Oecologia (2012) 170:1089–1098
DOI 10.1007/s00442-012-2363-3

COMMUNITY ECOLOGY - ORIGINAL RESEARCH

Nitrogen-fixing bacteria, arbuscular mycorrhizal fungi, and the productivity and structure of prairie grassland communities

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Received: 26 October 2011 / Accepted: 4 May 2012 / Published online: 10 June 2012

Abstract Due to their complementary roles in meeting plant nutritional needs, arbuscular mycorrhizal fungi (AMF) and nitrogen-fixing bacteria (N_2-fixers) may have synergistic effects on plant communities. Using greenhouse microcosms, we tested the effects of AMF, N_2-fixers (symbiotic: rhizobia, and associative: *Azospirillum brasilense*), and their potential interactions on the productivity, diversity, and species composition of diverse tallgrass prairie communities and on the productivity of *Panicum virgatum* in monoculture. Our results demonstrate the importance of AMF and N_2-fixers as drivers of plant community structure and function. In the communities, we found a positive effect of AMF on diversity and productivity, but a negative effect of N_2-fixers on productivity. Both AMF and N_2-fixers affected relative abundances of species. AMF shifted the communities from dominance by *Elymus canadensis* to *Sorghastrum nutans*, and seven other species increased in abundance with AMF, accounting for the increased diversity. N_2-fixers led to increases in *Astragalus canadensis* and *Desmanthus illinoense*, two legumes that likely benefited from the presence of the appropriate rhizobia symbionts. *Sorghastrum nutans* declined 44 % in the presence of N_2-fixers, with the most likely explanation being increased competition from legumes. *Panicum* monocultures were more productive with AMF, but showed no response to N_2-fixers, although inference was constrained by low *Azospirillum* treatment effectivity. We did not find interactions between AMF and N_2-fixers in communities or *Panicum* monocultures, indicating that short-term effects of these microbial functional groups are additive.

Keywords Symbiosis · Diversity · *Panicum virgatum* · *Azospirillum* · Rhizobia

Communicated by Catherine Gehring.

Electronic supplementary material The online version of this article (doi:10.1007/s00442-012-2363-3) contains supplementary material, which is available to authorized users.

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Introduction

Soil micro-organisms are increasingly appreciated as important drivers of the productivity and structure of plant communities (Bever et al. 2010; Reynolds et al. 2003). Mycorrhizal fungi (AMF) and nitrogen-fixing bacteria (N_2-fixers) are well-known microbial functional groups that are key in meeting plant nutritional needs for phosphorus (Smith et al. 2003) and nitrogen (Cleveland et al. 1999), respectively. However, while the independent effects of AMF and N_2-fixers on plant communities have been addressed, much less is known about how these two functional groups might interact to influence the productivity and structure of plant communities. These symbionts may have interactive effects on plant communities by mediating plant resource partitioning through their complementary roles in supplying limiting nutrients, or alternatively by competing for plant photosynthate (Larimer et al. 2010; Reynolds et al. 2003).

In experimental manipulations, plant productivity is generally higher in communities with AMF than in

communities without AMF or where AMF have been suppressed (Hoeksema et al. 2010), although this effect varies with soil fertility (Hoeksema et al. 2010; Johnson 1993). This increase in productivity is likely due to increased phosphorus uptake (van der Heijden et al. 1998), although AMF may have other benefits including improved pathogen resistance (Borowicz 2001). Plant species can vary widely in their response to AMF, however, and some studies find little change in productivity with AMF suppression as a result of compensation from species less reliant on mycorrhizae (Hartnett and Wilson 1999; O'Connor et al. 2002). Variation in the mycorrhizal dependence of plants can lead to shifts in plant competitive dominance and subsequently plant community diversity in the presence versus absence of AMF (Hartnett et al. 1993; Vogelsang et al. 2006). For example, in a grassland dominated by non-mycorrhizal C3 grasses, the presence of AMF increased diversity by promoting the subordinate mycorrhizal species (Grime et al. 1988; Stein et al. 2009). In contrast, in grasslands dominated by C4 grasses that are highly responsive to AMF, the presence of AMF may decrease diversity by allowing competitive suppression of subordinate species (Hartnett and Wilson 1999; Smith et al. 1998), an effect observed to be dependent on level of phosphorus (Vogelsang et al. 2006).

The presence of symbiotic (e.g., rhizobia) or associative (e.g., *Azospirillum* spp.) N_2-fixers can also increase productivity of plants and plant communities through increased availability of nitrogen (Cleveland et al. 1999). Rhizobia primarily associate with legumes, and the presence of rhizobia can increase total community nitrogen by direct supply to legumes (van der Heijden et al. 2006a) or indirectly via root exudates, common mycorrhizal networks. and litter decomposition associated with legumes (He et al. 2004; Johansen and Jensen 1996; Paynel and Cliquet 2003; Thomas and Asakawa 1993). This may increase total community productivity by increasing total ecosystem nitrogen (Thomas and Bowman 1998) and through increased nitrogen partitioning between legumes versus plants that rely on pools of soil inorganic nitrogen (Reynolds et al. 2003; van der Heijden et al. 2006a). The presence of rhizobia may also shift community composition by allowing legumes to compete with dominant species (van der Heijden et al. 2006a; Wurst and van Beersum 2009), shifting communities from nitrogen to phosphorus limitation (Thomas and Bowman 1998), and because of variation in species ability to benefit from increased nitrogen (Marty et al. 2009).

