

## MYCORRHIZAL COMMUNITY DYNAMICS FOLLOWING NITROGEN FERTILIZATION: A CROSS-SITE TEST IN FIVE GRASSLANDS

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**Abstract.** Arbuscular mycorrhizal fungi (AMF) are considered both ecologically and physiologically important to many plant communities. As a result, any alteration in AMF community structure following soil nitrogen (N) enrichment may impact plant community function and contribute to widespread changes in grassland productivity. We evaluated the responses of AMF communities to N fertilization ( $\geq 100 \text{ kg N} \cdot \text{ha}^{-1} \cdot \text{yr}^{-1}$ ) in five perennial grasslands within the Long-Term Ecological Research network to generate a broader understanding of the drivers contributing to AMF species richness and diversity with increasing soil N fertility, and subsequent effects to host-plant communities. AMF spore and hyphal community data at three mesic sites (Cedar Creek, Kellogg Biological Station, Konza Prairie) and two semiarid sites (Sevilleta, Shortgrass Steppe) were collected over two consecutive years and used to test four hypotheses about AMF responses to N fertilization. Under ambient soil N, plant annual net primary productivity and soil phosphorus (P) were strongly related to climatic differences in AMF communities (semiarid vs. mesic). Following N fertilization, the drivers of AMF community structure were soil N availability, N:P supply ratio, and host-plant photosynthetic strategy ( $C_3$  vs.  $C_4$ ) but not climate. In P-rich soils (low N:P), N fertilization reduced AMF productivity, species richness, and diversity and intensified AMF community convergence due to the loss of rare AMF species and the increased abundance of *Glomus* species. In P-limited soils (high N:P), AMF productivity, species richness, and diversity increased with N fertilization; the most responsive AMF taxa were *Acaulospora*, *Scutellospora*, and *Gigaspora*. Soil N or N:P  $\times$  host-plant ( $C_3$ ,  $C_4$ ) interactions further modified these responses: AMF hyphae (primarily Gigasporaceae) associated with  $C_3$  plants increased in abundance with N fertilization, whereas  $C_4$  plants hosted nitrophilous *Glomus* species. Such responses were independent of the duration or quantity of N fertilization, or the time since cessation of N fertilization. This synthesis provides a new understanding of AMF community patterns and processes, and it identifies three key drivers (soil N, N:P, host plant) of AMF community structure that may be tested in other communities.

**Key words:** AMF response to N fertilization; arbuscular mycorrhizal fungi, AMF;  $C_3$  vs.  $C_4$  species and AMF; community structure; diversity; extraradical hyphae; grassland productivity and AMF dynamics; LTER sites; soil N:P; species richness.

### INTRODUCTION

Human sources of nitrogen (N) now rival or exceed the natural inputs of N into many terrestrial ecosystems. Anthropogenic N enrichment impacts broad geographic regions and its consequences are clearly visible within terrestrial ecosystems in the alterations in plant community productivity, species diversity, and dominance (Vitousek et al. 1997). In contrast to the rich empirical and theoretical knowledge base for the aboveground biota, few comparable efforts have been undertaken on the less observable, but equally important, belowground biota (Jackson et al. 2000, Norby and Jackson 2000).

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This study examines the influence of N enrichment on one particular group of belowground organisms, arbuscular mycorrhizal fungi (AMF), a ubiquitous root symbiont. Mycorrhizae are considered both ecologically and physiologically important to plant communities (Smith and Read 1997). For example, a species-rich AMF community positively contributes to a diverse and productive plant community (van der Heijden et al. 1998b) because each fungal species has a unique functional trait and utilizes a different component of the resource base (Streitwolf-Engel et al. 1997, van der Heijden et al. 1998a). It follows that any changes in AMF community composition with N enrichment could have significant consequences for plant community function (Johnson 1993, Corkidi et al. 2002, Johnson et al. 2003).

TABLE 1. The long-term ecological research (LTER) sites, their geographic coordinates, annual precipitation, soil nutrient status, and host plants sampled for arbuscular mycorrhizal fungi (AMF) community structure.

LTER site (state)	Latitude (°N)	Longitude (°W)	Elevation (m)	Precipitation (mm/yr)	Fertilizer rate <sup>†</sup> (kg·ha <sup>-1</sup> ·yr <sup>-1</sup> )	
					Type	Period
Sevilleta National Wildlife Refuge (New Mexico), SEV	33°89′	106°56′	1403	255	N, 100	1998–present
Shortgrass Steppe (Colorado), SGS	40°49′	104°46′	1650	322	N, 100–150	1971–1975
Cedar Creek Natural History Area (Minnesota), CDR	45°29′	93°26′	279	660	N, 170	1986–present
W. K. Kellogg Biological Station (Michigan), KBS	42°24′	85°22′	288	890	N, 120	1989–present
Konza Prairie Research Natural Area (Kansas), KZA	39°18′	96°57′	340	814	N, 100 P, 10	1986–present

<sup>†</sup> Fertilizer type: “N” denotes nitrogenous fertilizer; “P” denotes phosphorus-containing fertilizer.

<sup>‡</sup> KCl-extractable NO<sub>3</sub> + NH<sub>4</sub>, and HCO<sub>3</sub>-extractable P; –N, no nitrogen fertilizer applied, and +N, nitrogen fertilizer applied.

Hayman (1970, 1982) first noted that AMF species richness decreased as soil resource availability and plant productivity increased. Subsequent N-fertilization experiments and studies of AMF community responses to anthropogenic N enrichment have mostly supported this pattern (Johnson et al. 1991, 2003, Johnson 1993, Egerton-Warburton and Allen 2000, Sigüenza 2000), but exceptions do exist. For example, Eom et al. (1999) and Johnson et al. (2003) found that N fertilization increased AMF species richness and biomass, respectively, in a tallgrass prairie with low soil-P availability. Similarly, experiments focusing on the individual or combined effects of N and P have indicated that AMF external hyphal abundance and composition, and root colonization patterns, may also demonstrate positive, negative, or even neutral responses to fertilization (Mosse and Phillips 1971, Bååth and Spokes 1989, Sylvia and Neal 1990, Corkidi et al. 2002, Treseder and Allen 2002, Johnson et al. 2003). Several factors, including host specificity, plant community composition, and soil N:P ratio have been used to explain these opposing responses (Eom et al. 1999, 2000, Miller et al. 2002, Johnson et al. 2003). This inability to disentangle the effects of various ecological and environmental drivers, however, impedes our ability to both explain the current patterns of AMF response to N or to predict future shifts in AMF community structure with increasing global change.

Our present study can clarify whether and how AMF community responses to N fertilization might contribute to widespread changes in grassland productivity with global change. Much of what is known about the AMF community in grasslands concerns the patterns of response to N within a single site or year (e.g., Johnson et al. 1991) or region (Egerton-Warburton and Allen 2000). Far less is known about the common drivers that contribute to AMF species richness and diversity or their temporal dynamics with increasing soil N fertility. In this study, we followed the responses of AMF communities to N fertilization in two semiarid and

three mesic grasslands over two consecutive years. We began by examining the relationship between N fertilization and AMF abundance and species richness to put our analyses within the context of previous studies. Next, we determined if AMF community responses to N might be explained by single factors including soil nutrient status (N, P, N:P), plant functional growth strategy (C<sub>3</sub>, C<sub>4</sub>) or the cumulative effects of local resources (plant community productivity), or higher-order interactions among these factors. We used these soil and plant traits since they correspond, to some degree, with the factors most frequently given importance in general theories of community structure, namely, disturbance, resources, abiotic filters, and heterogeneity (Tilman 1988). Finally, we consider the implications of our results for grasslands. Throughout, we used two measures of AMF community structure, namely, abundance and species richness, within the spore community and external hyphal pool.

To underpin AMF community responses to N fertilization across the sites, we tested four hypotheses. Our initial hypothesis (H1) was that AMF community structure under ambient soil N varies as a function of climatic variables so that AMF productivity will covary with annual net plant productivity (ANPP). We also hypothesized that the direction and magnitude of change in AMF species diversity to N fertilization would be related to the initial soil P fertility and any subsequent changes in soil N:P ratio (H2). Under P-limited conditions, the investment of a host plant in an AMF genus or species should be strengthened to maintain the uptake of plant-limiting nutrients (Johnson et al. 2003). Adding N fertilizer to low-P soils will therefore enhance belowground C allocation whereas applying N fertilizer to high-P soils will reduce C allocation belowground. These effects should persist, even after N fertilization has ceased, owing to the legacies of N on plant ANPP (H2a; Milchunas and Lauenroth 1995).

TABLE 1. Extended.

Ambient soil level‡			Sampled plant taxa (functional group)
N ( $\mu\text{g/g}$ )	P ( $\mu\text{g/g}$ )	N:P	
-N 19	7	3.4	<i>Bouteloua gracilis</i> (C <sub>4</sub> )
+N 27			<i>B. eriopoda</i> (C <sub>4</sub> )
-N 14	18	0.9	<i>B. gracilis</i> (C <sub>4</sub> )
+N 17			<i>Elymus elymoides</i> (C <sub>3</sub> )
-N 11	39	0.3	<i>Andropogon scoparium</i> (C <sub>4</sub> )
+N 13			<i>Agropyron repens</i> (C <sub>3</sub> )
-N 17	18	1.3	<i>Poa compressa</i> (C <sub>3</sub> )
+N 31			<i>Agropyron repens</i> (C <sub>3</sub> )
-N 42	11	4.1	<i>Andropogon gerardii</i> (C <sub>4</sub> )
+N 64			<i>Panicum virgatum</i> (C <sub>4</sub> )

If AMF communities respond similarly to N over multiple years, then one might hypothesize that increases in soil N supply will lead to the pronounced increase in the abundance of a suite of N-responsive AMF taxa with particular strategies, habitats, or growth rates across all sites (H3). Any sensitivity to N enrichment, as measured by species richness, should therefore be demonstrated by the loss of rare or unique species (H3a). Moreover, the responses of AMF spores and hyphae to N enrichment should show similar patterns because both structures reflect the AMF biomass of their respective species (H3b).

Finally, intrinsic differences in plant photosynthetic biochemistry and mycorrhizal dependency should lead to markedly different AMF responses to N fertilization in C<sub>3</sub> and C<sub>4</sub> plants (H4); C<sub>3</sub> grasses are generally more disturbance adapted and nitrophilic whereas C<sub>4</sub> grasses generally benefit more from AMF than do C<sub>3</sub> grasses (Wilson and Hartnett 1998). For our study, we proposed the hypothesis that AMF associated with C<sub>3</sub> grasses should respond more to increases in N supply than AMF associated with C<sub>4</sub> grasses (H4a; Wedin and Tilman 1996) because C<sub>3</sub> plants are more likely to reduce C allocation to their AMF with increasing soil fertility than C<sub>4</sub> plants (Graham et al. 1997). In addition, these differences may be larger in mesic than semiarid grasslands because mesic sites are generally N limited whereas semiarid systems are primarily water limited (H4b).

## METHODS

### Study sites

We conducted our research in 1997 and 1998 at five sites located within the Long-Term Ecological Research (LTER) network. The aboveground net primary productivity at each site tends to be limited by N, and the sites provide a range of environmental conditions with respect to annual precipitation, effective growing season, annual net plant productivity (ANPP), and intrinsic soil fertility (Table 1).

