocation to leaves, stems, and roots (Mooney 1972, Brouwer, 1983). Plants growing in nutrient-poor soils tend to allocate relatively more to roots, whereas those in nutrient-rich soils allocate relatively more to stems and leaves (Chapin 1980, Tilman 1988, Tilman and Cowan 1989). Within grassland systems, we may expect that resource availability should also control allocation to intraradical and extraradical AM structures because AM symbioses are integral to most root systems and are ultimately controlled by host carbon supplies. We hypothesize that relative allocation to arbuscules, coils, and extraradical hyphae should be reduced when soils are sufficiently fertilized so that AM delivery of soil resources is no longer of value to host plants; therefore, they allocate less photosynthate belowground (Fig. 1). If this hypothesis is correct, then we would predict terrestrial N eutrophication to reduce allocation to mycorrhizae if P is not a limiting nutrient but increase allocation to mycorrhizae if P is limiting. This shift can be expected at multiple scales; at the community scale, nutrient enrichment is predicted to change AM fungal species composition in response to increasing fungal competition for a more limited supply of host photosynthate. At the genotype scale, N enrichment can be expected to select for N-loving ecotypes of AM fungi; phenotypic changes are also expected as individual fungal clones exhibit plasticity in allocation. Changes in AM allocation to intraradical and extraradical structures is important because a reduction in extraradical hyphal networks is likely to impact soil structure and soil food webs (Miller and Jastrow 2000). Also, because AM fungal taxa vary tremendously in their mutualistic function (Smith and Smith 1996, Van der Heijden et al. 1998*b*), changing the composition of AM fungal communities is directly relevant to plant community structure and ecosystem function.

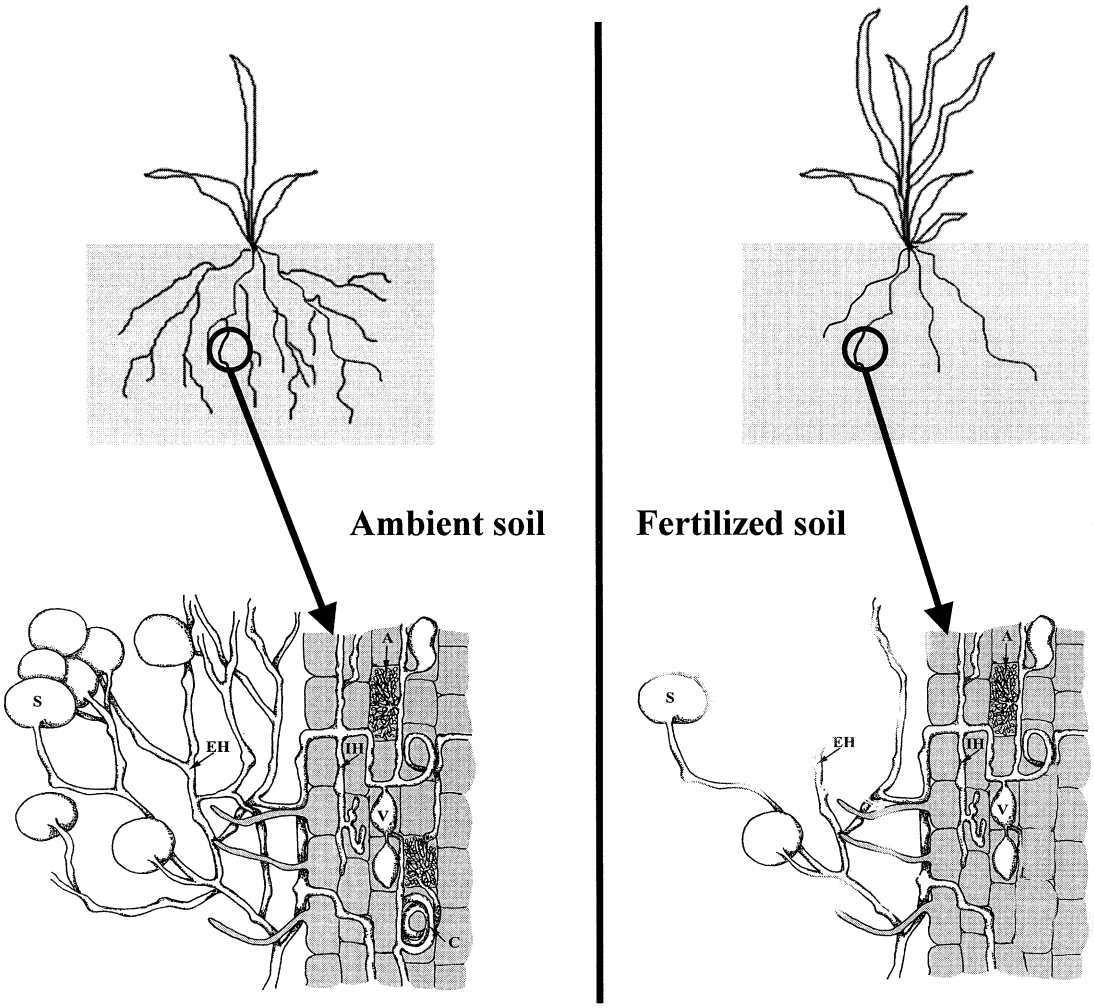
The goals of this study were to systematically measure AM fungi in five experimental grasslands with vastly different soil fertilities, climates, disturbance regimes, and host communities to: (1) assess allocation to AM structures in ambient soil conditions, (2) compare impacts of N enrichment on AM allocation, and (3) test the hypothesis that N enrichment causes AM fungi to reduce allocation to arbuscules, coils, and extraradical hyphae relative to total allocation to mycorrhizae.