Azospirillum spp. and other free-living nitrogen-fixing bacteria may also be important sources of nitrogen to soil (Eisele et al. 1989; Solheim et al. 1996). Most experimental work has focused on associative nitrogen-fixing bacteria associated with the rhizospheres of agriculturally important plants, especially grasses (Dobbelaere et al. 2003; James and Olivares 1998). Associative N_2-fixers associated with the rhizosphere of plants may lead to significant levels of N_2-fixation (Montanez et al. 2009; Wickstrom and Garono 2007), and studies have also shown increased plant growth as a result of growth hormones produced by the bacteria or enhanced mineral uptake associated with colonization by *Azospirillum* (Dobbelaere et al. 2003; Lin et al. 1983; Okon and Labandera-Gonzalez 1994).

As a result of their role in providing nutrients that are frequently limiting to plant productivity, AMF and nitrogen-fixing bacteria may have synergistic effects on plant hosts by reducing both phosphorus and nitrogen limitation (Larimer et al. 2010; Stanton 2003). However, these symbionts may also compete for photosynthate (Orfanoudakis et al. 2004), as AMF and rhizobia both place high demands on the carbon fixed by plants (Jakobsen and Rosendahl 1990; Schulze et al. 1999; Warembourg and Roumet 1989). A meta-analysis found that plant performance did not exceed additive effects when plants were co-infected with both rhizobia and AMF, although this result could be attributable to variation among studies in level of soil fertility, plant growth stage, or coevolutionary history of plants and symbionts (Larimer et al. 2010). In contrast, where studies manipulate soil fertility in addition to symbionts, conditions of low soil fertility produce cases of synergistic interactions between rhizobia and AMF (Jia et al. 2004) and between *Azospirillum* and AMF (Mishra et al. 2008; Subba Rao et al. 1985). Given the potential for increased resource partitioning in association with microbial symbionts (Reynolds et al. 2003; Temperton et al. 2007), synergies between plants, N_2-fixing bacteria, and AMF may be more likely to manifest at the level of plant communities rather than individual plant hosts. However, to our knowledge, synergies between AMF and N_2-fixing bacteria in plant communities have not been tested.

These synergies are potentially important in a number of applied settings, particularly in low nutrient soils. *Panicum virgatum* and diverse native grassland communities are being considered as potential feedstocks for cellulosic biofuel production (Tilman et al. 2006; Wright and Turhollow 2010). Including appropriate microbial symbionts in these systems is likely to be important for sustainably increasing productivity and increasing ecosystem services generated by these crops, such as increased carbon sequestration and improved soil quality (Rygiewicz and Andersen 1994; van der Heijden et al. 2006b). Mycorrhizae and nitrogen-fixing bacteria may also improve establishment of plants on degraded sites, such as mine spoils (Roy et al. 2007; Walker et al. 2004). Plant microbial symbionts are increasingly recognized as important for the successful establishment of desirable species in ecological restoration (Middleton and Bever 2011; Richter and Stutz 2002).

Here, we test the effects of AMF and N_2-fixers (rhizobia and *Azospirillum*) on the productivity, diversity, and

species abundances of nutrient-poor grassland microcosms. Using *Panicum virgatum* as a case study, we also examine the separate versus interactive effects of these microbial functional groups in monoculture as opposed to diverse communities. We tested the following hypotheses: (1) complementary roles of AMF and nitrogen-fixing bacteria will have a synergistic positive effect on total plant productivity and diversity; synergistic effects of microbial symbionts will be stronger in communities than monoculture due to increased resource partitioning; and (2) the presence of AMF and nitrogen-fixing bacteria will shift plant community composition in favor of plant species that benefit most from these associations.

Materials and methods

Study system

Our focus was native tallgrass prairie restoration on land reclaimed after strip mining for coal, a system that we expected to be impoverished in ambient microflora and levels of plant nutrients and thus responsive to manipulations of AMF and N_2-fixers (Corbett et al. 1996; Saxerud and Funke 1991). Modern coal strip mining in the US involves removal and stockpiling of surface soil layers, typically the A horizon and portions of the B and C horizons, for capping the mine pit after coal seams are removed, and the pit is backfilled with mine spoils (Indiana Code 2011). Mixing of richer A horizons with less fertile B and subsoil horizons dilutes soil fertility, and both mixing and stockpiling disrupts and reduces microbial communities (Saxerud and Funke 1991; Williamson and Johnson 1990). This study system is of interest given that many thousands of acres of lands are still being strip mined and reclaimed in the Midwest U.S. and elsewhere, and there is increasing interest in restoration with native grassland for its benefits to wildlife habitat, recreation, carbon sequestration, and water purification among other ecosystem services (Halofsky and McCormick 2005; MRCSP 2006; Scott and Lima 2004). If effective, restoring the soil fertility of these lands via microbial inoculations would be a more sustainable approach than application of chemical fertilizer, whose production and use entail substantial inputs of fossil fuels and greenhouse gas emissions, nutrient leaching and associated degradation of water quality, and inhibition of the soil microbial community (Broussard and Turner 2009; Robertson et al. 2000; Tilman et al. 2002).