Sevilleta (SEV) and the Shortgrass Steppe (SGS) are representative of semiarid ecosystems where evapotranspiration exceeds measured precipitation for most of the

year. The SEV grassland is dominated by *Bouteloua gracilis* (H.B.K.) Lag. and *B. eriopoda* (Torr.) Torr., while vegetation at SGS is a mosaic of shortgrasses composed primarily of *B. gracilis* with *Aristida purpurea* Nutt., *Pascopyrum smithii* Rydb. (Love), and *Elymus elymoides* (Rafin.) Swezey (Lauenroth et al. 1978).

Konza Prairie (KZA), Kellogg Biological Station (KBS), and Cedar Creek (CDR) are typically mesic environs where up to 75% of the annual precipitation is received during the growing season (May to October). Vegetation varies with topographic relief at KZA, but for the most part represents a tallgrass prairie dominated by *Andropogon gerardii* Vitm., *Schizachyrium scoparium* (Michx.) Nash, and *Panicum virgatum* L. (Freeman and Hulbert 1985). Cedar Creek incorporates a mosaic of ecosystems including successional grasslands and tallgrass prairies. Our study site was a successional grassland-old-field system last cropped to soybean in 1957 and dominated by *Schizachyrium scoparium* (Wilson and Tilman 1991). Kellogg Biological Station is primarily utilized to evaluate discrete cropping systems of corn-soybean-wheat, but our study sites were in successional old-field communities that were last cropped in 1989 (Huberty et al. 1998).

AMF communities were assessed in experimental fertilization plots maintained at CDR, KBS, KZA, and SGS, and in plots that we initiated in 1995 at SEV (Table 1). The fertilization regimes, summarized here, are detailed in Johnson et al. (2003). At SGS, we evaluated plots that were fertilized with  $\text{NH}_4\text{NO}_3$  at rates of  $100\text{--}150 \text{ kg N}\cdot\text{ha}^{-1}\cdot\text{yr}^{-1}$  between 1971 and 1975. At CDR, KBS, KZA, and SEV, N fertilization was applied annually as granular  $\text{NH}_4\text{NO}_3$  at rates of at least  $100 \text{ kg N}\cdot\text{ha}^{-1}\cdot\text{yr}^{-1}$ . At CDR, both N-fertilized and non-fertilized plots received K, Ca, Mg, S, and micronutrients, and P at an application rate of  $200 \text{ kg P}\cdot\text{ha}^{-1}\cdot\text{yr}^{-1}$  (as  $\text{P}_2\text{O}_5$ ). In addition, experimental N+P fertilization plots at KZA were sampled as part of this study. These plots received  $100 \text{ kg N}\cdot\text{ha}^{-1}\cdot\text{yr}^{-1}$  as  $\text{NH}_4\text{NO}_3$  and  $10 \text{ kg P}\cdot\text{ha}^{-1}\cdot\text{yr}^{-1}$  as  $\text{P}_2\text{O}_5$ .

Extractable inorganic soil N and P concentrations are given in Table 1. Ambient soil N levels varied from  $11 \mu\text{g/g}$  at CDR to  $42 \mu\text{g/g}$  at KZA. At SEV, SGS, and KZA, N was present predominantly in the reduced form ( $\text{NH}_4$ ), while soils at CDR and KBS contained N primarily as  $\text{NO}_3$ . Fertilization significantly increased soil N at SEV, KZA, and KBS and to a lesser extent at CDR, and the residual effects of N enrichment were still evident at SGS ( $P < 0.05$ ,  $t$  test; Table 1). The difference in soil inorganic-N levels ( $\text{NH}_4 + \text{NO}_3$ ) between non-fertilized and N-fertilized plots at SGS appeared to be small. Nevertheless, higher rates of soil N and carbon (C) mineralization, total soil N, and high levels of plant tissue N relative to C in the N-fertilized vs. non-fertilized plots indicate that the effects of N fertilization have indeed persisted over time (Vinton and Burke 1995, Johnson et al. 2003).

### Collection of soil samples

We sampled root and rhizosphere soil from two grass species at each site (Table 1). The species' selection included the warm-season C<sub>4</sub> (*Andropogon*, *Bouteloua*, *Panicum*, *Schizachyrium*) and cool-season C<sub>3</sub> grasses (*Agropyron*, *Poa*, *Elymus*) to take into account any differences in host mycorrhizal responses (Wilson and Hartnett 1998). Composite samples of soil and roots, each 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) to 15 cm deep, since the majority of AMF propagules can be recovered from this depth (An et al. 1990). Ten replicates (five from each grass species) from each treatment were collected. Soils were placed in plastic bags and frozen (−20°C) within 48 h of collection. In 1997 and 1998, samples were collected from each site in the middle of (June–July) and late in (August–December) the growing season so that AMF species with different sporulation times (cool- and warm-season species; Pringle and Bever 2002) would be included in our survey.

### AMF spore abundance and community composition

AMF abundance and community composition was evaluated by identifying the asexual spores to genus or species. Molecular methods have recently been used to estimate AMF community composition. However, these methods may lack sufficient sensitivity because they only detect the most abundant taxa (e.g., DNA arrays), or they introduce bias through PCR (polymerase chain reaction; e.g., single-strand conformation polymorphisms; Kirk et al. 2004). Such methods are also costly and are thus not yet practical for studies that require processing and analyzing a large number of samples.

Arbuscular mycorrhizal spores were extracted from 5-g subsamples of soil using sucrose density-gradient centrifugation, and mounted in a 1:1 solution of polyvinyl alcohol (PVA): Meltzer's solution under glass coverslips to make permanent slides (Egerton-Warburton and Allen 2000). The prepared spores were viewed under a compound microscope (400×–1000× with differential interference contrast optics) and identified to species level by comparison with authenticated samples held in a reference collection and using current taxonomic criteria (*available online*).<sup>6</sup> Spores that could be positioned phylogenetically but not identified to species level were labeled by genus and in numerical sequences. For example, unmatched *Glomus* species became *Glomus* 1, 2, 3, and so on. Spore samples are maintained in permanent collections.

The abundance of each species within a sample was then converted into biovolume, since biovolume can be a proxy for biomass (Van Veen and Paul 1977). The biovolume of each AMF spore species was calculated using the equation for a prolate spheroid (Bever and

Morton 1999). In turn, the total spore biovolume of an AMF species within each sample was calculated by multiplying its biovolume with the corresponding relative abundance (RA). RA was calculated as the number of spores of an individual AMF taxon divided by the total number of AMF spores within a sample. In total, 75 890 spores, representing 63 species from six genera (Table 2), were recovered and identified from soil samples during the study.

### Abundance and community composition of AMF hyphae

The abundance and community composition of extraradical hyphae (mycorrhizal fungal hyphae that occur outside the root, either on the surface of the root or in the soil) was evaluated using direct immunofluorescence. This technique identifies AM fungi to genus level, and only live hyphae will fluoresce after incubation in immune serum. Polyclonal antibodies were raised against each of the four major genera of AM fungi using whole-spore fractions of *Glomus deserticola* Trappe, Bloss & Menge, *Acaulospora laevis* Gerdemann & Trappe, *Gigaspora margarita* Becker & Hall, and *Scutellospora calospora* (Nicol. & Gerd.) Walker & Sanders, and each conjugated to fluorescein isothiocyanate (FITC; detailed in Egerton-Warburton and Allen [2000]).

Extraradical hyphae were extracted from duplicate subsamples of each soil core. For an individual subsample, 5 g of soil were suspended in 200 mL of sodium hexametaphosphate (39.5 g/L) for one hour, washed through a 250-μm mesh, then resuspended in 300 mL of distilled water, left to settle for 15 s, and decanted through a 28-μm sieve (Frey and Ellis 1997). Hyphae were rinsed out of the sieve into 15 mL of distilled water and then concentrated by pulse centrifuging, to produce a final volume of 1 mL of hyphal suspension in double-distilled water. We placed 200-μL of hyphal suspension in each of five microfuge tubes. To each tube we added 200 μL of an undiluted single antiserum so that each set of four tubes represented screening for *Glomus*, *Scutellospora*, *Gigaspora*, and *Acaulospora*. The fifth tube contained deionized water (control) to detect any autofluorescence in AM hyphae.

The tubes were wrapped in foil and incubated for 24 h at room temperature, after which each sample was filtered and rinsed over an individual 1.2-μm pore membrane, and the membrane mounted in glycerol containing propyl gallate (0.1% mass/volume) to prevent fading. Each sample was viewed using a Zeiss Axioskop 2 microscope (200×–400×) equipped with an FITC filter set (480-nm excitation; 535-nm emission; 505-nm dichroic mirror), and scored for the presence and abundance of positively labeled (intense green fluorescence) and unlabeled (non-fluorescent) hyphae. Forty randomly located fields of view were scored using the gridline intercept method and converted to hyphal length (in meters) of each AMF genus per gram dry mass of soil (Tennant 1975). Hyphal-length data were

<sup>6</sup> (<http://invam.caf.wvu.edu/>)

TABLE 2. The abundance of AMF spore species in semi-arid and mesic grassland sites, by family.