Materials and Methods

Study sites

This three-year study was conducted from 1996 through 1998 at five grasslands within the Long Term Ecological Research (LTER) network in the United States: Kellogg Biological Station in Michigan, Cedar Creek Natural History Area in Minnesota, Konza Prairie Biological Station in Kansas, Shortgrass Steppe in Colorado, and Sevilleta National Wildlife Refuge in New Mexico. These five grasslands include examples of successional old fields, tallgrass prairie, shortgrass prairie, and desert grassland with mean annual precipitation ranging from 890 to 244 mm (Table 1). Except for Sevilleta, all sites had ongoing, long-term N-enrichment experiments. At each site, N fertilizer was applied as granular NH_4NO_3 to replicated N-enriched and control plots at least 25 m^2 in size, much larger at most sites. We sampled root and rhizosphere soil from two grass species at each site. Table 1 summarizes the location, climate, soil parameters, and the experimental treatments at each site. Additional details about the LTER sites follow.

Kellogg Biological Station is a 1600-ha agricultural/successional grassland, 15 km northeast of Kalamazoo, Michigan. We sampled roots and rhizosphere soils from *Poa compressa* in N-enriched and ambient subplots



Intraradical structures	Functions
Vesicles (V)	Stores fungal lipids and other compounds
Arbuscules (A)	Area of resource exchange between plants and fungi
Coils (C)	Area of resource exchange between plants and fungi
Intraradical hyphae (IH)	Spreads colonization intraradically, transports resources within fungus
Extraradical structures	**Functions**
Extraradical hyphae (EH)	Sites of mineral and water uptake, spreads colonization extraradically
Spores (S)	Asexual reproductive structures

FIG. 1. The functional equilibrium model predicts that plant photosynthate is preferentially allocated toward structures that acquire resources that are most limiting. Thus, fertilization is expected to cause plants to allocate less photosynthate to roots and mycorrhizae. We suggest that this model can also apply to mycorrhizal allocation, and we predict that fertilization should reduce allocation to extraradical hyphae, arbuscules, and coils relative to other structures.

within five replicate plots of an old field last cropped in 1989 (T7; Huberty et al. 1998) and from *Agropyron repens* in N-enriched and ambient subplots within uncropped sections of a no-till treatment (T2).

Cedar Creek Natural History Area is a 2200-ha successional grassland–forest mosaic, 50 km north of Minneapolis, Minnesota. We sampled roots and rhizosphere soils from *Agropyron repens* and *Andropogon scoparius* in N-enriched and ambient plots (five replicates of each) in an old field, last cropped in 1957 (Wilson and Tilman 1991, 1993). Unlike the four other sites, all of the Cedar Creek plots (both N-enriched and ambient)

TABLE 1. Location, climate, soil parameters, and experimental treatments at each of the five Long Term Ecological Research (LTER) study sites in the U.S.

LTER site†	Latitude, longitude	Climate	Mean annual precipitation and pattern	Mean July/January temperature (°C)	Soil type, texture	Soil parent material	Soil bulk density (g/cm^3)
Kellogg Biological Station	42°24′ N, 85°22′ W	mesic	890 mm, spread fairly evenly throughout the year	22.0°/−5.0°	Typic Hapludalfs, sandy loam-silty clay loam	glacial till	1.5
Cedar Creek	45°24′ N, 93°12′ W	mesic	660 mm, spread fairly evenly throughout the year	22.2°/−10.0°	Typic Udipsamments sandy	glacial outwash	1.6
Konza Prairie	39°05′ N, 96°35′ W	mesic	835 mm, ~75% during the growing season	26.6°/−2.7°	Pachic Argiustolls silty clay loam	chert-bering limestone	1.0
Shortgrass steppe	40°49′ N, 107°47′ W	semiarid	311 mm, ~70% during the growing season	22.0°/−5.0°	Ustollic Haplargids fine loam	Holocene alluvium	1.3
Sevilleta	34°24′ N, 106°40′ W	semiarid	244 mm, ~60% during June–September	33.1°/−5.0°	Typic Calciorthid sandy loam	Holocene alluvium and eolian	1.2

† Web sites: Kellogg Biological Station, ⟨http://lter.kbs.msu.edu⟩; Cedar Creek, ⟨http://www.lter.umn.edu⟩; Konza Prairie, ⟨http://www.konza.ksu.edu⟩; Shortgrass Steppe, ⟨http://sgs.cnr.colostate.edu⟩; Sevilleta, ⟨http://sev.lternet.edu⟩.

received K, Ca, Mg, S, micronutrients, and P (200 kg $P \cdot ha^{-1} \cdot yr^{-1}$ as P_2O_5).

Konza Prairie Biological Station is a 3400-ha tallgrass prairie in the Flint Hills, 20 km south of Manhattan, Kansas. We sampled roots and rhizosphere soils from *Panicum virgatum* and *Andropogon gerardii* growing in burned and unmowed plots (five replicates of each) that were enriched with N or left as ambient controls (Gibson et al. 1993). In 1997 and 1998, we also sampled plots that were enriched with both N and P (10 kg $P \cdot ha^{-1} \cdot yr^{-1}$ as P_2O_5).

Shortgrass Steppe is an 84 380 ha tract of shortgrass prairie, 60 km northeast of Fort Collins, Colorado. We sampled roots and rhizosphere soils from *Elymus elymoides* and *Bouteloua gracilis* in N-enriched and ambient plots (five replicates of each; Lauenroth et al. 1978). Although N had not been applied to these plots since 1975, plots with a history of N enrichment continue to have significantly higher N contents and altered plant communities (Milchunas and Lauenroth 1995).

Sevilleta National Wildlife Refuge is a 100 000-ha tract of desert grassland, ~95 km south of Albuquerque, New Mexico. Unlike the other four LTER sites, Sevilleta had no ongoing N-enrichment study. In December of 1995, we established 20 10 × 5 m experimental plots in a *Bouteloua*-dominated grassland with 10 plots enriched with N and 10 left as ambient controls. We sampled roots and rhizosphere soils from *Bouteloua gracilis* and *B. eriopoda*.

Sampling protocol

Composite samples of soil and roots, composed of four cores, were collected from the base of individual clumps of the specified grass species using a soil corer (2.5 cm diameter × 15 cm deep). Ten replicates (five from each grass species) from each treatment were collected. Soils were placed in plastic bags and frozen within 48 h of collection. In 1996, samples were collected from each site on three dates: early (April–May), middle (June–July), and late (August–December). In 1997 and 1998, samples were collected during the middle of the growing season and again late in the growing season.