Tallgrass prairie microcosms were established in 11.3-L nursery pots (Hummert International, Earth City, MO, USA) filled with Cory and Iva series soil obtained from the Lewis Mine (Solar Sources, Vigo County, IN, USA). This soil included the top 2.1 m of alluvial silt and clay loam A, B, and C horizons, which had been removed, partially mixed, and stockpiled, then tilled to 0.3-m depth before delivery (Boyles, personal communication). These mixed layers had a pH (H_2O) of 5.8, contained 1.4 % organic matter, and tested at 1.2 ppm NO_3^-–N, 2.7 ppm NH_4^+–N, and 5 ppm PO_3^-–P (1 N KCL-extractable N, Bray 1-extractable P; Michigan State University, Soil and Plant Nutrient Laboratory, East Lansing, MI, USA). These low levels of available N and P in stockpiled soils, where plant uptake is not a large factor, are consistent with low soil fertility (Binkley and Vitousek 1989). Using a cement mixer (Gilson mixer 59015B; CF Gilco, Grafton, WI, USA), we mixed this soil 4:1 with coarse river sand to further reduce fertility and to lighten texture to promote root recovery at harvest. In order to eliminate background microbes, the soil mixture was pasteurized at 90 °C over two consecutive days for 120 min each day, using an electric soil sterilizer (Model SS-60; Pro-Grow Supply, Brookfield, WI, USA).

Plant treatments

Microcosms were planted either with monocultures of 24 individuals of *Panicum virgatum* or with mixtures of 2 individuals each of the following 12 species: *Elymus canadensis*, *Panicum virgatum*, *Sorghastrum nutans* (Poaceae); *Astragalus canadensis*, *Desmanthus illinoensis*, *Desmodium canadense* (Fabaceae); *Echinacea purpurea*, *Liatris spicata*, *Symphyotrichum novae-angliae*, *Ratibida pinnata* (Asteraceae); *Asclepias tuberosa* (Apocynaceae); and *Monarda fistulosa* (Lamiaceae). Seeds were purchased from Prairie Moon Nursery, Winona, MN, USA. All these species associate with AMF, though they vary in the benefits they gain from this association (Hartnett et al. 1993; Wilson and Hartnett 1998). The three legumes form associations with rhizobia, but the importance of associations between our study plant species and free-living N_2-fixers is unknown. Plants were initially grown from surface-sterilized seed (2 min soak in 50 % ethanol followed by 1 min in 5 % Clorox bleach and two rinses in sterile water for 1 min) in 2 × 2 × 4 cm cells containing pasteurized Metro Mix 360 (Sun Gro Horticulture) potting soil (except for *Panicum*, which is described below). Seedlings were grown for 1 month and then transplanted into experimental microcosms. Tools were sterilized between replicates by washing in soap and water, rinsing, and immersing in 95 % ethanol. To further minimize cross-contamination, we planted mesocosms in order of no-microbe controls to single-microbe treatments to AMF × N2-fixer treatments. Plants were regularly spaced in the microcosms, and in the case of the 12-species mixtures, a randomly generated arrangement of species was consistently used across microbial treatments.

Microbial treatments

AMF and N_2-fixers (rhizobia and *Azospirillum*) treatments were imposed in a 2 × 2 factorial design (sterile control, AMF, N_2 fixers, AMF × N_2 fixers). The AMF treatment consisted of a mixture of fungal cultures isolated from Indiana and Illinois prairie soils and cultured with *Sorghum bicolor*, and included *Acaulospora spinosa*, *Entrophospora infrequens*, *Scutellospora fulgida*, *Glomus claroideum*, *G. lamellosum*, and *G. mosseae*. The soil and coarsely chopped roots from these cultures were used as inocula for this experiment. Uninoculated controls received an equivalent volume of sterilized soil of the same type used for the AMF cultures. AMF were identified by species-defining characteristics of their asexually produced spores. AMF are generally thought to have low specificity of association, but growth of an individual plant species may depend upon the particular fungal partner. We inoculated with a diverse community as previous work has demonstrated that a diverse community provides the benefit of the best fungus (Vogelsang et al. 2006). A microbial wash is often included in uninoculated treatments to control for other micro-organisms introduced in the AMF cultures, but we did not include this background wash to reduce the potential for contamination with N_2-fixing bacteria. A meta-analysis of AMF studies found that the absence of this microbial wash provides a conservative test of the benefits of AMF and recommends this approach for factorial manipulation of AMF and rhizobia (Hoeksema et al. 2010). The N_2-fixer treatment included cysts of wild-type *Azospirillum brasilense*, ATCC 29145, suspended in 10 mM KPO_4 buffer (pH 6.8; 1×10^7 cfu/mL) and rhizobia as cultures attached to granular peat (~1×10^8 rhizobia per g). *Azospirillum* cysts were induced on minimal salts media (MSM; as described by Neyra et al. 1997) in the laboratory of Carl Bauer at Indiana University. Rhizobia strains were obtained from the *Rhizobium* Research Laboratory at the University of Minnesota where they were originally isolated from tallgrass prairie in Minnesota. A strain of *Rhizobium giardinii* and *Mesorhizobium huakuii* were used as the inoculants for *Desmanthus* and *Astragalus*; respectively. The strain used as inocula for *Desmodium* was not identified to species, but had previously been identified as "inoculant quality" for *Desmodium* (Beyhaut et al. 2006; Tlusty et al. 2004, 2005). Pots were filled two-thirds full with sterile background soil, then 80 mL of AMF inocula or a control of sterile soil of the same soil type was added. Five grams of rhizobia inocula for each of the three legume species (15 g total) or 15 g of a sterile granular peat control were then added to each pot which was topped with a layer of sterile soil. Pots were watered lightly, and then a template was used to create regularly spaced holes for plugs of the appropriate plant species. Before plants were added, 1 mL of *Azospirillum* or sterile buffer solution was added to each hole. Then, seedling plugs were planted into the holes and the pots were watered thoroughly.