AMF species	Species abundance (no./g dry soil)†		
	Semiarid	Mesic	Significance
<b>Acaulosporaceae</b>			
<i>Acaulospora appendicula</i> Spain, Sieverding & Schenck	ND	0.18 ± 0.02	
<i>Ac. elegans</i> Trappe & Gerdemann	ND	0.29 ± 0.08	
<i>Ac. foveata</i> Trappe & Janos	ND	0.11 ± 0.02	
<i>Ac. laevis</i> Gerdemann & Trappe	ND	0.06 ± 0.01	
<i>Ac. longula</i> Spain & Schenck	ND	0.05 ± 0.01	
<i>Ac. morrowiae</i> Spain & Schenck	ND	0.06 ± 0.01	
<i>Ac. scrobiculata</i> Trappe	0.05 ± 0.01	0.07 ± 0.01	
<i>Ac. spinosa</i> Walker & Trappe	ND	0.06 ± 0.02	
<i>Acaulospora</i> sp. 1	ND	0.05 ± 0.01	
<i>Enterophospora infrequens</i> (Hall) Ames & Schneider	0.12 ± 0.03	0.06 ± 0.02	*
<b>Archaeosporaceae</b>			
<i>Archaeospora gerdemannii</i> (Rose, Daniels & Trappe) Morton & Redecker	ND	0.02 ± 0.02	
<i>Ar. leptoticha</i> (Schenck & Smith) Morton & Redecker	1.06 ± 0.10	0.70 ± 0.08	*
<b>Gigasporaceae</b>			
<i>Gigaspora albida</i> Schenck & Smith	ND	0.07 ± 0.01	
<i>Gi. margarita</i> Becker & Hall	ND	0.15 ± 0.03	
<i>Gi. gigantea</i> (Nicol. & Gerd.) Gerd. & Trappe	ND	0.47 ± 0.06	
<i>Scutellospora erythropha</i> (Koske & Walker) Walker & Sanders	ND	0.20 ± 0.04	
<i>Sc. persica</i> (Koske & Walker) Walker & Sanders	ND	0.24 ± 0.03	
<i>Sc. calospora</i> (Nicol. & Gerd.) Walker & Sanders	ND	0.28 ± 0.03	
<i>Sc. pellucida</i> (Nicol. & Schenck) Walker & Sanders	ND	1.40 ± 0.18	
<i>Scutellospora</i> sp. 1	ND	0.29 ± 0.05	
<b>Glomaceae</b>			
<i>Glomus aggregatum</i> Schenck & Smith	18.96 ± 1.50	3.63 ± 0.49	***
<i>Gl. albidum</i> Walker & Rhodes	0.45 ± 0.05	0.30 ± 0.01	*
<i>Gl. ambisporum</i> Smith & Schenck	0.15 ± 0.03	1.04 ± 0.09	***
<i>Gl. claroideum</i> Schenck & Smith	0.82 ± 0.08	0.60 ± 0.03	*
<i>Gl. clarum</i> Nicol. & Schenck	1.64 ± 0.15	0.43 ± 0.03	***
<i>Gl. constrictum</i> Trappe	0.01 ± 0.01	ND	
<i>Gl. coremioides</i> (Berk. & Broome) Redecker & Morton	0.02 ± 0.02	0.07 ± 0.01	*
<i>Gl. deserticola</i> Trappe, Bloss & Menge	4.51 ± 0.59	ND	
<i>Gl. diaphanum</i> Morton & Walker	0.13 ± 0.02	0.02 ± 0.01	***
<i>Gl. eburneum</i> Kennedy, Stutz & Morton	0.16 ± 0.03	0.24 ± 0.06	*
<i>Gl. etunicatum</i> Becker & Gerdemann	5.20 ± 0.34	0.63 ± 0.08	***
<i>Gl. fasciculatum</i> (Thaxter) Gerd. & Trappe emend. Walker & Koske	1.28 ± 0.16	3.69 ± 0.42	***
<i>Gl. fistulosum</i> Skou & Jakobsen	0.07 ± 0.01	2.47 ± 0.40	***
<i>Gl. geosporum</i> (Nicol. & Gerd.) Walker	2.57 ± 0.21	0.25 ± 0.03	***
<i>Gl. halonatum</i> Rose & Trappe	ND	0.46 ± 0.11	
<i>Gl. intraradices</i> Schenck & Smith	0.08 ± 0.03	0.37 ± 0.06	***
<i>Gl. macrocarpum</i> Tulasne & Tulasne	0.22 ± 0.03	0.71 ± 0.09	***
<i>Gl. microcarpum</i> Tulasne & Tulasne	0.08 ± 0.02	0.19 ± 0.03	*
<i>Gl. mortonii</i> Bentivenga & Hetrick	ND		
<i>Gl. mosseae</i> (Nicol. & Gers.) Gerd. & Trappe	0.42 ± 0.07	0.17 ± 0.02	*
<i>Gl. pulvinatum</i> (Henn.) Trappe & Gerdemann	0.86 ± 0.10	0.05 ± 0.01	***
<i>Gl. rubiforme</i> (Gerd. & Trappe) Almeida & Schenck	0.02 ± 0.01	0.97 ± 0.12	***
<i>Gl. scintillans</i> Rose & Trappe	0.19 ± 0.04	0.03 ± 0.01	***
<i>Gl. sinuosum</i> (Gerd. & Bakshi) Almeida & Schenck	0.62 ± 0.09	0.28 ± 0.04	*
<i>Gl. spurcum</i> Pfeffer, Walker & Bloss	0.85 ± 0.09	0.14 ± 0.02	***
<i>Gl. tenue</i> (Greenhall) Hall	1.13 ± 0.13	0.66 ± 0.10	*
<i>Gl. tortuosum</i> Schenck & Smith	0.23 ± 0.06	0.34 ± 0.06	
<i>Gl. aff. heterosporum</i> Smith & Schenck	0.08 ± 0.03	0.10 ± 0.03	
<i>Gl. aff. melanosporum</i> Gerd. & Trappe	0.71 ± 0.13	ND	
<i>Gl. aff. invermaium</i> Hall	0.04 ± 0.01	0.75 ± 0.22	***
<i>Gl. aff. monosporum</i> Gerdemann & Trappe	0.05 ± 0.01	0.07 ± 0.01	
<i>Gl. aff. multicaule</i> Gerd. & Bakshi	0.41 ± 0.12	0.04 ± 0.01	**
<i>Glomus</i> sp. 1	0.12 ± 0.02	ND	
<i>Glomus</i> sp. 2	0.04 ± 0.01	ND	
<i>Glomus</i> sp. 3	0.07 ± 0.02	ND	
<i>Glomus</i> sp. 4	0.08 ± 0.2	ND	
<i>Glomus</i> sp. 5	0.41 ± 0.07	1.93 ± 0.41	**
<i>Glomus</i> sp. 6	1.03 ± 0.12	0.49 ± 0.08	**
<i>Glomus</i> sp. 7	0.31 ± 0.06	0.13 ± 0.02	*
<i>Glomus</i> sp. 8	0.01 ± 0.01	0.03 ± 0.02	
<i>Glomus</i> sp. 9	0.02 ± 0.02	ND	
<i>Glomus</i> sp. 10	0.10 ± 0.02	ND	
<i>Glomus</i> sp. 11	0.18 ± 0.03	0.02 ± 0.02	***
<i>Paraglomus occultum</i> (Walker) Morton & Redecker	1.39 ± 0.09	1.03 ± 0.09	*

† Values are means ± SE, averaged over 1997 and 1998. ND means spore species were not detected during sampling. Mean spore abundances differ significantly between semiarid and mesic sites at: \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

acquired from a total of 1160 soil samples collected in 1997 and 1998. Hyphae of *Glomus* and *Acaulospora* were detected at SEV, *Glomus* at SGS, and all four AMF genera at KZA, KBS, and CDR. These data were used to calculate the total hyphal biovolume per sample. For each fungal genus, hyphal biovolume was calculated assuming that hyphae were cylindrical. The total biovolume of live hyphae per sample was calculated by summing the biovolumes of each genus present.

#### Data analysis

All statistical analyses were performed using JMP 5.0.1 (SAS Institute 2002) or SYSTAT 11 (SYSTAT Software 2004) software. Spore and hyphal data sets were ordinated using nonmetric multidimensional scaling (NMDS), a nonlinear dimensionality reduction, which is one of the most robust unconstrained ordination methods in community ecology (McCune and Grace 2002). This approach circumvents the linearity assumption of metric ordination methods (e.g., CCA), and reflects the environment and the way in which it interacts with an individual sample. The NMDS method searches for the best positions of  $n$  individuals (AMF samples) along  $k$  dimensions (axes) that minimize the departure from monotonicity in the relationship between the original dissimilarities ( $\delta$ ) among the  $n$  samples and those in the reduced  $k$ -dimensional ordination space. For our study, NMDS was performed with the Bray-Curtis dissimilarity measure, so that the abundances of dominant and rare species contributed equally to the resultant matrix (Legendre and Legendre 1998). Nonparametric correlations (Spearman rank) were then used to examine the relationship between the measured environmental or biotic variables (e.g., soil N, ANPP) and (1) each of the axes of the final two-dimensional ordination space, and (2) AMF sample scores. For interpretability, the correlated environmental and biotic variables were plotted as vectors to show their relationships with the AMF sample scores (McCune and Grace 2002).

NMDS was initially undertaken on spore or hyphal data sets from SEV, SGS, KBS, and CDR since the responses of AMF fungi to N at KZA differed considerably from those in the other four sites (Johnson et al. 2003). The NMDS analyses were then repeated using data from all five sites to determine the similarities and differences in groupings between the KZA samples and those identified previously using the SEV, SGS, KBS and CDR data sets; results from both ordinations are presented.

AMF spore or hyphal community response to the combined effects of host-plant functional category, ANPP, and soil N:P with N fertilization was analyzed using multivariate repeated-measures analysis of variance (MANOVA). All data sets were  $(1 + \ln(x))$  transformed prior to analysis to meet the assumptions of normality. Pillai's trace was used as the multivariate test of significance, and it is the statistic reported.

Because the initial MANOVA indicated a significant effect of year on both AMF spore (Pillai's trace,  $F = 7.27$ ,  $df_{1071, 6171}$ ,  $P < 0.0001$ ) and hyphal communities ( $F = 4.606$ ,  $df_{42, 735}$ ,  $P < 0.0001$ ), separate MANOVA were subsequently undertaken on spore and hyphal data from each year. Univariate tests were performed when MANOVA revealed significant effects of plant functional category, ANPP, or soil N:P as a main effect or interaction term.

We evaluated the effects of N on AMF richness using species richness ( $S_{\text{obs}}$ ), defined as the mean number of AMF species recovered per fertilizer treatment, and nonparametric extrapolation methods to estimate AMF species richness (first-order Jackknife,  $\text{Chao}_2$ ) and diversity (Shannon's  $H'$ , Fisher's  $\alpha$ ). We used the first-order Jackknife ( $\text{Jack}_1$ ) and  $\text{Chao}_2$  (Chao 1984) since these estimators indicate the influence of rare species on  $S_{\text{obs}}$ .  $\text{Jack}_1$  is the simplest jackknife species richness estimator and a function of the number of rare species found in a community. The calculation uses the number of AMF species that occur in one and only one sample (singletons). Thus,

$$\text{Jack}_1 = S_{\text{obs}} + [L(N - 1)]/n$$

where  $N$  is the number of singletons, and  $n$  is the sample size. The  $\text{Chao}_2$  estimator uses the observed number of AMF species combined with the number of singletons and doubletons (species detected twice). Thus,

$$\text{Chao}_2 = S_{\text{obs}} + (N^2/2M^2)$$

where  $S_{\text{obs}}$  is the number of observed species,  $N$  is the number of singletons, and  $M$  is the number of doubletons. Comparative tests have identified these indices as robust estimators of species richness (Colwell and Coddington 1994, Brose and Martinez 2004). Estimates of  $\text{Jack}_1$ ,  $\text{Chao}_2$ ,  $H'$  and  $\alpha$  were computed with EstimateS software (Colwell 2002) on rarified data sets to account for differences in sample size among sites and years. The order of sample input was randomized 50 times to remove the effects of spatial patchiness on species richness and diversity. The theoretical variance of  $\text{Jack}_1$ ,  $\text{Chao}_2$ , and Fisher's  $\alpha$  was computed using the methods given in Gimaret-Carpentier et al. (1998). Statistical comparisons among diversity indices were undertaken using Wilcoxon signed-rank tests with the null hypothesis that non-fertilized and N-fertilized AMF communities do not differ in species diversity or richness.

Evenness ( $E$ ) of the AMF species richness in each site and in non-fertilized and N-fertilized plots was calculated as the Gini coefficient ( $G$ ), a measure of inequality within a community. This coefficient is based on the Lorenz curve, in which a cumulative frequency curve for a specific variable is compared with a uniform distribution that represents equality, usually represented by a diagonal line. Specifically,  $G$  represents the ratio between the area enclosed by the line of equality and

the Lorenz curve, and the total triangular area under the line of equality. Values of  $E_G$  range from 0 to 1 and are analogous to other evenness measures, i.e., values approaching 0 indicate the greater role of each AMF species in the community (equality), whereas values approaching 1 denote inequality within the community because a few individual AMF species dominate or comprise a very large proportion of the community composition. For the Lorenz curve, we plotted the cumulative proportion of total AMF biovolume ( $y$ -axis) against the cumulative number of AMF species ( $x$ -axis) for each site and fertilizer treatment. Calculated  $G$  values were multiplied by  $n/(n-1)$  to provide unbiased values. Departures of  $E_G$  from a null hypothesis of equal species' representation were tested using the  $\chi^2$  distribution with  $2^k - k - 1$  degrees of freedom.