Soil chemistry

Soil samples collected in May, July, and October of 1996 were analyzed by the Analytical Laboratory at the University of California, Davis, California, USA. Available NH_4^+ and NO_3^- were determined using diffusion-conductivity analysis following KCl extraction (Carlson et al. 1990). Available PO_4^{3-} was determined colorimetrically with an autoanalyzer following bicarbonate extraction (Page 1986). Soil pH was measured from a 1:1 soil : water paste.

Staining and quantification of intraradical colonization

Roots were removed from the soil samples, gently washed, stained with trypan blue using the method of Koske and Gemma (1989), and scored for intraradical AM colonization using the magnified gridline intersect method developed by McGonigle et al. (1990). This method uses a compound microscope (200–400×) to measure the percentage of root length colonized by intraradical hyphae, vesicles, arbuscules, and coils.

TABLE 1. Continued.

N enrichment history (plot size)	Vegetation type	Species sampled
1989–present: 120 kg N·ha^{-1}·yr^{-1} (0.9 ha)	old field, 7 yr post agriculture	*Agropyron repens* (C_3)
		Poa compressa (C_3)
1988–present: 170 kg N·ha^{-1}·yr^{-1} (25 m^2)	old field, 40 yr post agriculture	*Andropogon scoparius* (C_4)
		Agropyron repens (C_3)
1986–present: 100 kg N·ha^{-1}·yr^{-1} (156 m^2)	native tallgrass	*Andropogon gerardii* (C_4)
		Panicum virgatum (C_4)
1971–1975: 100–150 kg N·ha^{-1}·yr^{-1} (1 ha)	native shortgrass	*Bouteloua gracilis* (C_4)
		Elymus elymoides (C_3)
1995–present: 100 kg N·ha^{-1}·yr^{-1} (50 m^2)	native desert grassland	*Bouteloua gracilis* (C_4)
		Bouteloua eriopoda (C_4)

Extraction and quantification of extraradical hyphae

Extraradical hyphae were extracted from 10 g of Cedar Creek and Sevilleta soil or 5 g of soil from the other three sites using a modification of the procedure of Frey and Ellis (1997). Soil was suspended in 200 mL of sodium hexametaphosphate (39.5 g/L) for 1 h, washed through a 250-μm mesh, then resuspended in 500 mL of distilled water, left to settle for 15 s, and decanted through a 20-μm sieve. This process was repeated three times. Hyphae were rinsed out of the 20-μm sieve and placed in 50 mL of distilled water. Duplicate 3-mL aliquots were pipetted after 30 s and filtered through a 1.2-μm gridded membrane. The membranes were mounted on slides with Permount (Fisher Scientific, Hanover Park, Illinois, USA) and viewed using a compound microscope (200–400×). Forty randomly located fields of view were scored using the gridline intercept method and converted to hyphal lengths per dry mass of soil (Newman 1966, Tennant 1975).

Calculation of allometric ratio

An allometric ratio was calculated to test the hypothesis that N enrichment should reduce allocation to arbuscules, coils, and extraradical hyphae relative to total colonization (all intra- and extraradical structures, excluding spores). This ratio was calculated as (arbuscules + coils + extraradical hyphae)/(intraradical hyphae + vesicles + arbuscules + coils + extraradical hyphae). The units for intraradical structures and extraradical hyphae differ, but both units are in the numerator and denominator so that they cancel one another, making a unitless ratio. All of the 1997 and 1998 data were combined for this analysis; the 1996 data was not included because extraradical hyphae was not measured that year.

Spore extraction and calculation of spore biovolumes

Spores were recovered from 5 g of air-dried soil using dry sieving and sucrose-sodium hexametaphosphate centrifugation (Allen et al. 1979). The extracted sample was then filtered evenly over a gridded membrane, and all spores present on the membrane were counted. All of the large spores (>100 μm) were removed from the membrane using a fine needle, mounted in 1:1 polyvinyl alcohol (PVA) : Melzer's reagent, and gently crushed under a glass coverslip for identification. A subsample of the smaller spores (<100 μm) was collected from 50–100% of the membrane area, mounted in PVA : Melzer's reagent, and crushed under a coverslip for identification. All spores on the prepared slides were examined, counted, and identified using a light microscope (100–400×) equipped with Nomarski interference optics. Voucher specimens are maintained in a collection of permanent slides held in storage at the University of California, Riverside. For each AM fungal species, the mean length (*l*, measured on the longest axis of the spore) and width (*w*, at the widest

TABLE 2. Available soil N and P and pH in ambient experimental plots at each Long Term Ecological Research (LTER) site.

LTER site	NH_4^+ (mg/kg)	NO_3^- (mg/kg)	PO_4^{3-} (mg/kg)	N:P	pH
Kellogg Biological Station	10.8^{bc}	6.6^{a}	17.7^{b}	1.0^{b}	7.4^{b}
	(1.3)	(1.3)	(2.7)	(0.12)	(0.7)
Cedar Creek	8.8^{c}	2.1^{b}	39.8^{a}	0.3^{c}	6.4^{c}
	(1.6)	(1.6)	(2.8)	(0.02)	(0.5)
Konza Prairie	33.7^{a}	5.4^{a}	11.1^{bc}	3.5^{a}	6.6^{c}
	(1.5)	(1.6)	(1.0)	(0.35)	(0.7)
Shortgrass Steppe	11.4^{bc}	4.9^{ab}	18.4^{b}	0.9^{b}	7.1^{b}
	(1.1)	(1.1)	(2.1)	(0.10)	(0.7)
Sevilleta	14.0^{b}	5.3^{ab}	6.6^{c}	2.9^{a}	8.9^{a}
	(1.9)	(2.0)	(0.7)	(0.36)	(0.5)

Notes: Values are the means (with 1 SE in parentheses) of early-, mid-, and late-season samples collected in 1996. Different letters within columns indicate that sites differ at $P \leq 0.05$.

TABLE 3. Results of repeated-measures ANOVA for intraradical and extraradical arbuscular mycorrhial (AM) structures: total intraradical colonization, intraradical hyphae, arbuscules, vesicles, coils extraradical hyphae, and spore biovolume.