We included a *Panicum* endophyte inoculation treatment with six endophytic fungi that we had previously identified as having beneficial effects on the growth of *Panicum* seedlings (Kleczewski et al. 2012). Briefly, *Panicum* seeds were cleared of preexisting endophytes by heat treating in 50 °C water for 30 min. Seeds were germinated in autoclaved sand and then inoculated with endophytes (Kleczewski et al. 2012). After 14 days, seedlings were transferred to Metro Mix immediately prior to their incorporation into the experimental microcosms. *Panicum* seedlings were smaller and less vigorous than those of the other study plant species, which did not receive hot water treatments and were sown directly into Metro Mix. In tests for presence of endophytic fungi in *Panicum* leaves after several months of growth, we were not able to consistently reculture inoculated fungi from *Panicum.* Furthermore, we did not detect significant effects of endophyte treatments in our initial statistical analyses of harvested plant biomass. Thus, the endophyte treatment was removed from the final analysis and will not be discussed further.

For both *Panicum* monocultures and prairie communities, each microbial treatment was replicated 6 times. However, due to the lack of an endophyte effect, our replication was effectively doubled so that each treatment in both communities and monocultures was replicated 12 times.

Data collection

Plants were allowed to grow in microcosms for 16 weeks, from June 6 to September 28, 2009. At this point, we clipped all aboveground biomass plus any root crowns. Plant tissue was then dried at 60 °C to a constant mass and weighed. Belowground biomass was sampled from each microcosm by taking three 5.1-cm diameter cores the full depth of the microcosm. The three cores from each microcosm were bulked, sealed in plastic bags, and held at 4 °C until processing. Bulked soil was subsampled to test for the abundance of associative N_2-fixers in the soil by plating a dilution series of 1 g soil in 10 mL water, then further diluting 10, 100, and 1,000×. Dilutions were plated on N-free media containing Congo Red, which binds to the cell walls of *Azospirillum* and changes color in the presence of nitrogen-fixing bacteria (Caceres 1982). Colony morphology was also compared to reference strains for identification as *Azospirillum*. Roots were washed and nodules present on the roots were counted to assess rhizobia inoculation. Roots were then clipped into 1-cm segments and a representative subsample from three replicates of each treatment was weighed and placed in tissue cassettes (HistoPrep OmniSette; Fisher Scientific Healthcare, Pittsburgh, PA, USA).

Following standard protocols (McGonigle et al. 1990), roots were cleared with KOH and stained with trypan blue for analysis of percentage fungal colonization (number of hyphae, arbuscules, vesicles or coils found at 30–40 root intersections per root subsample). The remaining roots were then dried at 60 °C and weighed. Dry weights of root subsamples were estimated using a wet to dry weight conversion factor obtained from the main root mass.

Data analysis

Data were analyzed using Proc GLM in SAS (v.9.3.1, 2010; SAS Institute, Cary, NC, USA). For each 12-species microcosm, total aboveground productivity was determined by adding the biomass of all species and the Shannon Diversity Index (H′) was calculated using the biomass of each species. We tested for microbial treatment effects on each of these response variables and root biomass, percent colonization of roots by AMF, and root nodulation using two-way ANOVA.

Shifts in plant community composition in the 12-species microcosms were analyzed with non-metric multidimensional scaling (NMS) using a Bray Curtis distance measure and starting coordinates from a Bray Curtis Ordination. The resulting axis scores were used as a response variable in a MANOVA to test for effects of AMF, N_2-fixers, and interactions on community composition. Changes in the abundance of each species were further tested using a MANOVA with AMF, N_2-fixers, and AMF × N_2-fixers interactions as fixed effects and the biomass of each species as response variables.