Species turnover, or replacement, was analyzed in SGS and CDR where  $C_3$  and  $C_4$  host plants co-occurred. Species turnover ( $t$ ) was calculated as:

$$t = T / [(n_{\text{non}} + n_{+\text{N}}) / 2]$$

where  $T$  is the number of AMF species in common between non-fertilized and N-fertilized plots, and  $n_{\text{non}}$  and  $n_{+\text{N}}$  are the number of AMF species retrieved from non-fertilized and N-fertilized plots, respectively. Values of  $t$  approaching 1 indicated a complete turnover of species following N fertilization, whereas  $t$  values approaching 0 indicated that AMF community composition in N-fertilized soils was largely similar to those in non-fertilized soils. Differences in  $t$  between  $C_3$  and  $C_4$  plants for each site were analyzed using the  $\chi^2$  distribution and a null hypothesis of equal species' turnover.

## RESULTS

### *AMF spore community responses to N fertilization*

A solution with two dimensions was achieved for nonmetric multidimensional scaling (NMDS) of spore biovolume in both years of the study (final stress: 1997, 0.082,  $R^2 = 0.903$ ; 1998, 0.028,  $R^2 = 0.998$ ). Based on these analyses, arbuscular mycorrhizal fungi (AMF) samples from Sevieta (SEV), Shortgrass Steppe (SGS), Kellogg Biological Station (KBS), and Cedar Creek (CDR) could be separated into three groups in ordination space (Fig. 1A, C): (1) non-fertilized mesic sites (KBS, CDR), (2) non-fertilized semiarid sites (SEV, SGS), and (3) N-fertilized samples from all four sites. The extent of group overlap between semiarid and mesic site groups in 1997 (no overlap) and 1998 (overlap) indicates interannual variations in sporulation and community composition. In both years, however, vectors showing the most significant correlations ( $P < 0.05$ ) with the ordination sample scores on axis 1 were those indicating increasing annual net plant productivity (ANPP), and soil P as factors separating non-fertilized mesic and semiarid AMF communities. AMF communities and ANPP are correlated, of course, because

vegetation influences its mycorrhizae (through C transfer) and is influenced by AMF by the extent of fungal resource acquisition and transfer on plant establishment, growth, and productivity. A posteriori comparisons of the AMF communities showed the presence of Gigasporaceae and Acaulosporaceae in mesic (high ANPP) but not semiarid conditions, and an abundance of Glomaceae and Paraglomaceae under semiarid conditions (low ANPP; Table 2). Soil P also influenced AMF communities because higher levels of soil P occurred in mesic than semiarid sites (Table 1).

The vectors showing a significant positive correlation ( $P < 0.05$ ) with ordination scores on axis 2 were soil N, soil N:P ratio, and plant photosynthetic strategy (Fig. 1A, C). Consequently, AMF community composition was influenced by increasing soil N levels and N:P ratios, and further modified by the functional category of the host plant; these results were confirmed by MANOVA (Table 3). Sample scores parallel to axis 2 comprised a gradient from non-fertilized (SEV, SGS, KBS, CDR) to N-fertilized (high soil N or N:P in SEV, SGS, KBS, CDR). Nitrogen-fertilized samples from SEV, SGS, KBR, and CDR coalesced as a single group so that site identity was effectively lost (Fig. 1A, C).

The exact opposite response was found in Konza Prairie (KZA) so that non-fertilized KZA samples were grouped with N-fertilized samples from SEV, SGS, KBS, and CDR, and N or N+P-fertilized KZA samples were grouped with non-fertilized semiarid or mesic samples (Fig. 1B, D). The alignment of KZA scores parallel to axis 2 indicated that AMF responses were also the outcome of soil N and N:P, and plant functional category. These findings confirm the unique responses of AMF fungi at KZA to fertilization, as has been noted previously (Eom et al. 1999, Johnson et al. 2003).

### *Spore species richness, diversity, evenness, and turnover*

The rarified indices of richness and diversity revealed that overall AMF spore species richness and diversity, and evenness, declined following N fertilization at SEV, SGS, KBS, and CDR in comparison to non-fertilized samples (Table 4). Such shifts were detectable in AMF communities of  $C_3$  and  $C_4$  plants (Table 4) and in both years of the study (Appendix). These changes could be attributed to the loss of rare or unique AMF species (Chao<sub>2</sub>, Jack<sub>1</sub>; see *Methods: Data analysis*, above) and subsequent changes in the proportional abundance or dominance of the remaining taxa ( $H'$ ,  $\alpha$ ,  $E_G$  [evenness, calculated as the Gini coefficient,  $G$ ]). In particular,  $C_4$  plants demonstrated a significant loss of singletons after N fertilization, whereas  $C_3$  plants did not (Jack<sub>1</sub>). Even in the most diverse AMF community (KBS), observed AMF species richness declined from 38 to 23 following N fertilization (Appendix). Together with the NMDS ordination (Fig. 1), these data suggest that N fertilization has significantly weakened site or regional differences among AMF communities irrespective of plant community, the initial diversity of the AMF community,

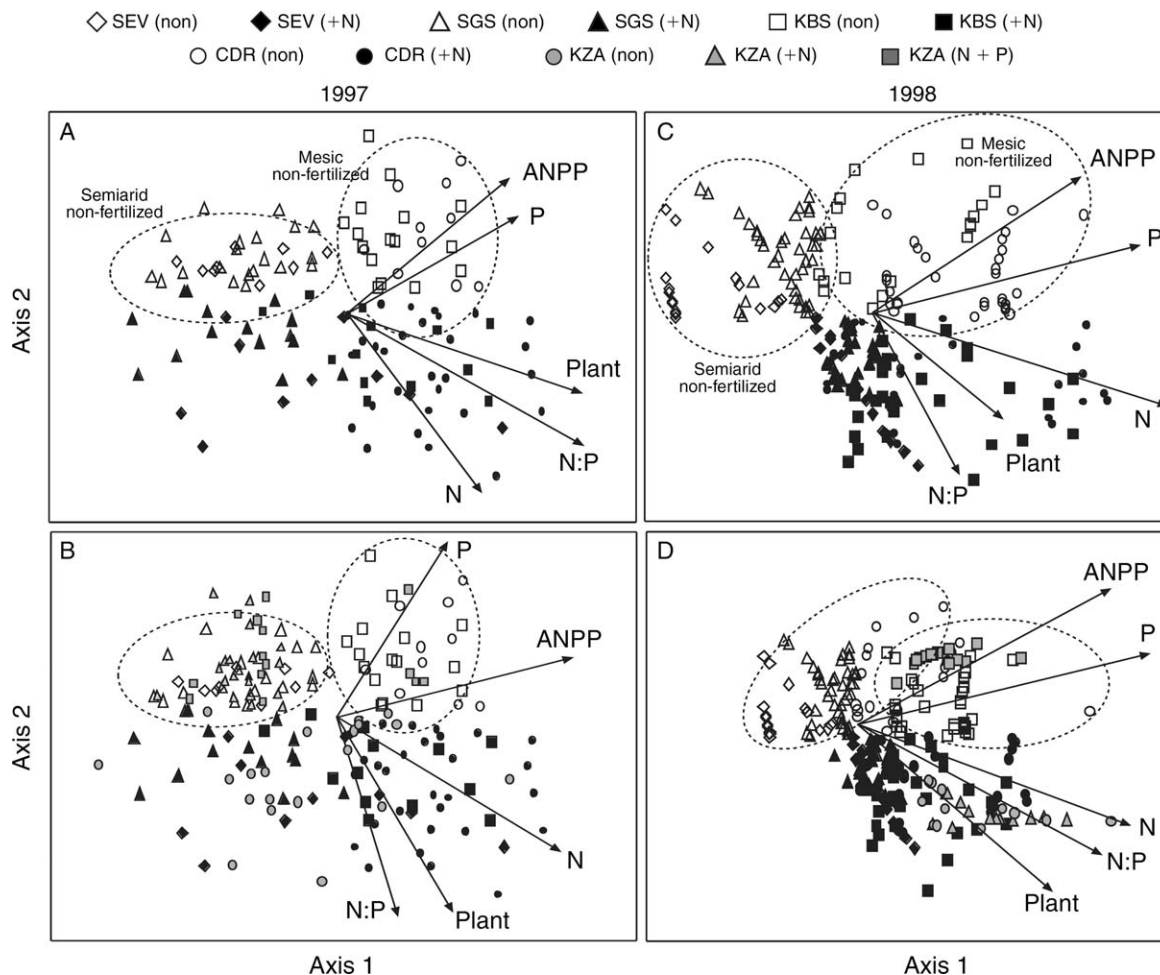


FIG. 1. Joint plot of NMDS ordination scores of arbuscular mycorrhizal fungi (AMF) spore community samples and vectors of the significant environmental, soil, and plant community factors across sites ( $P < 0.05$ ). LTER site abbreviations in the key at the top correspond to those given in Table 1; non, non-fertilized, and +N, fertilized with nitrogen. (A, B) 1997 significant  $R^2$  values: Axis 1, ANPP 0.667; Axis 2, soil N 0.665, soil N:P 0.687, plant photosynthetic category 0.360. (C, D) 1998 significant  $R^2$  values: Axis 1, ANPP 0.433; Axis 2, soil N 0.385, soil N:P 0.238, plant functional category 0.397.

time since the onset (3 yr, SEV) or cessation of fertilization (20 yr, SGS), or duration (3–16 yr) or quantity of fertilizer applied ( $100\text{--}170\text{ kg N}\cdot\text{ha}^{-1}\cdot\text{yr}^{-1}$ ; Table 1). On the contrary, N or N+P fertilization significantly increased AMF species richness and diversity in KZA. Estimators of species richness and diversity, and evenness were all significantly higher in N- or N+P-fertilized plots than in the non-fertilized plots (Table 4) and could be attributed to a significant increase in the abundance of unique taxa ( $\text{Chao}_2$ ,  $\text{Jack}_1$ ), and the decreasing dominance of the most abundant spore species ( $E_G$ ).

Species turnover was estimated for SGS and CDR where  $C_3$  and  $C_4$  grasses co-occur (Tables 1 and 5). In both SGS and CDR, species turnover was higher in  $C_3$  than  $C_4$  plants. In SGS, this difference was significant only in 1998, whereas it was significant in both years of the study in CDR (Table 5).

#### *Effects of N fertilization, soil N:P, and plant functional category on abundances of AMF spore species*

The MANOVA illustrated that AMF spore species' abundances were altered by N fertilization, plant photosynthetic strategy, soil N:P, or a combination of these factors (Table 3). Nitrogen fertilization resulted in a significant increase in sporulation in  $C_3$  plants within a matrix of  $C_4$  plants (SGS, CDR) but not in communities comprising  $C_3$  plants alone (KBS; Fig. 2). In contrast, N fertilization resulted in a significant decline in spore abundance in  $C_4$  plants in SEV, SGS, and CDR.