Source of variation	df†	Total intraradical colonization			Intraradical hyphae			Arbuscules		
		MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Between subjects										
Site	4	1.631	68.79	**<0.0001**	1.760	105.73	**<0.0001**	0.018	3.22	**0.0129**
Fertilization‡	1	0.055	3.12	**0.0842**	0.142	11.56	**0.0014**	0.017	2.94	**0.0953**
Site × fertilization	4	0.084	3.56	**0.0073**	0.087	5.23	**0.0004**	0.003	0.47	0.7553
Replicate (fertilization)	20	0.016	0.67	0.8593	0.011	0.65	0.8716	0.006	1.00	0.4585
Within subjects										
Year	1	1.767	74.52	**<0.0001**	0.581	34.89	**<0.0001**	0.217	38.14	**<0.0001**
Season (Year)	2	0.081	3.43	**0.0332**	0.023	1.41	0.2451	0.023	4.11	**0.0172**

Note: Boldface type indicates the factor was significant at $P < 0.05$.
† The numerator degrees of freedom for all factors except Fertilization was 365.
‡ The numerator degrees of freedom for Fertilization was 45.

point of the spore) were measured using an optical graticule in a light microscope. Spore length and width data were used to calculate biovolume (V) using the equation for a prolate spheroid, $V = (\pi l^2 w)/6$ (Bever and Morton 1999). The biovolume of an individual AM fungal species per sampling date, host plant, and treatment was calculated by multiplying spore abundance in the sample (number of spores per gram of soil) by the corresponding biovolume. Data within each size category were then summed for each sample.

Statistical analyses

Statistical analyses were performed using SAS JMP (SAS 1997) and SPSS 10.0 (SPSS 1999). Yearly and seasonal variation in AM structures among sites was assessed using repeated-measures mixed-model analyses of variance (ANOVA). Replicate was nested within N treatment as between-subject effects, and season was nested within year as within-subject effects. Replicate was treated as a random effect, and all other effects were treated as fixed. All intraradical AM structures were arcsine square-root transformed before analysis (Sokal and Rohlf 1995). Mycorrhizal structures were examined in the roots of two host plants per site. At no site was there a significant interaction between host species and N treatment, so data from the two species were combined. Soil nutrient data were analyzed using fixed ANOVAs followed by Tukey-Kramer honestly significant difference multiple comparison tests.

RESULTS

Ambient soil chemistry

When averaged across season, available soil NH_4^+ and NO_3^- were highest at Konza and lowest at Cedar Creek (Table 2). In contrast, available PO_4^{3-} was highest at Cedar Creek (fertilized annually with P_2O_5) and lowest at Konza and Sevilleta (Table 2). Accordingly, available soil N:P ratios were lowest at Cedar Creek, highest at Konza, and intermediate at the other sites. Soil pH was lowest at Cedar Creek and Konza and highest at Sevilleta.

Arbuscular mycorrhizal structures

Allocation to intra- and extraradical AM structures was significantly influenced by site and year (Table 3). Intraradical colonization varied greatly across the sampling dates and the sites (Fig. 2). Allocation to extraradical hyphae was greatest at Konza (Fig. 3), and allocation to spores was greatest at Cedar Creek (Fig. 4). Nitrogen enrichment accounted for a significant amount of the variance in intraradical hyphae ($P = 0.001$), vesicles ($P = 0.02$), and spore biovolume ($P < 0.0001$; Table 3). Depending upon the site and season, allocation to these structures increased, decreased, or were unaffected by N enrichment. There was a significant site × N treatment interaction for all of the AM structures except arbuscules and coils (Table 3). At Cedar Creek, dual N and P enrichment decreased total intraradical colonization ($P = 0.03$), arbuscules ($P < 0.0001$), vesicles ($P = 0.001$), extraradical hyphae ($P = 0.001$), and spores ($P < 0.0001$; Table 4, Figs. 2b, 3b, 4b). Nitrogen enrichment also decreased spore biovolume at Kellogg ($P = 0.001$), Shortgrass Steppe ($P < 0.0001$), and Sevilleta ($P = 0.007$), but it did not significantly affect total intraradical colonization at these three sites (Table 4, Figs. 2, 3, 4). In contrast, AM structures at Konza generally increased with N enrichment; total intraradical colonization ($P < 0.0001$), intraradical hyphae ($P < 0.0001$), and spore biovolume ($P = 0.05$) were higher in plots enriched with N and with both N and P (Table 4, Figs. 2c, 3c, 4c). The allometric ratio indicates that relative allocation to arbuscules, coils, and extraradical hyphae is significantly lower in N-enriched plots compared to ambient plots at Kellogg, Cedar Creek, and Sevilleta, but not at Konza or Shortgrass Steppe (Fig. 5).

No Gigasporaceae spores were recovered from the semiarid sites, Shortgrass Steppe and Sevilleta; however, spores from this family of AM fungi were par-

TABLE 3. Extended.

	Vesicles			Coils				Extraradical hyphae			Spore biovolume		
df†	MS	*F*	*P*	MS	*F*	*P*	df†	MS	*F*	*P*	MS	*F*	*P*
4	0.259	24.25	<**0.0001**	0.419	61.24	<**0.0001**	4	163 910	64.09	<**0.0001**	1.5×10^{14}	22.51	<**0.0001**
1	0.042	5.42	**0.0244**	0.004	0.52	0.4779	1	7746	3.06	**0.0889**	3.8×10^{14}	22.38	<**0.0001**
4	0.029	2.72	**0.0297**	0.009	1.35	0.2518	4	12 344	4.83	**0.0009**	8.1×10^{13}	12.20	<**0.0001**
20	0.007	0.63	0.8936	0.008	1.13	0.3120	20	2520	0.99	0.4797	2.1×10^{13}	3.20	<**0.0001**
1	0.264	24.76	<**0.0001**	0.093	13.62	**0.0003**	1	51 748	20.23	<**0.0001**			
2	0.024	2.29	0.1026	0.059	8.66	**0.0002**	2	4947	1.93	**0.1463**			

ticularly sensitive to N enrichment at the three mesic sites. At Cedar Creek, N enrichment decreased spore biovolume by more than 80% (Table 5). At Kellogg, Gigasporaceae were completely absent from N-enriched plots in mid-season, and late in the season these spores were reduced by more than 95% in the N-enriched plots. In contrast, both N and dual N and P enrichment at Konza dramatically increased Gigasporaceae spore volumes (Table 5).