Results

Treatment effectivity

Percent colonization of roots by AMF was significantly higher in the microcosms inoculated with AMF (44 ± 3 %, mean ± SE) than in controls (7 ± 1 %, $p < 0.0001$). Counts of nodules on harvested roots found that microcosms inoculated with N_2-fixers had more nodules per root sub-sample than controls (N_2-fixers: 4.25 ± 1.2 nodules; control: 1.32 ± 0.3 nodules; ANOVA $p = 0.03$). We did not find evidence that AMF affected nodulation or that rhizobia affected AMF colonization (non-significant AMF × N_2-fixer interactions). There was extensive colonization of most pots by *Azospirillum* by the end of the experiment so that counts of plated colonies did not differ significantly between the N_2-fixers treatment and controls ($p = 0.20$). We therefore make the conservative assumption that any effects of the N_2-fixer treatment on plant responses in this experiment were due to rhizobia, and thus limited to the 12-species communities where legumes were also present.

Productivity

AMF and N_2-fixer treatments significantly affected above-ground productivity in communities, with productivity increasing in response to AMF (Fig. 1a) and decreasing in response to N_2-fixers relative to controls (Fig. 1b). There were no interactive effects of AMF and N_2-fixers on community productivity. *Panicum virgatum* monocultures produced more above-ground biomass with AMF, but, consistent with the poor *Azospirillum* treatment effectivity, we did not detect an effect of N_2-fixers or an AMF × N_2-fixers interaction on above-ground biomass (Fig. 1c; Table 1). Above-ground productivity in *Panicum* monocultures increased by 71 % in response to inoculations with AMF, in contrast to the 12 % increase observed in communities. We did not detect any treatment effects on root biomass in either *Panicum* monocultures or in communities (Table 1).

Community composition and diversity

Inoculation with AMF had a positive effect on the diversity (H′) of community microcosms, whereas inoculation with N_2-fixers did not significantly affect H′ (Fig. 2). For the NMS ordination, a three-axis solution was significantly different from random data ($p = 0.005$) and accounted for 96.3 % of the variation in our data. The ordination showed differences in composition of community microcosms due to AMF and N_2-fixer treatments, with axes 1 and 2 explaining 84 and 8.9 % of the variation, respectively (Fig. 3; axis 3 explained 3.6 % of variation). The significance of these

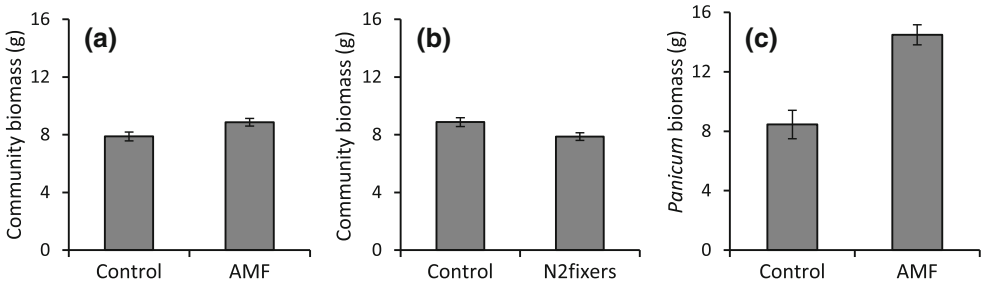

Fig. 1 The effect of **a** AMF and **b** N_2-fixers on total above-ground biomass in community microcosms and the effect of **c** AMF on above-ground productivity in *Panicum* monocultures

Table 1 Results of ANOVA for main effects and interactions of AMF and N_2-fixers on above-ground biomass in 12 species community microcosms, above-ground biomass in *Panicum* monocultures, root biomass and on diversity (H′) in 12 spp. microcosms

Productivity	*df*	*F*	*P*
12 spp. communities			
AMF	1,44	6.33	0.0156
N_2-fixers	1,44	6.70	0.0130
AMF × N_2-fixers	1,44	0.05	0.8204
Panicum monocultures			
AMF	1,44	35.88	<0.0001
N_2-fixers	1,44	2.10	0.1565
AMF × N_2-fixers	1,44	1.70	0.2012
Root biomass			
AMF	1,44	1.33	0.2550
N_2-fixers	1,44	0.10	0.7547
AMF × N_2-fixers	1,44	0.00	0.9630
Diversity—H′			
AMF	1,44	15.99	0.0002
N_2-fixers	1,44	0.95	0.3348
AMF × N_2-fixers	1,44	1.39	0.2456

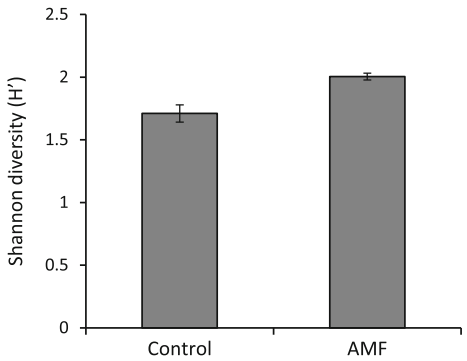

Fig. 2 Effect of AMF inoculation on species diversity, as measured by the Shannon Index (H′), in community microcosms

community shifts were established by MANOVA on axis scores, which indicated significant overall effects of AMF ($F_{3,42} = 13.12$, $p < 0.0001$) and N_2-fixers ($F_{3,42} = 7.83$, $p = 0.0003$), but no interaction ($F_{3,42} = 0.73$, $p = 0.73$). Shifts in community composition along axis 1 were due to both AMF ($F_{1,44} = 14.6$, $p < 0.0001$) and N_2-fixers ($F_{1,44} = 2.63$, $p = 0.012$), with standardized canonical coefficients indicating a stronger and opposite effect of AMF treatment (1.387) versus N_2-fixers (−0.722) on plant species abundances (see species MANOVA results below). N_2-fixers had a significant effect on axis 2 scores ($F_{1,44} = 1.32$, $p = 0.0065$), but there was no effect of AMF ($F_{1,44} = 0.35$, $p = 0.15$). Neither treatment had a significant effect on axis 3 scores ($F_{3,44} = 0.13$, $p = 0.94$).