The MANOVA also demonstrated that AMF spore species' responses to N fertilization, plant, and soil N:P also differed significantly between each year of the study (Table 3). One reason for this response is the interannual variations in sporulation (Fig. 1) and turnover among AMF species (Table 5). Only 17 AMF species, or 27% of



TABLE 3. *F* values from multivariate repeated-measures analysis of variance (MANOVA) of N fertilization, soil, and plant factors on the biovolume of AMF spores or hyphae.

Factor	Hyphae		Spores	
	1997	1998	1997	1998
Between subjects				
Soil P	2.84	<b>14.56</b>	0.03	3.66
Soil N:P	<b>11.71</b>	1.07	0.74	<b>17.80</b>
Plant	0.85	0.46	<b>5.96</b>	0.72
ANPP	3.12	0.26	3.02	0.02
Within subjects				
Fertilization	4.29	2.81	<b>6.64</b>	<b>24.04</b>
Fertilization × plant	2.67	<b>5.85</b>	<b>4.23</b>	<b>15.68</b>
Fertilization × soil P	1.73	<b>21.61</b>	1.36	<b>10.66</b>
Fertilization × soil N:P	<b>4.09</b>	<b>10.41</b>	<b>2.53</b>	<b>24.47</b>
Fertilization × ANPP	<b>3.99</b>	<b>5.52</b>	1.89	<b>32.88</b>
Fertilization × plant × soil N:P	0.64	<b>6.06</b>	<b>4.17</b>	<b>23.72</b>
Fertilization × plant × ANPP	<b>3.66</b>	2.64	1.23	<b>27.87</b>
Fertilization × soil N:P × ANPP	2.76	3.74	1.85	<b>18.41</b>

Notes: *F* values in plain text are not significant,  $P > 0.05$ ; in bold, significant at  $P < 0.001$ ; in italics, significant at  $P < 0.01$  or  $P < 0.05$ . For Hyphae, for both years, between-subjects  $df = 1, 119$ , and within-subjects  $df = 3, 117$ ; for Spores, for 1997, between-subjects  $df = 1, 169$ , and for 1998, between-subjects  $df = 1, 280$ ; for 1997, within-subjects  $df = 63, 107$ , and for 1998, within-subjects  $df = 60, 220$ .

the species pool, common to all sites sporulated in 1997 and 1998, meaning that only a quarter of the spore community might signal the effects of N fertilization from one year to the next. Such consistently detectable species included *Glomus aggregatum*, *Gl. etunicatum*, *Gl.*

TABLE 5. Percentage turnover of AMF community species composition following N-fertilization in C<sub>3</sub> and in C<sub>4</sub> host plants at SGS and CDR.

Site†	Host plant	Year	
		1997	1998
SGS	C <sub>3</sub>	35.5	57.4
	C <sub>4</sub>	31.8	25.4
	Significance	NS	*
CDR	C <sub>3</sub>	43.5	36.2
	C <sub>4</sub>	32.1	22.8
	Significance	*	*

Note: Mean species turnover differs significantly between C<sub>3</sub> and C<sub>4</sub> plants at \*  $P < 0.05$ ; NS indicates that turnover did not differ significantly ( $P > 0.05$ ).

† LTER site abbreviations correspond to those given in Table 1.

*fasciculatum*, and *Archaeospora leptoticha*. Along with these species of *Glomus* and *Archaeospora*, an additional eight species sporulated consistently in mesic sites, including *Scutellospora calospora* and *Aculospora laevis*.

Five AMF species were influenced only by host-plant identity alone (Fig. 3A), and an additional nine species were sensitive to the effects of N fertilization alone (Fig. 3B, plus *Ac. morrowiae*, *Enterophospora*, and *Gl. monosporum*). The greatest influences on AMF spore species abundance, however, were the result of interactions between N fertilization, soil N:P, and plant photosynthetic strategy. To limit the confounding effects of early- and late-season samplings, we used pooled data to evaluate these factors on AMF sporulation. Overall,

TABLE 4. Distribution of species richness, diversity, and evenness in AMF communities in five grassland sites following N fertilization.

LTER site†	Plant (fertilization)	Species richness			Species diversity		Evenness, $E_G$
		No. observed species, $S_{obs}$	Chao <sub>2</sub>	Jack <sub>1</sub>	Fisher's $\alpha$	$H'$	
SEV	C <sub>4</sub> (Non)	25.8 (1.9)	42.6 (1.3)	30.0 (8.5)	5.46 (1.42)	1.98 (0.17)	0.283
	C <sub>4</sub> (N-fert)	17.1 (2.1)	25.3 (4.2)	25.3 (3.4)	2.80 (0.59)	1.58 (0.18)	0.455
	Significance	*	*	*	*	*	*
SGS	C <sub>3</sub> (Non)	14.1 (2.6)	18.7 (1.5)	28.0 (10.9)	7.26 (2.23)	1.85 (0.16)	0.089
	C <sub>3</sub> (N-fert)	11.3 (1.7)	11.8 (0.3)	17.6 (5.8)	5.99 (1.32)	1.71 (0.07)	0.277
	Significance	*	*	NS	NS	NS	*
	C <sub>4</sub> (Non)	26.8 (3.8)	27.8 (0.3)	36.7 (10.8)	7.86 (1.88)	1.86 (0.09)	0.301
	C <sub>4</sub> (N-fert)	16.7 (2.4)	17.7 (3.8)	20.5 (5.9)	6.46 (1.89)	1.76 (0.08)	0.414
Significance	*	*	*	NS	*	*	
KBS	C <sub>3</sub> (Non)	35.4 (1.7)	37.1 (3.3)	42.6 (6.8)	7.84 (1.81)	1.92 (0.19)	0.199
	C <sub>3</sub> (N-fert)	23.4 (2.8)	23.5 (0.1)	30.1 (7.8)	5.17 (1.24)	1.03 (0.18)	0.381
	Significance	*	*	NS	NS	*	*
CDR	C <sub>3</sub> (Non)	27.0 (1.7)	31.1 (9.3)	35.6 (10.8)	5.36 (1.10)	1.95 (0.10)	0.053
	C <sub>3</sub> (N-fert)	20.3 (1.7)	20.4 (0.2)	30.9 (12.8)	3.67 (0.60)	1.36 (0.19)	0.190
	Significance	*	*	NS	*	*	*
	C <sub>4</sub> (Non)	27.4 (1.1)	31.9 (0.2)	35.9 (10.0)	5.86 (1.18)	2.27 (0.07)	0.191
	C <sub>4</sub> (N-fert)	22.4 (0.8)	28.5 (0.1)	33.4 (12.8)	4.42 (0.79)	1.79 (0.03)	0.594
Significance	*	*	NS	NS	*	*	
KZA	C <sub>4</sub> (Non)	23.3 (2.2)	30.5 (0.2)	23.8 (3.9)	5.23 (1.20)	1.90 (0.10)	0.469
	C <sub>4</sub> (N-fert)	32.3 (2.2)	39.0 (0.9)	34.4 (5.9)	7.34 (1.68)	2.42 (0.09)	0.422
	C <sub>4</sub> (NP-fert)	33.3 (1.8)	39.8 (0.25)	33.6 (4.2)	6.44 (1.44)	2.23 (0.05)	0.432
	Significance	*	*	*	NS	*	*

Notes: Values represent the mean with standard error ( $S_{obs}$ ,  $H'$ ) or variance (Chao<sub>2</sub>, Jack<sub>1</sub>, Fisher's  $\alpha$ ) given in parentheses. Mean AMF species richness, diversity, or evenness differs significantly between non-fertilized and N-fertilized C<sub>3</sub> or C<sub>4</sub> plants in each site at \*  $P < 0.05$ ; NS indicates that the means do not differ significantly ( $P > 0.05$ ).

† Site abbreviations correspond to those given in Table 1.

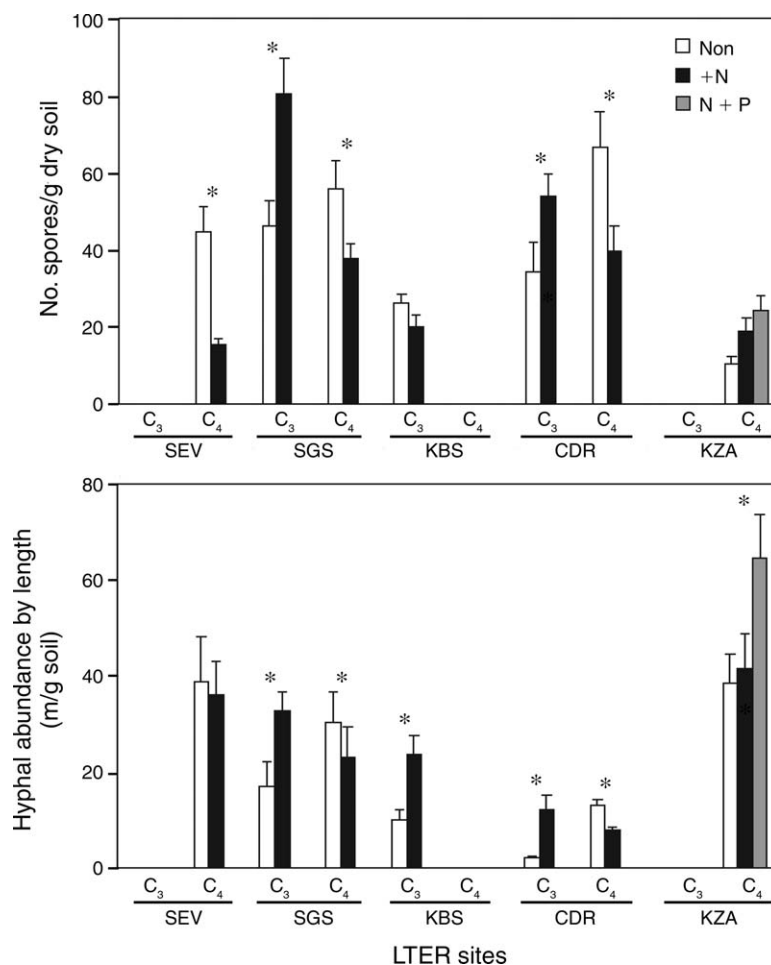


FIG. 2. Relative abundance of AMF spores and hyphae in C<sub>3</sub> and C<sub>4</sub> plants in non-fertilized and N-fertilized (+N) soils at each site. Data are means and SE and represent the average across both years of the study. For spores or hyphae, the mean abundance between non-fertilized and N-fertilized soils and each plant host designated with an asterisk differ significantly at  $P < 0.05$ . LTER site abbreviations correspond to those given in Table 1.

the abundances of 43 AMF species (or 68% of the species pool) were significantly influenced by these interactions, meaning that about three quarters of the spore community could be expected to be controlled by both plant and soil factors. Although total spore abundance increased in response to N in C<sub>3</sub> plants at SGS and CDR (Fig. 2), the abundances of 19 AMF spore species declined significantly in C<sub>3</sub> plants but increased in abundance or were not significantly influenced by N fertilization in C<sub>4</sub> plants (Fig. 4). These responsive taxa included several species of *Glomus* including the N-responsive taxa, *Gl. aggregatum* and *Paraglomus occultum* (Fig. 4A, B), along with *Ac. laevis*, *Ac. spinosa*, and *Sc. erythropha* (not shown). Only seven species associated with C<sub>4</sub> plants declined significantly in abundance with N fertilization (Fig. 4C plus *Ac. Scrobiculata* [not shown]).