DISCUSSION

Our findings indicate that across a wide range of grassland ecosystems, allocation to AM fungal structures is dynamic over time and may either increase or decrease in response to N enrichment. The seasonal and annual variability in the abundance of AM structures that we observed corroborates other studies (Bentivenga and Hetrick 1991) and cautions against extrapolating too much from studies based upon a single sampling date. The five LTER sites differ tremendously in climate, management history, plant composition, and soil fertility (Table 1). However, in terms of AM responses to N enrichment, the semiarid sites did not consistently differ from the mesic sites, nor did the old fields contrast with the native grasslands, or the C_4-dominated communities differ from the C_3-dominated communities. Ambient soil fertility, namely N:P ratio, was the overall best predictor of the effect of N enrichment on AM fungal allocation patterns. This finding is consistent with recent studies showing that AM fungal responses to N and P enrichment are mediated by ambient N and P availability (Miller et al. 2002, Treseder and Allen 2002).

Nitrogen enrichment increased allocation to AM structures at Konza where ambient soil N was highest and P availability lowest, and it tended to decrease AM fungal structures at the other sites and most strongly at Cedar Creek, the site with the lowest soil N:P ratio (Tables 2, 4). Many greenhouse trials have found that N enrichment increases AM colonization when P is limiting and decreases it when P is not limiting (Smith et al. 1986, Bååth and Spokes 1989, Sylvia and Neal 1990, Liu et al. 2000). Furthermore, P deficiency is known to cause plants to allocate more photosynthate to mycorrhizae (Hepper 1983, Schwab et al. 1991). Based upon these findings, N enrichment would be expected to increase plant allocation to mycorrhizae at Konza because adding N to the high N:P soils (N:P = 3.5) exacerbates P limitation and further increases the value of mycorrhizae for P uptake. The observation that AM structures often had intermediate levels in the Konza plots enriched with *both* N and P further supports the idea that the balance between N and P is involved in this response. In terms of soil N:P, Konza and Cedar Creek represent opposite extremes. At Cedar Creek *both* control and N enriched plots had been enriched with P, K, Ca, Mg, S, and micronutrients thus generating P-rich, N-poor soils (N:P = 0.3), so adding N to these soils eliminated nutrient limitation and caused plants to reduce their root:shoot ratio (Wilson and Tilman 1991). This shift in plant allocation would reduce photosynthate available to AM fungi, and consequently a N-induced reduction in AM fungal structures is expected at Cedar Creek.

If the functional equilibrium model applies to both plants and their associated AM fungi, then we predict that allocation to arbuscules, coils, and extraradical hyphae relative to total AM structures should decrease when plant reliance on AM uptake of nutrients is reduced by fertilization. Our results generally support this hypothesis. Nitrogen enrichment significantly reduced allocation to arbuscules, coils, and extraradical hyphae at Kellogg, Cedar Creek, and Sevilleta but not at Konza, where N enrichment is expected to accentuate the role of mycorrhizae for P uptake, or at Shortgrass Steppe, a relatively fertile site that has not received additional N fertilizer in over 20 years (Fig. 5). There is currently no method to link AM structures to their functioning in field studies. Consequently, finding an allometric pattern that corresponds to a functional prediction is noteworthy because it suggests that relative allocation to AM structures could be a useful approach for linking AM structure and functioning. However, many more studies are necessary to thoroughly develop this approach. This method could be further refined if AM colonization is measured as centimeters of colo-