Different plant species responses to AMF versus N_2-fixer treatments help explain changes in NMS scores (Fig. 4). Accordingly, a MANOVA of individual species' performance found significant main effects of AMF ($F_{12,33} = 7.83$, $p < 0.0001$) and N_2-fixers ($F_{12,33} = 5.17$, $p < 0.0001$) treatments, but no significant interaction ($F_{12,33} = 1.14$, $p = 0.36$, ESM Resource 1). Most strikingly, *Elymus* and *Sorghastrum* switched dominance based on AMF treatment (Fig. 4a). *Elymus* was the most important species in microcosms without AMF, whereas *Sorghastrum* dominated microcosms with AMF. Additionally, *Desmodium* increased dramatically with AMF along with positive responses of *Symphyotrichum*, *Liatris*, *Ratibida*, *Echinacea*, and *Monarda* (Fig. 4a). In contrast, *Elymus* and *Sorghastrum* were codominant in microcosms without N_2-fixers, whereas *Elymus* alone was dominant in microcosms with N_2-fixers, reflecting a significant negative effect of N_2-fixers on *Sorghastrum* abundance (Fig. 4b). *Liatris*, *Desmodium*, and *Symphyotrichum* also responded negatively to N_2-fixers, although this effect was significant only for *Liatris* (Fig. 4b). The legumes *Desmanthus* and *Astragalus* responded positively to N_2-fixers, as did *Ratibida* (Asteraceae) (Fig. 4b).

Discussion

We hypothesized that our low fertility microcosms, including co-occurring tallgrass prairie plants and microbial symbionts, would be an ideal context for synergistic effects of AMF and N_2-fixers on plant productivity and community structure. We expected greater interactive effects of AMF and N_2-fixers in communities versus monocultures due to increased resource partitioning (Reynolds et al. 2003). However, this hypothesis was not supported. AMF and N_2-fixers did not have interactive effects on productivity in communities or *Panicum* microcosms, although our ability to test for interactive effects in *Panicum* microcosms was constrained by poor treatment effectivity of associative N_2-fixing bacteria. Our results suggest that Larimer et al.'s (2010) conclusions, from a meta-analysis of single-species responses, that the response of plants to dual inoculation with microbial symbionts does not exceed the additive effects of either symbiont independently can also apply to plant communities.

We did observe significant main effects of AMF and N_2-fixers on productivity. AMF increased the productivity of community microcosms and *Panicum* monocultures. The effect of AMF was greater in *Panicum* monocultures than in community microcosms, which may be a result of *Panicum*'s strong positive response to AMF inoculation and the variable response of the other species included in our microcosms (Vogelsang et al. 2006; Wilson and

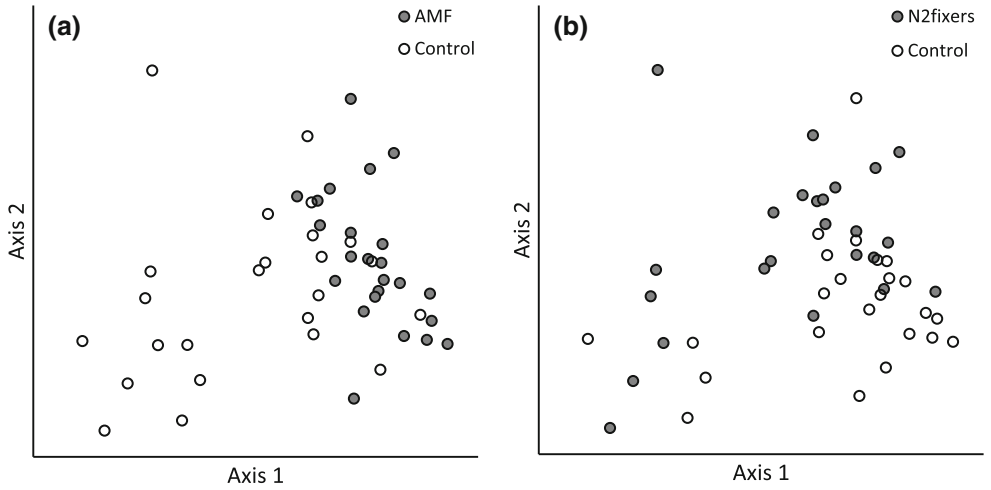

Fig. 3 NMS ordination of community microcosms showing main effects of **a** AMF treatment and **b** N_2-fixers treatment on composition of community microcosms. *Control* in (**a**) includes both control and N_2-fixers treatments and *AMF* includes both the AMF and AMF + N_2-fixers treatments. Similarly, *Control* in (**b**) includes both control and AMF treatments and *N_2fixers* includes both N_2-fixers and AMF + N_2-fixers treatments