Spore abundance also varied with soil N:P (Fig. 5). Data from KZA most effectively illustrated this result. An increase in soil N:P was matched by a progressive,

and significant, increase in spore abundance in *Gl. aggregatum*, *Gl. tenue*, *Gl. etunicatum*, *Gl. ambisporum*, *Gl. clarum*, *Paraglomus occultum*, and *Ar. leptotichum*. Only at the highest N:P supply ratio, did the abundances of *Gigaspora gigantea*, and two species of *Scutellospora* increase significantly.

#### AMF hyphal community composition

A solution with two dimensions was also achieved for the NMDS of the hyphal community and in both years of the study (final stress: 1997, 0.064,  $R^2 = 0.982$ ; 1998, 0.074,  $R^2 = 0.976$ ). NMDS of the hyphal samples from SEV, SGS, KBS, and CDR produced two groups formed by non-fertilized mesic sites (KBS and CDR), or non-fertilized semiarid sites (SEV and SGS; Fig. 6A, C). Unlike the spore community (Fig. 1), the N-fertilized hyphal scores from these four sites were widely dispersed throughout the ordination space, and formed a continuum of response with the non-fertilized samples (e.g., Fig. 6C). Accordingly, considerable variation

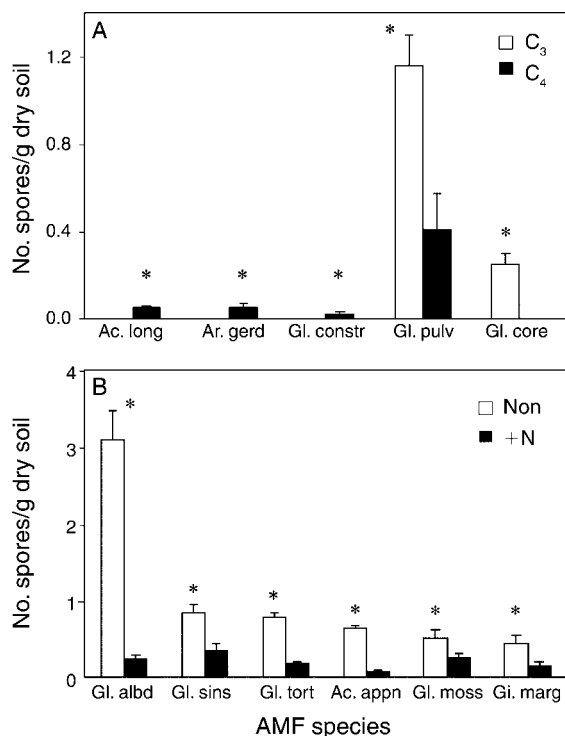


FIG. 3. Relative abundance of (A) five AMF spore species that were influenced only by N fertilization, and (B) six AMF species that were responsive only to the photosynthetic category of the host plant. Data are means and SE; asterisks denote AMF spore species whose abundance differed significantly between N-fertilized and non-fertilized soils, or C<sub>3</sub> and C<sub>4</sub> plants, respectively ( $P < 0.05$ ). Abbreviations: Ac. long, *Acaulospora longula*; Ar. Gerd, *Archaeospora gerdemanni*; Gl. constr, *Glomus constrictum*; Gl. pulv, *Gl. pulvinatum*; Gl. core, *Gl. coremioides*; Gl. albd, *Gl. albidum*; Gl. sins, *Gl. sinuosum*; Gl. tort, *Glomus tortuosum*; Ac. appn, *Ac. appendicula*; Gl. moss, *Gl. mosseae*; Gi. marg, *Gigaspora margarita*.

exists within the live hyphal community in response to N fertilization.

Those environmental variables showing significant correlations ( $P < 0.05$ ) with hyphal scores were ANPP and soil P (axis 1). As in the spore community (Fig. 1), sites with higher ANPP (CDR, KBS) were characterized by a greater abundance of hyphae than semiarid sites (SEV, SGS; Fig. 7). Such differences were reinforced by the presence of *Scutellospora*, *Gigaspora*, and *Acaulospora* in mesic sites, and a predominance of *Glomus* in semiarid sites (Fig. 7). Those variables with significant correlations ( $P < 0.05$ ) on axis 2 were soil N:P and plant functional category. Thus, the significant predictors of the hyphae mirrored those for spores.

Analogous to the observations on spore community (Fig. 1), KZA hyphal samples formed a discrete group (1997; Fig. 6B) or were slightly overlapping with non-fertilized mesic samples (1998; Fig. 6D). The unique community traits in KZA were therefore detectable in both the spore (reproductive) and hyphal (vegetative) structures.

#### Effects of soil N:P and plant on hyphal community composition

The MANOVA confirmed the significant response of the hyphal community to plant photosynthetic strategy, soil N:P, or ANPP with N fertilization (Table 3). Since these results were similar in each year of the study, hyphal responses to soil fertility appear to be relatively consistent from year to year (see also Fig. 7).

We pooled data from both years to evaluate the significance of each factor on AMF hyphal abundance.

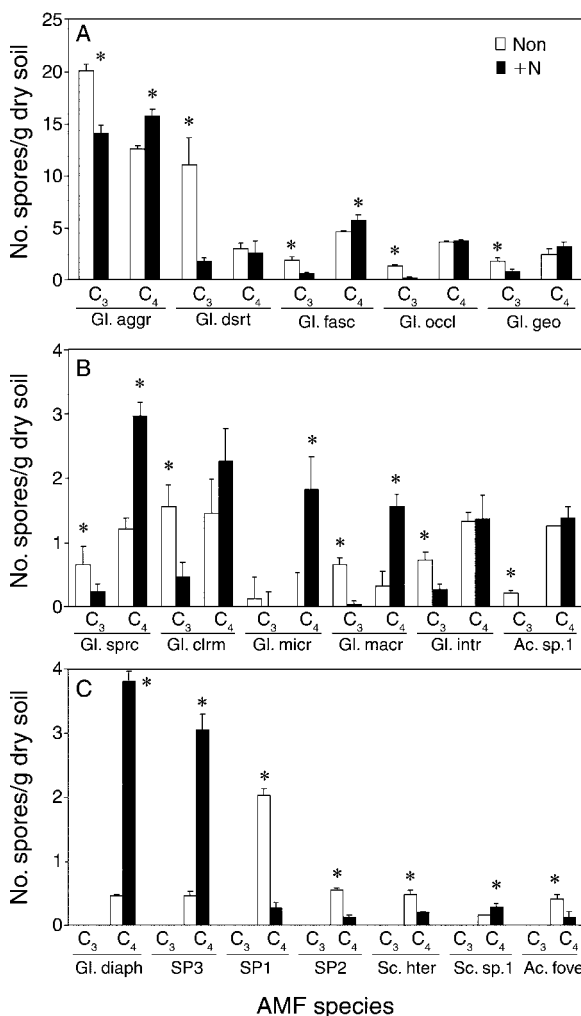


FIG. 4. Relative abundance of AMF species in response to N-fertilization treatments and plant functional category. Data are means and SE. For each species, spore abundance between non-fertilized and N-fertilized soils and each host plant designated with an asterisk differed significantly at  $P < 0.05$ . Abbreviations: (A) Gl. aggr, *Glomus aggregatum*; Gl. dsrt, *Gl. deserticola*; Gl. fasc, *Gl. fasciculatum*; Gl. occl, *Paraglomus occultum*; Gl. geo, *Gl. geosporum*; (B) Gl. sprc, *Gl. spurcum*; Gl. clrm, *Gl. clarum*; Gl. micr, *Gl. microcarpum*; Gl. macr, *Gl. macrocarpum*; Gl. intr, *Gl. intraradices*; Ac. sp. 1, *Acaulospora* sp. indet. (sp. 1); (C) Gl. diaph., *Gl. diaphanum*; Gl. sp. 3, 1, 2, *Glomus* species indeterminate (sp. 3, sp. 1, sp. 2); Sc. hter, *Scutellospora heterogama*; Sc. sp. 1, *Scutellospora* species indeterminate (sp. 1); Ac. fove, *Acaulospora foveata*.

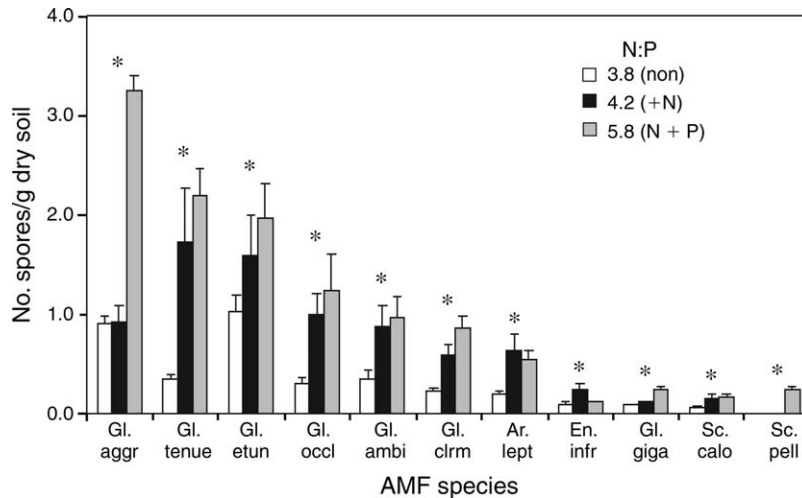


FIG. 5. Mean relative abundance of AMF species in response to soil N:P supply ratio at KZA. Data are means and SE. For each AMF, spore abundances designated with an asterisk indicate that fertilizer treatments differ significantly at  $P < 0.05$ . Abbreviations: Gl. aggr, *Gl. aggregatum*; Gl. tenue, *Gl. tenue*; Gl. etun, *Gl. etunicatum*; Gl. occl, *Paraglomus occultum*; Gl. ambi, *Gl. ambisporum*; Gl. clrm, *Gl. clarum*; Ar. lept, *Ar. leptotichum*; En. freq, *Enterophosphora infrequens*; Gi. giga, *Gigaspora gigantea*; Sc. calo, *Sc. calospora*; Sc. pell, *Sc. pellucida*.

The abundance of AMF hyphae increased in N-fertilized  $C_3$  plants, but not  $C_4$  plants, in comparison to non-fertilized plants (Fig. 2). The interaction of soil N:P and plant functional category produced distinct responses in AMF hyphal community composition (Fig. 8). In both  $C_3$  and  $C_4$  plants, the abundances of *Scutellospora* and *Acaulospora* hyphae increased with increasing soil N:P ratio; the abundance of *Gigaspora* increased only in  $C_3$  plants (Fig. 8). In all three genera, the magnitude of effect was several times greater, and initiated at lower soil N:P ratios in  $C_3$  than  $C_4$  plants. In contrast, the abundance of *Glomus* hyphae was significantly greater in  $C_4$  than  $C_3$  plants, and at intermediate soil N:P ratios. Overall, the greatest effects of increasing soil N and N:P supply ratio might be expected to occur in  $C_3$  plants. These observations are made stronger because we detected and measured the active AMF hyphal community by immunofluorescence instead of the standing biomass (live plus dead hyphae) as is customary.