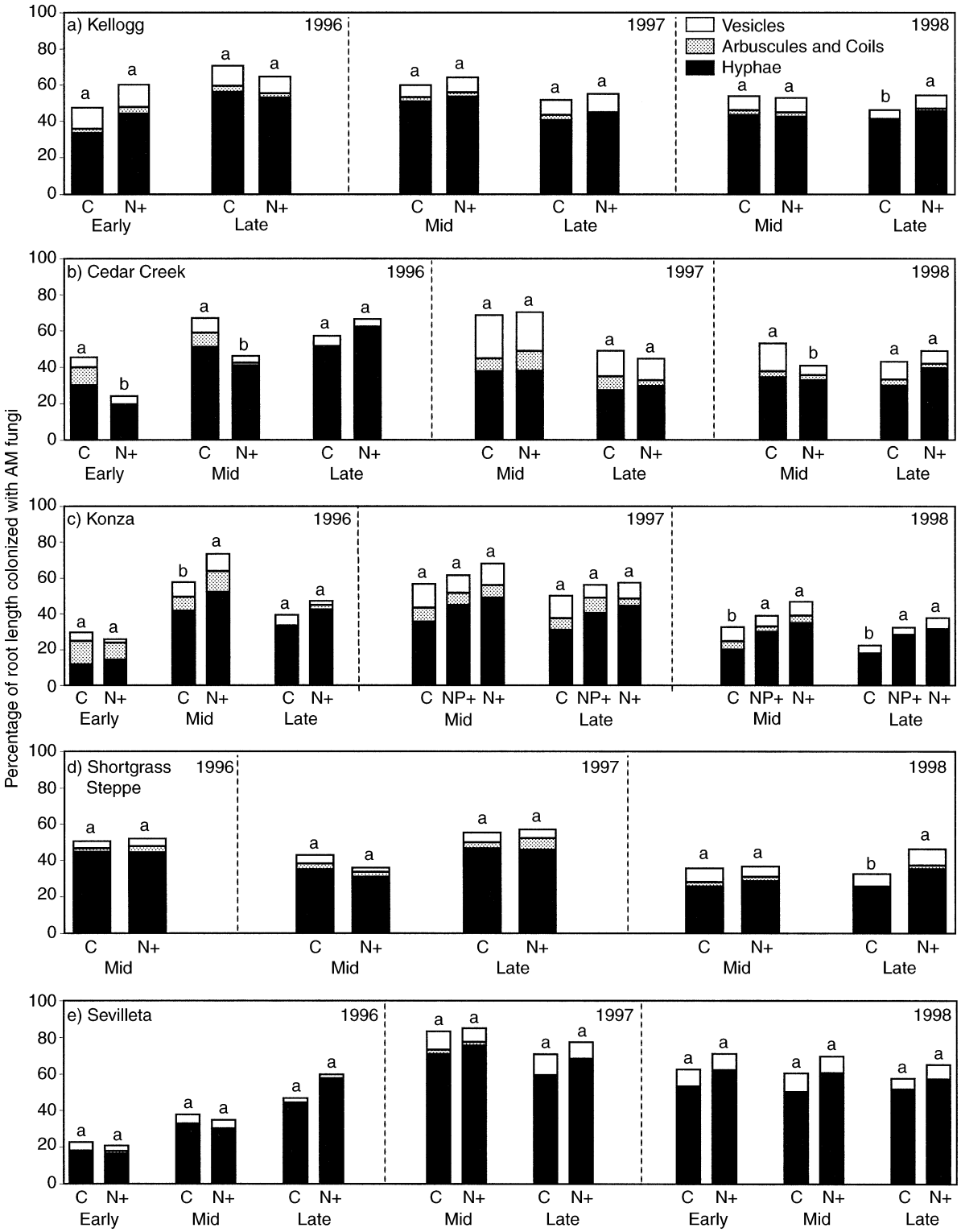


FIG. 2. Three years of intraradical arbuscular mycorrhizal (AM) structures in N-enriched (N+), N- and P-enriched (at Konza, NP+), and control (C) plots at five Long Term Ecological Research (LTER) sites in the United States. Analyses were made early (April–May), in the middle (June–July), and late (August–December) in the growing season. Within each bar, the percentage of colonization by hyphae, arbuscules and coils, and vesicles are represented by solid, shaded, and open sections, respectively. Within each site and sampling date, different lowercase letters indicate that the control plots and N-enriched plots differ at $P \leq 0.1$.

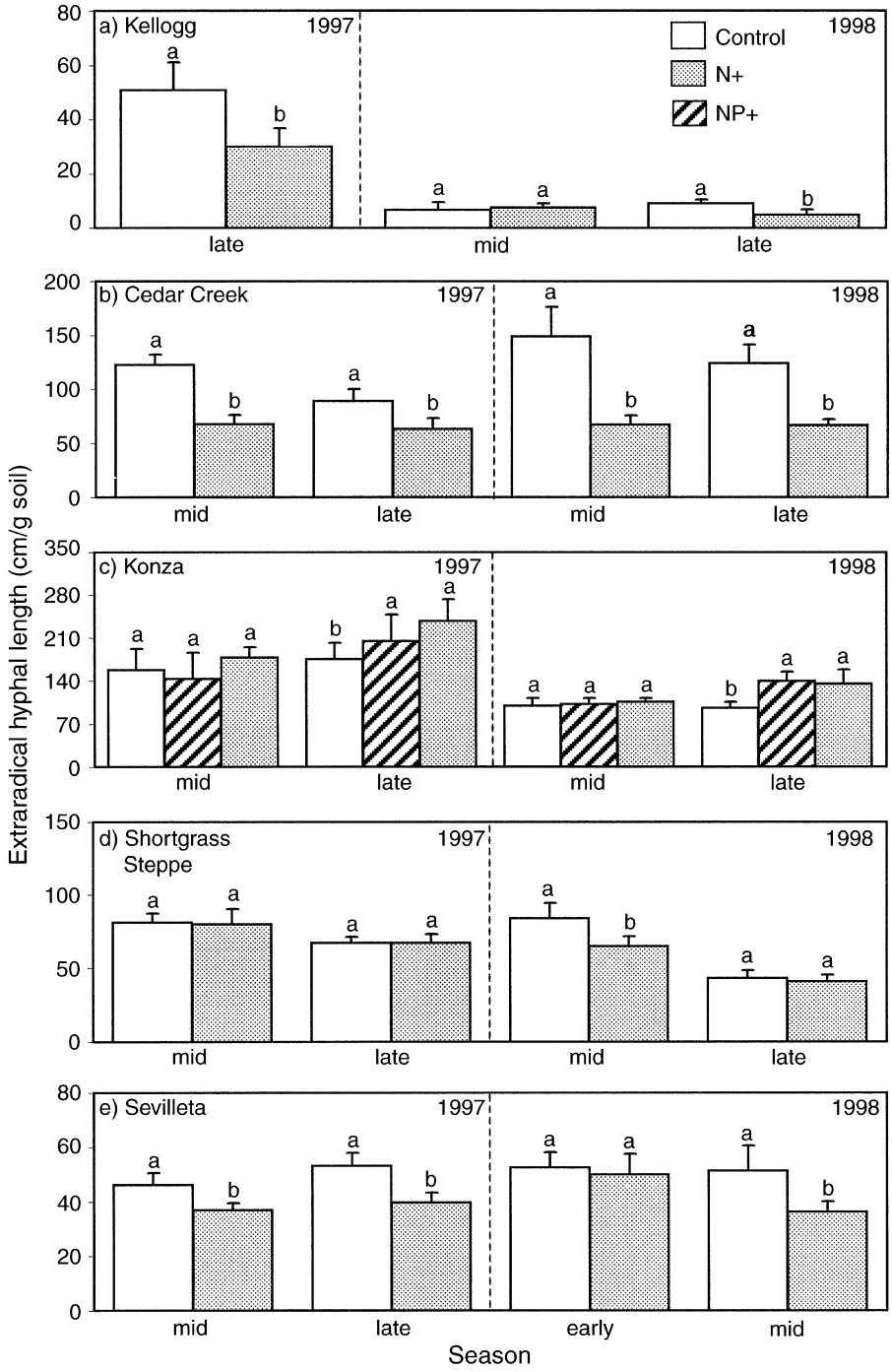


FIG. 3. Two years of extraradical hyphal lengths in N-enriched (N+), N- and P-enriched (at Konza, NP+), and control plots at five Long Term Ecological Research (LTER) sites in the United States (means + 1 SE). Analyses were made early (April–May), in the middle (June–July), and late (August–December) in the growing season. Within each site and sampling date, different lowercase letters indicate that the control plots and N-enriched plots differ at $P \leq 0.1$. Note that the *y*-axis scales vary among graphs.

nized root length per gram of soil (instead of percentage of colonized root length) so that both intraradical and extraradical structures can be expressed using the same units. Also, mechanistic studies are required to determine the scale(s) (phenotypic, genotypic, and/or community) at which AM allocation responds to perturbations and the functional ramifications of these responses.