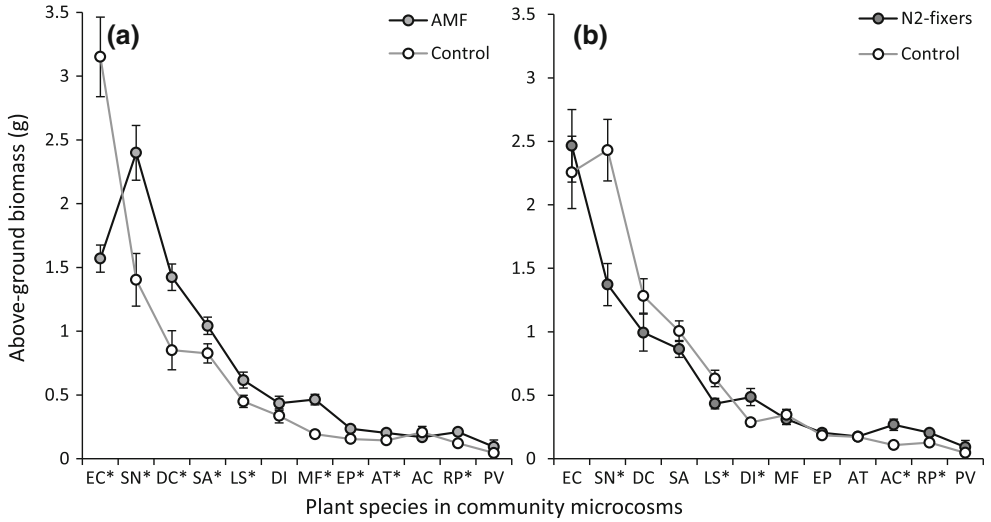

Fig. 4 Rank abundance diagrams showing the response of species within 12 species microcosms to **a** AMF and **b** N_2-fixers (*$p < 0.05$, full statistics in "Electronic Supplementary Material" (ESM) Resource 1). Abbreviations are as follows: *Elymus canadensis* (EC), *Sorghastrum nutans* (SN), *Desmodium canadense* (DC), *Symphyotrichum novae-angliae* (SA), *Liatris spicata* (LS), *Desmanthus illinoensis* (DI), *Monarda fistulosa* (MF), *Echinacea purpurea* (EP), *Asclepias tuberosa* (AT), *Astragalus canadensis* (AC), *Ratibida pinnata* (RP), *Panicum virgatum* (PV). As in Fig. 3, *Control* in (**a**) includes both control and N_2-fixers treatments and *AMF* includes both the AMF and AMF + N_2-fixers treatments. Similarly, *Control* in (**b**) includes both control and AMF treatments and *N_2fixers* includes both N_2-fixers and AMF + N_2-fixers treatments

Hartnett 1998). In contrast, N_2-fixers had no effect in *Panicum* monocultures. Again, the lack of an N_2-fixer treatment effect in *Panicum* monocultures is not surprising because *Azospirillum* colonized all treatments and *Panicum* does not associate with rhizobia. We did observe a negative main effect of N_2-fixers on productivity in community microcosms, indicating that the costs of maintaining this symbiosis during the establishment of these communities outweighed the benefits of increased nutrient inputs (Schulze et al. 1999; Warembourg and Roumet 1989). Although we found a negative effect of N_2-fixing bacteria on productivity in communities, other studies have found positive effects on plant community productivity, even within a single growing season, due to reduced competition for nitrogen and rapid transfer of nitrogen to other species (Marty et al. 2009; Temperton et al. 2007; van der Heijden et al. 2006a). However, other pathways for nitrogen input from N_2-fixers into ecosystems, such as decomposition of fine roots and leaf litter, may have positive effects over longer time scales than our experiment tested (Hogh-Jensen and Schjoerring 1997; Walley et al. 1996). Consequently, we may have been able to observe beneficial effects of N_2-fixing bacteria

had our experiment been conducted over a longer period of time. Further, nitrogen addition generally increases the beneficial effects of AMF on plants (Hoeksema et al. 2010) so that long-term nitrogen inputs in communities through the legume–rhizobia symbiosis could lead to AMF–rhizobia synergies.

We also expected synergistic effects of AMF and N_2-fixers on plant community composition and diversity. For example, AMF and N_2-fixers might be expected to have interactive effects on legumes, which can be highly responsive to both groups of microbial symbionts (van der Heijden et al. 2006a; Wilson and Hartnett 1998). Further, the potential for AMF and N_2-fixers to mediate resource partitioning could lead to synergistic effects on diversity. However, we did not find evidence for interactive effects of AMF and N_2-fixers in our analyses of diversity, overall plant community composition (NMS), or response of individual species within communities. As suggested above, synergistic effects may require more time for development than afforded in one growing season. Alternatively, it is possible that opportunities for resource partitioning or other complementary species interactions were constrained by the physical size of our microcosms.