#### DISCUSSION

Our findings indicate that ecological patterning in arbuscular mycorrhizal fungi (AMF) communities following N fertilization is unlikely to arise from single-factor explanations that operate identically across grassland sites or within AMF communities. Not only did AMF sporulation and hyphal abundance vary both positively and negatively with N fertilization, but AMF taxa also demonstrated host dependence, so that significant and distinct patterns of sporulation and hyphal abundance were detected between  $C_3$  and  $C_4$  plants following N fertilization. Such effects could not be attributed to the duration of N fertilization, or differences in precipitation, annual net plant productiv-

ity (ANPP), or soil P alone. Here, we explore our results and compare them to existing explanations for AMF community responses to increasing soil N fertility.

We initially hypothesized that host-plant species identity might determine AMF community structure (H4). Our results strongly support this possibility and also illustrate that N or N:P  $\times$  host plant interactions can differentially influence AMF communities. In fact, few AMF species were influenced by N or host plant alone. This outcome is consistent with the concept of plant-AMF specificity, and that feedbacks between plant and soil conditions control AMF community structure through the differential sporulation, growth, and survival of AMF taxa (Johnson et al. 1991, 1992, Bever et al. 1996, Eom et al. 2000). The differential responses in the hyphal and spore communities to plant species  $\times$  N:P, as well as among individual AMF species to soil N fertility support this possibility. For example, AMF spore and hyphal abundance in  $C_3$  plants increased several fold with increasing soil N:P supply, but declined in  $C_4$  host plants.

Discrepancies in fungal growth in N-enriched soils have been linked to changes in the allocation of photosynthates between the plant root and shoot (Graham and Eissenstat 1990, Peng et al. 1993, Graham et al. 1996), or differences in C use among fungal taxa (Douds and Schenck 1990). A parsimonious explanation, based on the increase in hyphal abundance and sporulation we observed in  $C_3$ , is that AMF likely allocate C resources (photosynthates) towards both active hyphal exploration for nutrient uptake and transfer (soil, root colonization; Sylvia and Neal 1990), and the sporulation of small-spored AMF species. Further, the abundance of *Gigaspora*, *Acaulospora*,

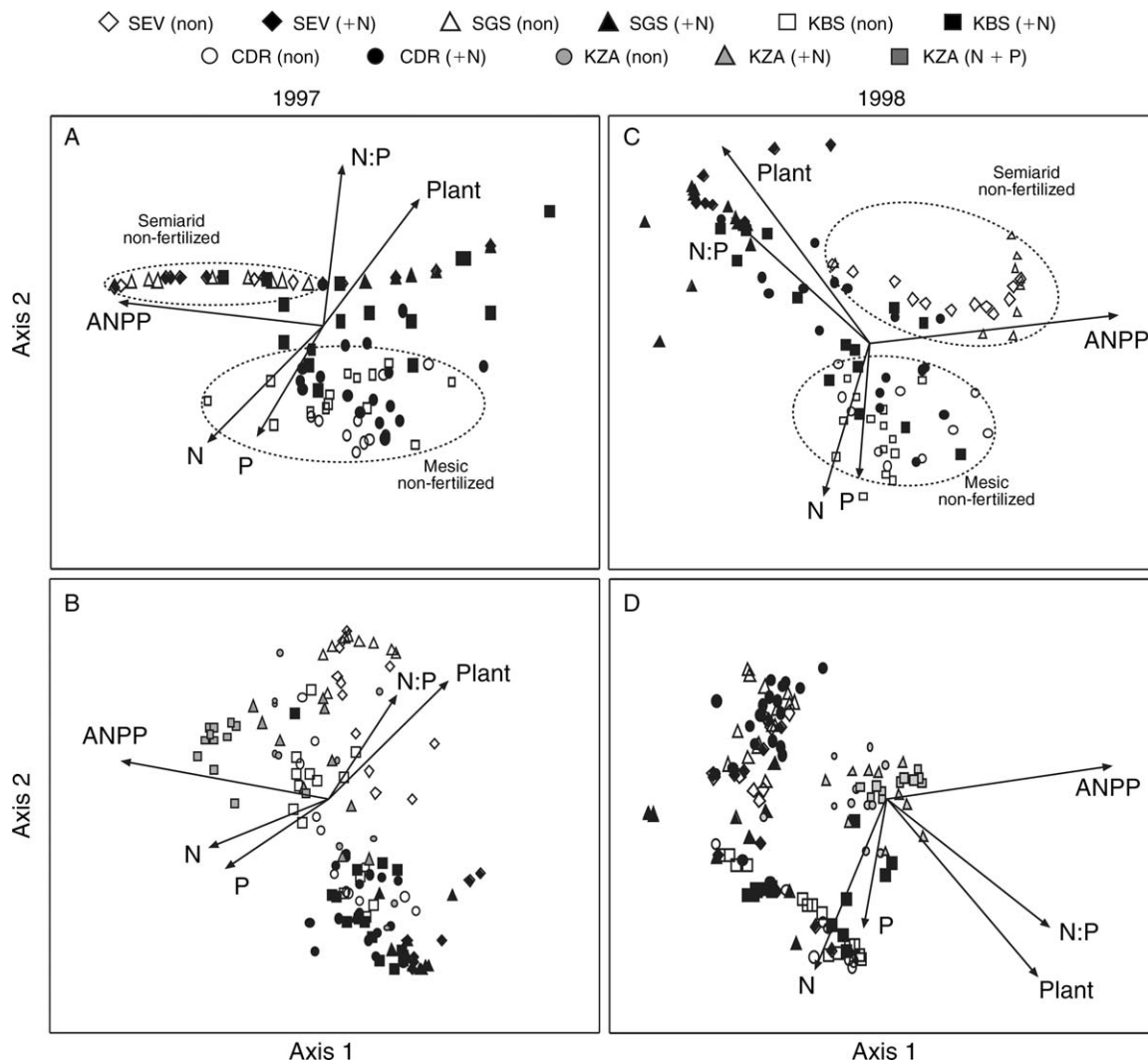


FIG. 6. Joint plot of NMDS ordination scores of AMF hyphal community samples and vectors of the significant environmental, soil, and plant-community factors across sites ( $P < 0.05$ ). Site abbreviations correspond to those given in Table 1. Data are means and SE. For 1997, significant  $R^2$  values: Axis 1, ANPP 0.795, soil P 0.619; Axis 2, soil N  $-0.943$ , soil P 0.584, soil N:P  $-0.898$ , plant functional category 0.495. For 1998, significant  $R^2$  values: Axis 1, ANPP 0.831, soil P 0.619; Axis 2, soil N  $-0.924$ , soil N:P  $-0.917$ , plant functional category 0.274.

and *Scutellospora* in  $C_3$  plants, and to a lesser extent in  $C_4$  plants, may fulfill the host-plant's requirement for limiting nutrients, especially for P acquisition. Specifically, soil N enrichment may induce or reinforce plant P deficiency (high N:P), and P nutrition in many grasses is linked to AMF (e.g., Hetrick et al. 1990). As a result, N fertilization may heighten the importance of AMF for plant P uptake (Anderson et al. 1994). AMF also vary in their capacity to take up, transport, and transfer P to plants (Jakobsen et al. 1992a, b), and, in particular, *Acaulospora* species tend to be more effective at providing P to host plants than are *Scutellospora* or *Gigaspora*. *Glomus* is possibly the least effective at P transfer (Jakobsen et al. 1992b, Boddington and Dodd 1998, 1999), but requires less C to maintain hyphal growth and sporulation (C efficient; Douds and Schenck

1990). These findings are consistent with the theoretical prediction that differences in AMF species composition are the basis of biodiversity effects on the plant community (van der Heijden et al. 1998b).

The differential responses of AMF to  $C_3$  or  $C_4$  host plants and N fertilization in our study is particularly interesting because we chose to sample the codominant plant species in each grassland site. Our results therefore suggest that coexistence among dominant plant species might be altered by the abundance and compositional changes of AMF taxa. Two other outcomes of our study, however, need to be considered before any conclusions about the effects of N and AMF on plant community structure can be contemplated.

First, the high degree of compositional similarity among the semiarid (Seviletta [SEV] and Shortgrass

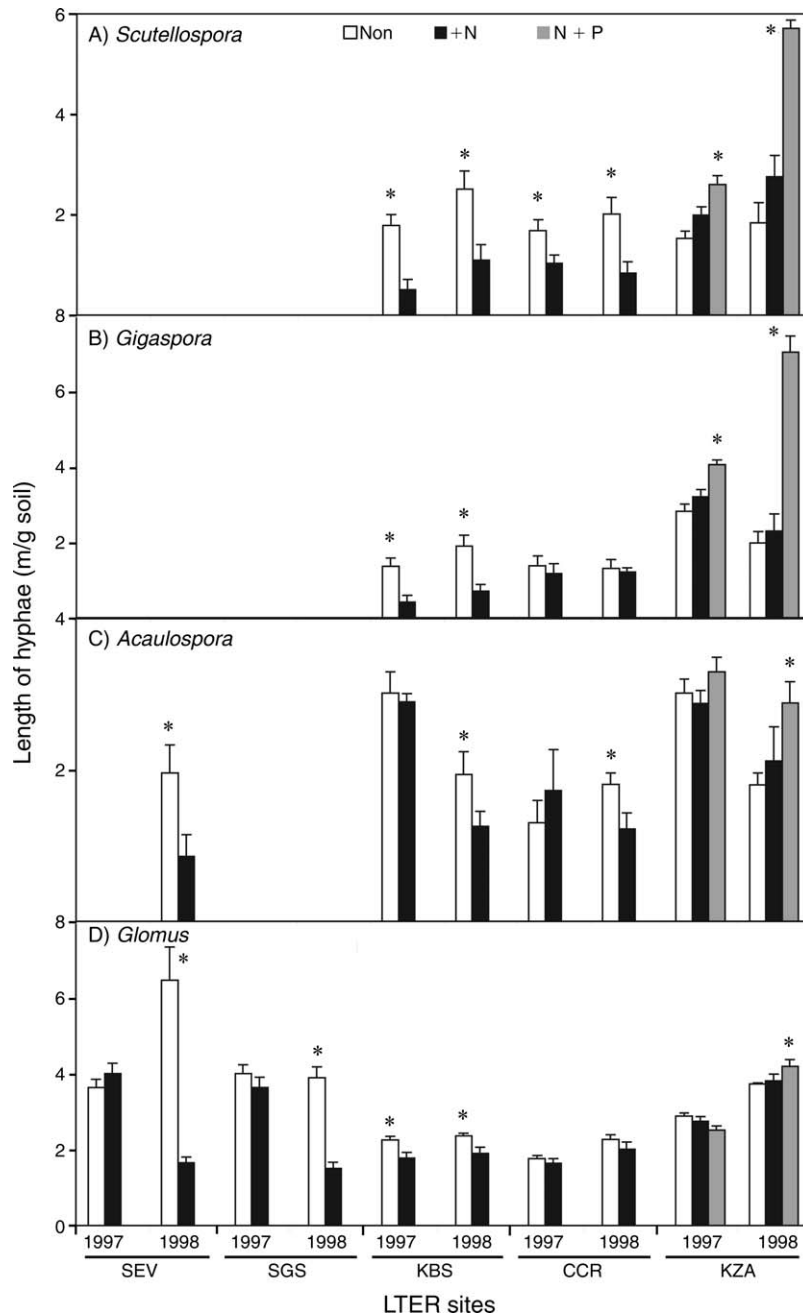


FIG. 7. The abundance of live AMF hyphae (mean ± SE) from each of the four major AMF genera for each study site and fertilization treatment. Site abbreviations correspond to those given in Table 1. Note that AMF hyphae of *Scutellospora* and *Gigaspora* were not recovered from SEV or SGS, and *Acaulospora* was not detected at SGS. Asterisks indicate significant differences ( $P < 0.05$ ) in mean hyphal abundance at SEV, SGS, KBS, CCR, and KZA by year. For KZA, asterisks indicate that the N + P treatment differs significantly ( $P < 0.05$ ) from the other fertilizer treatments.