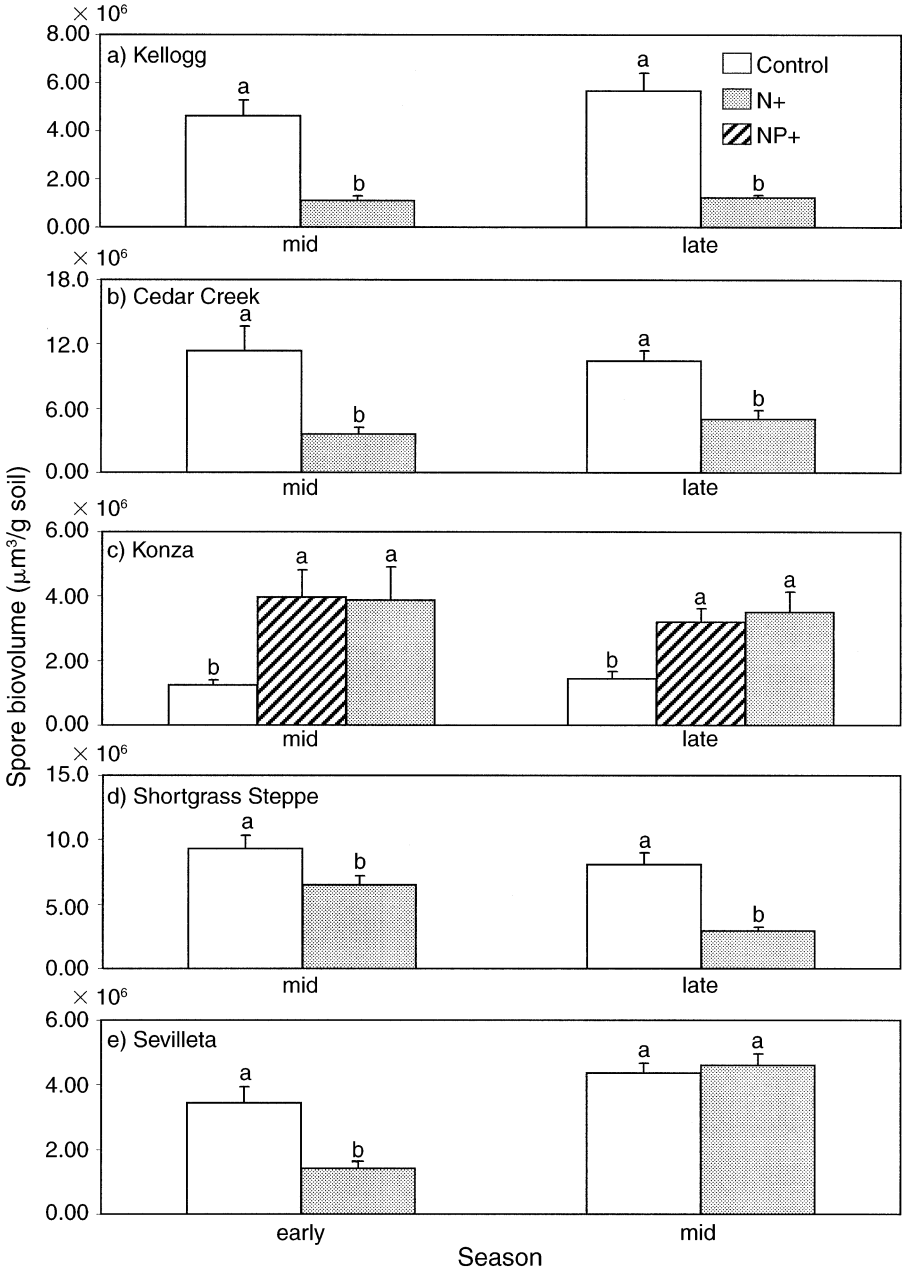


FIG. 4. Total volume of arbuscular mycorrhizal (AM) fungal spores in N-enriched (N+), N- and P-enriched (at Konza, NP+), and control plots at five Long Term Ecological Research (LTER) sites in the United States (values are expressed as millions of cubic microns per gram of soil; means + 1 SE). Analyses were made early (April–May), in the middle (June–July), and late (August–December) in the 1998 growing season. Within each site and sampling date, different lowercase letters indicate that the control plots and N-enriched plots differ at $P \leq 0.05$. Note that *y*-axis scales vary among graphs.

Grassland communities of AM fungi are composed of dozens of species (Walker et al. 1982, Johnson et al. 1991, Eom et al. 2000) with vastly different morphologies and life history characteristics (Abbott and Robson 1985, Hart et al. 2001). Our spore analysis indicates that N enrichment significantly altered the species composition of AM fungal communities at all five LTER sites (L. M. Egerton-Warburton and N. C. Johnson, *unpublished data*). Gigasporaceae were very sensitive to N enrichment at the three mesic sites, decreasing with N at Cedar Creek and Kellogg and increasing with N at Konza (Table 5). This finding corroborates spore analyses in separate studies at Cedar Creek and Konza (Johnson 1993, Eom et al. 1999) and also helps explain patterns with extraradical hyphae and vesicles. A study of 21 AM fungal taxa showed

TABLE 4. Responses of intraradical arbuscular mycorrhizal (AM) structures, extraradical hyphae, spore biovolume, and non-AM fungi to long-term N enrichment at the five Long Term Ecological Research (LTER) study sites.