We found support for our second hypothesis, that AMF and N_2-fixers would shift the composition of grassland plant communities. Major shifts in community composition occurred as a result of inoculation with AMF, with dominance of the communities shifting from *Elymus* towards *Sorghastrum*. Along with this increase in *Sorghastrum,* seven other species increased in biomass in the presence of AMF. This shift increased the evenness of the plant communities, explaining the increase in diversity (H′) associated with AMF. N_2-fixers did not significantly affect diversity, but did affect plant community composition through increases in the legumes *Astragalus* and *Desmanthus*, and decreases in *Sorghastrum* and *Liatris*.

Previous work has reported mixed results for the effect of AMF on species diversity. When the dominant plant is non-mycorrhizal, as in European calcareous grasslands, AMF promote diversity by increasing subordinate species and increasing the evenness of the plant community (Grime et al. 1988). In contrast, previous work in tallgrass prairie has found that when the dominant plants (e.g., C4 grasses) are mycorrhizal, AMF reduce diversity (Hartnett and Wilson 1999). In our study, the dominant species in uninoculated control microcosms was *Elymus*, a C3 grass with a weak response to AMF (Wilson and Hartnett 1998). The increase in diversity we observed supports the general framework that AMF can promote diversity when subordinate species are more dependent on mycorrhizae than dominants, but AMF suppresses diversity when dominant species are mycorrhizal (Hartnett and Wilson 2002; Urcelay and Diaz 2003; Vogelsang et al. 2006).

Our findings are consistent with others that have found that many legumes require rhizobia to grow and persist with competitive dominants (van der Heijden et al. 2006a; Wurst and van Beersum 2009). In our case, two legumes, *Astragalus* and *Desmanthus*, both had increased biomass in association with N_2-fixing bacteria, although a third legume, *Desmodium*, tended to decrease in biomass. We expected N_2-fixing bacteria to increase total productivity due to increased nitrogen inputs, but we found reduced productivity in the N_2-fixers treatment. This can largely be attributed to the 44 % decline in productivity of *Sorghastrum*. We did not detect any effect of N_2-fixers on *Panicum*, the other C4 grass included in our study. The N_2-fixers treatment included free-living *Azospirillum* in addition to rhizobia, but our treatment confirmation data indicate that all treatments were similarly colonized by *Azospirillum*. Rhizobia and the legumes they associate with thus appear to be the most likely drivers of the observed decline in *Sorghastrum* yield in microcosms treated with N_2-fixers. One possibility is that *Sorghastrum* experienced increased above- or belowground competition from the two legumes whose productivity was enhanced in the N_2-fixer treatment. Although *Sorghastrum* overtopped the legumes by the end of the experiment, it is possible that light competition was important early on, at the seedling stage. Alternatively, previous studies have reported reduced abundance of grasses as a result of phosphorus limitation associated with the presence of legumes (Thomas and Bowman 1998). Increased productivity of legumes and perhaps increased demand for phosphorus associated with the presence of rhizobia may thus have increased competition for phosphorus and decreased productivity in our experiment. *Sorghastrum* and other C4 prairie grasses are sensitive to phosphorus limitation, explaining their strong dependence on AMF (Hartnett et al. 1993; Hetrick et al. 1986). Therefore, *Sorghastrum*'s positive response to AMF and negative response to N_2-fixers may both be driven by demand for phosphorus.

The consistent, positive main effect of AMF on both *Panicum* and 12-species communities in our experiment supports further consideration of this symbiont as a means to improve the sustainability of biofuel crops and increase diversity and ecosystem services in prairie restoration and remediation. In addition to improved productivity, AMF have other benefits including increased carbon inputs into the soil and improved soil aggregation (Jakobsen and Rosendahl 1990; Wilson et al. 2009). Although our experiment found reduced plant community productivity in response to the presence of rhizobial N_2-fixers, long-term nitrogen inputs from legumes and associated rhizobia have the potential to improve the overall sustainability and productivity of a site over time (Hogh-Jensen and Schjoerring 1997).

Conclusions

Although we expected that our experimental conditions were ideal for synergies between AMF and N_2-fixers, we did not observe interactions between these two functional groups, supporting the conclusion that the effects of these symbionts will typically be additive rather than synergistic, at least over the short-term. However, we did observe significant main effects of AMF and N_2-fixers on plant productivity and community structure, which indicates the importance of soil microbial communities as drivers of ecosystem properties and plant community structure. These effects are likely to be important for improving productivity or species establishment in a number of applied settings including agricultural grasslands, remediation of degraded areas, and ecological restoration.

Acknowledgments We appreciate the contributions of the following research assistants to the set-up and harvest of our experiment: Sarah Svendson, Jessica Walter, Chris Brown, Adam Mercer, Amanda Posto, Rebecca Stoops, Erica Waters, and Julia Ferguson. Soil from the Lewis Mine was provided by George Boyles, Director of Reclamation for Solar Sources, Inc. Members of the Reynolds lab provided helpful comments on the design, results and interpretation of this work. Funding was provided to H.L.R. by Indiana University's Office of the Vice Provost for Research and the Biology Department, and to H.L.R., J.D.B. and K.C. by the Center for Research in Environmental Sciences (CRES). These experiments comply with current laws where the experiments were performed and the authors have no conflicts of interest.

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