Steppe [SGS]) and among the mesic (Kellogg Biological Station [KBS] and Cedar Creek [CCR]) sites (as indicated by nonmetric multidimensional scaling [NMDS]), and correlation between community structure and soil N fertility and N:P supply ratio further indicate that the equilibrium between soil N and P supply was important in controlling the AMF community response

to N fertilization. This outcome is consistent with Brown's (1984) argument that spatial gradients of a few important environmental variables determine the major patterns of species' distribution and abundance. Despite the large initial differences in AMF species composition between the mesic and semiarid sites (Table 2), or high species richness (e.g., KBS), the composition

of the AMF communities became increasingly similar following N fertilization, due to the loss of rare or unique species. Thus, increasing soil N fertility and N:P had a strong homogenizing influence on the AMF community (McKinney and Lockwood 1999), in much the same way that N fertilization promotes species convergence in plant communities (Inouye and Tilman 1988). The direction and magnitude of this response is also in agreement with the conversion of natural plant communities to agricultural fields (Hayman 1970, 1982, Sieverding 1990, Helgason et al. 1998). Further, AMF community homogenization was both relatively rapid (SEV, after 3 years), and remarkably persistent (SGS, 22 years post-fertilization). In the formerly N-fertilized plots at SGS, only a portion of the possible historic AMF community had returned following the cessation of fertilization (H3). This relative inertia in community recovery suggests that AMF species continue to respond to the residual, possibly nonlinear, effects of N within the host root or soil microenvironment, or that dispersal and recolonization may generate spatially clumped (Bever et al. 1996) or localized patterns that were smaller than the scale at which we sampled (Hart et al. 2003). As a consequence, AMF community assembly (species gain) and disassembly (species loss) in natural ecosystems following soil N enrichment has components of both chance and historical contingency.

In Konza Prairie (KZA), fertilization magnified the differences in species composition and abundance so that spore species richness and diversity increased in response to N or N+P fertilization. This contrasting response is best explained in terms of the combined responses of AMF and their host plants to soil fertility (Table 1; H2). In the low-P (high N:P) soils of KZA, plants are highly dependent on AMF for P acquisition (Hetrick et al. 1988), but not in the high-P soils elsewhere (Anderson et al. 1994, Schultz et al. 2001). Fertilization with N thus further exacerbates plant P limitation and increases the importance of AMF for P uptake. On the other hand, SEV, SGS, KBS, and CDR are primarily N limited and relatively P rich, particularly CDR, where P fertilizers have been applied for almost a decade. Under these conditions, N fertilization decreases AMF abundance and species richness (Johnson et al. 2003; our present study).

Second, it was apparent that after N fertilization a number of AMF spore taxa became extremely abundant, in direct contradiction to the more general trend of declining spore species' abundance following N enrichment (H2; Johnson et al. 1991, Egerton-Warburton and Allen 2000). Typically, a number of *Glomus* species, including *Gl. aggregatum*, *Paraglomus occultum*, and *Gl. microcarpum*, proliferated with  $C_4$  plant species, and *Archaeospora leptoticha* and *Gl. tenue* with  $C_3$  and  $C_4$  plants. These results are particularly illuminating because they show that the AMF spore species considered indicators of N-enriched soils, such as *Paraglomus occultum* (Johnson 1993, Egerton-Warbur-

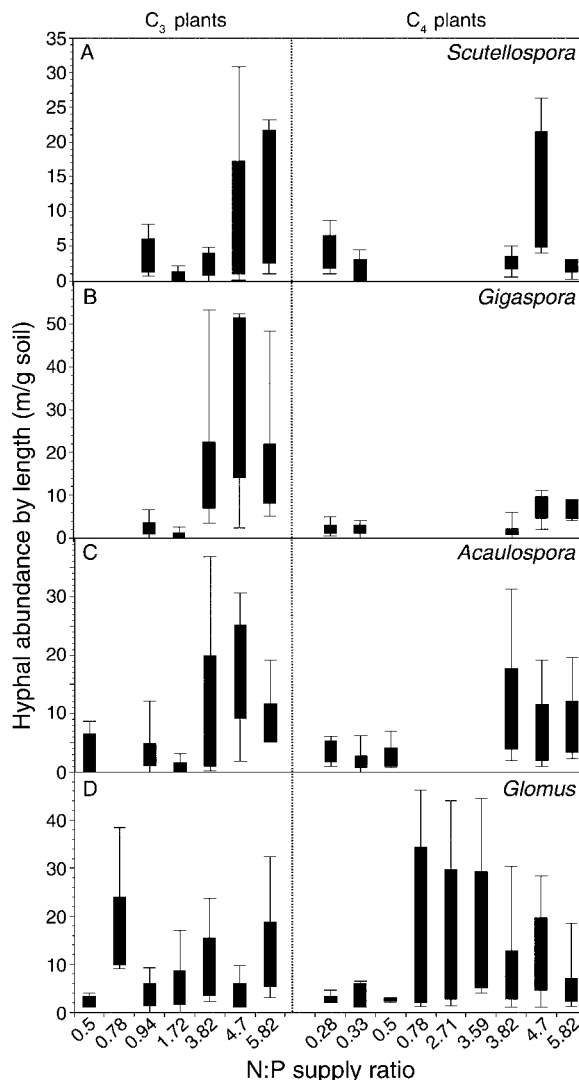


FIG. 8. The abundance of each genus of AMF hyphae in response to soil N:P levels and host-plant functional category. Abundance is represented as hyphal length (in meters) per gram of soil. Plots show the 25th and 75th percentiles (box ends), and 10th and 90th percentiles (whiskers). Hyphal data represent the pooled results of 1997 and 1998 assays. Note the scale differences among AMF genera.

ton and Allen 2000), largely sporulated in N-fertilized  $C_4$  plants. These AMF spore species also had a disproportionately large effect on AMF community evenness because their preexisting (high) abundance in non-fertilized soils was either retained (*Gl. intraradices*) or amplified in N-fertilized soils (e.g., *Gl. aggregatum*). By comparison, most of the AMF species lost from the community occurred with  $C_3$  host plants, a response that explained the overall loss of AMF community diversity.

Individual AMF spore species abundances also varied significantly from year to year. AMF communities have a reputation for high interannual variability in sporula-

tion, and indeed, our results indicated that, on average, only ~25% of the AMF community signaled the effects of N fertilization from one year to the next (see also Bever et al. [1996]). Many of these species have been previously classed as “N-indicator taxa” (e.g., *Gl. aggregatum*) in grass- and shrub-land communities (Egerton-Warburton and Allen 2000). Such taxa might be “indicators” simply because they have a high probability of producing spores from year to year in N-fertilized soils. Alternatively, these fungi may constitute a “functional group” owing to their collective responses to increasing soil N fertility. For instance, the N-responsive *Glomus* taxa have similar life histories (rapid root colonization, sporulation, small spores), life forms (root > soil colonization; Hart and Reader 2002), physiological requirements for photosynthate (modest; Douds and Schenck 1990), and ratio of P to C exchange (low; Jakobsen et al. 1992a, b). This stochasticity in species’ responses, rather than absolute variation, means that a single sampling or one-year study might fail to adequately capture the variability in response to N within an AMF community.

Under ambient soil N, it can therefore be argued that ANPP is one of the strongest predictors of AMF community structure (H1). AMF species richness also covaried with rainfall, thereby supporting the general contention that local variations in resource availability are important to AMF dynamics. Following N fertilization, however, AMF community structure appears to be controlled by the soil N:P supply ratio (H2) and plant photosynthetic strategy (H4), both of which presumably relate to host-plant specificity and changes in C allocation. In this context, it is useful to consider the relative importance of certain factors as drivers of AMF community change. These include the loss of rare or unique species (H3a), increases in the abundance of certain N-responsive AMF taxa (H3b), and a high turnover of AMF species (H3). It also appears to be true that these drivers are highly persistent over time (SGS: H2a) and that mesic sites were more sensitive to N fertilization than semiarid sites (H4b). On the contrary, we found less evidence to suggest that AMF spores and hyphal communities responded similarly to N enrichment (H3b). Instead, the local N:P supply ratio was more closely tied to AMF responses in both the spore and hyphal communities (H2).

While this study offers a unique perspective on AMF community responses to N, the results may have more immediate and practical implications for grassland systems. In particular, our findings indicate that variations in the AMF community could strongly influence the competitive balance between C<sub>3</sub> and C<sub>4</sub> plants in N-enriched soils. Previous studies have established that C<sub>3</sub> grasses, such as *Agropyron* and *Poa*, typically replace C<sub>4</sub> grasses in N-fertilized soils (e.g., *Andropogon*; Wedin and Tilman 1996). Similarly, the suppression of AMF in C<sub>4</sub> plants promotes the growth, reproduction, and relative densities of C<sub>3</sub> plants

(Hartnett and Wilson 1999). Our study suggests that a component of this competitive replacement is the shift in the physiological dependence of C<sub>3</sub> and C<sub>4</sub> plants on AMF. For example, the significant increase in hyphal length and shift in composition implies that C<sub>3</sub> plants invest more C into soil exploration and P acquisition than C<sub>4</sub> plants in N-fertilized soils. Another possibility is the phenological differences between C<sub>3</sub> and C<sub>4</sub> that enhance the preemption of space, i.e., AMF in C<sub>3</sub> plants take over any soil space vacated by AMF associated with C<sub>4</sub> plants. Conversely, both hyphal and spore abundance declined significantly in C<sub>4</sub> plants following N fertilization. In addition, the decline in hyphal length and diversity, and the abundance of N-responsive AMF taxa in the spore pool implies a reduction in AMF functional diversity in C<sub>4</sub> plants. Together, these interactions could feed back to alter plant community structure by shifting the distribution of resources among neighboring plants, either through common mycorrhizal networks or rates of nutrient cycling, since the photosynthetic pathway is assumed to represent the quality of plant resources (including root tissues). Notably, the differences in litter quality between C<sub>3</sub> and C<sub>4</sub> plants, high and low, respectively, could lead to different rates of N mineralization (Wedin and Tilman 1990) and because the belowground food web relies heavily on C originating from plant residues, produce a shift towards bacterial-based soil food webs rather than fungal-based food webs. As a result, we suggest that understanding how AMF communities respond to N should constitute the first step for predicting and understanding changes in both aboveground and belowground communities with increasing soil N availability.

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#### APPENDIX

A table listing measures of AMF diversity and species richness in each site and the N-fertilization treatments in 1997 and 1998 (*Ecological Archives* M077-015-A1).