Site	Total intraradical colonization	Intraradical hyphae	Arbuscules and coils	Vesicles	Extraradical hyphae	Spores
Kellogg Biological Station	NS (0.14)	↑ (0.07)	NS (0.57)	NS (0.45)	↓ (0.07)	↓ (0.001)
Cedar Creek	↓ (0.03)	NS (0.99)	↓ (0.0001)	↓ (0.001)	↓ (0.001)	↓ (0.0001)
Konza Prairie	↑ (0.0001)	↑ (0.0001)	NS (0.60)	↓ (0.01)	↑ (0.10)	↑ (0.048)
Shortgrass Steppe	NS (0.46)	NS (0.38)	↑ (0.06)	NS (0.15)	NS (0.19)	↓ (0.0001)
Sevilleta	NS (0.17)	↑ (0.06)	NS (0.19)	↓ (0.04)	↓ (0.02)	↓ (0.0007)

Notes: The table presents a summary of repeated-measures ANOVAs combining all three years of data for each site. The symbol ↓ indicates that a structure significantly decreased with N enrichment, ↑ indicates a significant increase with N enrichment, and NS indicates there was not a significant ($P \leq 0.1$) effect of N on the abundance of the structure. Probability values for the F ratios of the N effect are included in parentheses.

that Gigasporaceae produced four times more extraradical hyphae than Glomaceae and Acaulosporaceae (Hart and Reader 2002). Both Gigasporaceae spores and extraradical hyphae decreased with N enrichment at Kellogg and Cedar Creek, and both increased with N enrichment at Konza, suggesting that shifts in the prevalence of Gigasporaceae may be an important driver of this response. Furthermore, N enrichment at Konza significantly decreased allocation to vesicles, yet it consistently increased allocation to all other AM structures. This finding might be explained by the fact that members of the Gigasporaceae do not form vesicles, and thus, as Gigasporaceae become more abundant in Konza's N-enriched plots, vesicles should decrease in abundance.

At three of the sites, long-term N enrichment caused replacement of the dominant grass species: from *Andropogon scoparius* to *Agropyron repens* at Cedar Creek, from *Andropogon gerardii* to *Panicum virgatum* at Konza, and from *Bouteloua gracilis* to *Elymus elymoides* at Shortgrass Steppe (Wilson and Tilman 1991, Gibson et al. 1993, Milchunas et al. 1990). Are these plant community shifts related to altered mycorrhizal functioning? Results of a series of greenhouse experiments indicate that N enrichment diminishes the mutualistic functioning of communities of AM fungi at Sevilleta and Shortgrass Steppe (Corkidi et al. 2002), at Cedar Creek (Johnson 1993), and at Kellogg (N. C. Johnson, D. L. Rowland, L. Corkidi, and E. B. Allen, *unpublished manuscript*). Furthermore, in California shrublands, three years of N enrichment resulted in a significant decline in species richness of AM fungi (Egerton-Warburton and Allen 2000, Egerton-Warburton et al. 2001) and alterations in mycorrhizal function (Sigüenza 2000). Importantly, AM changes were observed before any shifts in the composition of the aboveground plant community could be perceived.

Conclusions

Arbuscular mycorrhizae conform to the functional equilibrium model; allocation to arbuscules, coils, and extraradical hyphae relative to total AM fungal allocation are reduced when fertilization reduces the value of AM for nutrient uptake. Mycorrhizal response to N enrichment is mediated by ambient soil fertility. Nitrogen enrichment generally decreases allocation to AM structures at sites with ample soil P, but it increases allocation to mycorrhizae at sites with P-deficient soils. Extraradical structures (hyphae and spores) are more responsive to N enrichment than are intraradical structures. This response could be caused by allocation plasticity within individual AM fungal taxa, changes in the species composition of the AM fungal community, or both. Allometric analyses appear to be a promising approach for linking measurements of mycorrhizal structures with their function. Mechanistic experiments are needed to more thoroughly develop this approach.

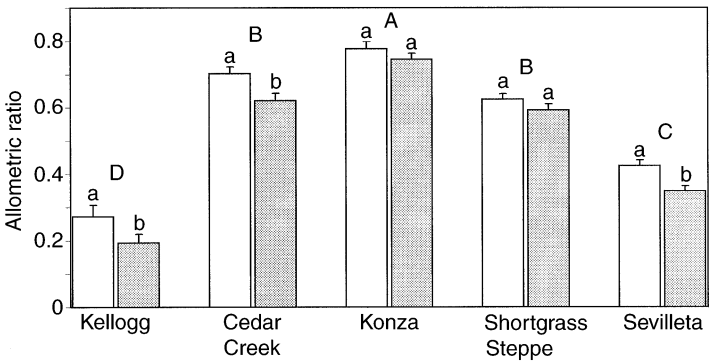


FIG. 5. Fungal allocation to structures involved with nutrient uptake and transfer relative to total colonization: (arbuscules + coils + extraradical hyphae)/(total intraradical colonization + extraradical hyphae) at five Long Term Ecological Research (LTER) sites in the United States (means + 1 SE). Open bars are from control plots, and shaded bars are from N-enriched plots. Within each site, different lowercase letters indicate that plants grown in soils from the control plots and the N-enriched plots differ at $P \leq 0.1$. Different uppercase letters indicate that the sites differ at $P \leq 0.1$.

TABLE 5. Biovolume of Gigaspora spores (>200 μm diameter) in the N-enriched (N+, NP+) and control plots at the three mesic sites in the middle of and late in the 1998 growing season.

Site and season	Size (μm)	Spore biovolume ($\mu g^3/g$)			
		Control	N+	NP+	*P*
Kellogg Biological Station					
Mid	200–250	727	0	···	<0.0001
	>250	6174	0	···	<0.0001
Late	200–250	865	24	···	<0.0001
	>250	4603	216	···	0.0562
Cedar Creek					
Mid	200–250	2562	273	···	<0.0001
	>250	6914	1531	···	<0.0001
Late	200–250	2673	546	···	<0.0001
	>250	7179	1247	···	<0.0001
Konza Prairie					
Mid	200–250	110	0	121	0.0466
	>250	184	662	253	0.0044
Late	200–250	0	0	31	<0.0001
	>250	0	395	519	<0.0001

Note: A paired *t* test was used to determine differences in spore biovolume between N-enriched and control plots, except at Konza, where one-way ANOVA was used.

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