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N addition undermines N supplied by arbuscular mycorrhizal fungi to native perennial grasses

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) form associations with plants and are ubiquitous in grassland and agriculture ecosystems. AMF are known to contribute to plant nitrogen (N) uptake, but the importance of AMF to ecosystem N cycling and overall plant N nutrition remains unclear, particularly in the context of agroecosystems. AMF abundance typically declines under N addition, but how this affects AMF function and subsequent N transfer to plants is unknown. We measured plant yield and plant N content in relation to AMF abundance and function under different soil N conditions, using both an N-addition experiment and a survey across perennial grassland sites with varying soil N levels. We used AMF root colonization to assess AMF abundance, but the presence of AMF does not necessarily relate to *function* (*i.e.* nutrient transfer with host plant), so we also used an allometric ratio of AMF structures and AMF fatty acid biomarkers as an index of AMF function. N addition significantly decreased AMF abundance by an average of 27%, and decreased function by an average of 42%, as measured by the allometric ratio. This pattern was supported by our survey study where soil N was negatively correlated with AMF abundance and function. In addition, plant N was positively related to higher levels of AMF allocation to nutrient transfer structures within host roots. Demonstrating these relationships across varying soil N levels at eight sites supported the hypothesis that AMF benefit perennial grasses by increasing N uptake. This is particularly important for perennial grasses grown for bioenergy because managing for higher AMF abundance and function may reduce or eliminate incentives for costly and environmentally problematic N addition.

1. Introduction

One factor that may contribute to the conservative N-use of perennial warm-season grasses are symbiotic fungi – mycorrhizae – that increase plant access and uptake of water and soil nutrients, including N (Smith and Read, 2008). Symbiotic mycorrhizae can provide their plant host with soil nutrients in exchange for photosynthate, influencing nutrient cycling above- and below-ground (Smith and Read, 2008). Since the mycorrhizal association costs the plant carbon, mycorrhizae may become more parasitic than symbiotic in fertile environments (Reynolds et al., 2005; Smith and Read, 2008). The exchange of nutrients has implications for plant nutrition, but mycorrhizae also affect many ecosystem processes by altering plant communities (Wilson et al., 2012), carbon cycling (Miller et al., 2002), and N cycling (Veresoglou et al., 2012). N cycling in an ecosystem may be regulated by mycorrhizal-N uptake (de Vries et al., 2011). While there is evidence for AMF-mediated N uptake by plants (Govindarajulu et al., 2005; Hodge et al., 2001; Veresoglou et al., 2012), other studies have found that AMF do not improve plant N gain (Reynolds et al., 2005). In cases where AMF are not enhancing N-uptake, N-limitation of plants may not be sufficient for the plant to give C to the mycorrhizae in exchange for N.

Mechanisms for how AMF benefit plants via N nutrition are still debated. Although evidence suggests that AMF are able to transfer N to plant hosts, it has not yet been determined whether AMF contribute to a significant amount of plant N (Corrêa et al., 2015). It was previously thought that only ectomycorrhizal fungi were able to secrete enzymes to aid in SOM mineralization to release N from substrates. However, there is evidence that AMF are also able to mineralize N from organic residues, therefore, AMF may also be directly involved with SOM degradation (Atul-Nayyar et al., 2009; Barrett et al., 2011; Hodge et al., 2001). Although lacking specific organic acids and chelators to degrade SOM directly, AMF aid in the weathering of soil minerals simply through their exudates and high rates of respiration. Additionally, AMF associate with bacteria that can further aid in the weathering of soil minerals (Taylor et al., 2009). AMF hyphal exudates may also aid soil N mineralization by providing a source of carbon for microbes, thereby

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Table 1
Site descriptions with soil properties.

Site			Soil properties						
Name	Latitude	Longitude	Texture	C:N	pH	Sand [%]	Silt [%]	Clay [%]	Bulk density [g cm^{-3}]
ARL	43° 17′ 45″ N	89° 22′ 48″ W	silt loam	8.24	6.6	9	66	25	1.30
KBS	42° 23′ 47″ N	85° 22′ 26″ W	sandy loam	7.82	6.1	63	31	6	1.60
Rocky Run	43° 27′ 30″ N	89° 19′ 54″ W	loamy sand	9.38	6.1	81	13	6	1.26
Fairfield	43° 28′ 32″ N	90° 21′ 11″ W	clay	7.14	6.5	19	20	61	1.10
Lund	42° 50′ 40″ N	90° 42′ 19″ W	sandy loam	8.76	6.4	54	28	18	1.45
Manthey	43° 26′ 32″ N	90° 48′ 41″W	sandy loam	10.65	6.9	77	13	11	1.42
Rowe	43° 25′ 24″ N	90° 40′ 44″ W	sandy loam	10.21	6.4	58	25	17	1.46
Becker	43° 33′ 44″ N	90° 41′ 32″ W	sandy loam	9.35	5.7	77	9	14	1.57
Becker Lake	43° 36′ 46″ N	90° 44′ 34″ W	sandy loam	11.22	6.8	82	1	17	1.47
Shinners	43° 17′ 58″ N	89° 21′ 22″ W	silt loam	10.90	6.8	13	71	16	1.30

reducing carbon limitation and increasing N mineralization and available N in the soil surrounding the hyphae (Hodge and Storer, 2014).

The role of AMF in plant N-uptake is even more uncertain when N availability is high, which is typical of many agroecosystems. Some have shown that N addition decreases AMF abundance (Leff et al., 2015; Staddon et al., 2004; Treseder, 2004; van Diepen et al., 2010) and AMF diversity (Emery et al., 2017), but N addition may also reduce (Johansen et al., 1994), stimulate (Tu et al., 2006), or show no effect (Schroeder-Moreno et al., 2011) on AMF-mediated N acquisition. Hence, increased AMF abundance does not necessarily translate to increased function. Moreover, mycorrhizal response to N addition can depend on nutrient stoichiometry. For instance, phosphorus (P)-limited grasslands may respond to N addition with increases in mycorrhizae because plants become even more P-limited, making it more beneficial for plants to invest in mycorrhizae to increase P-uptake (Johnson et al., 2003). However, in P-polluted soils, AMF colonization rates were higher in N-deficient soil (Blanke et al., 2011). These outcomes illustrate the complexities in determining how important AMF are in plant N uptake and growth, especially with N addition. Corrêa et al. (2015) hypothesized a curvilinear relationship between N availability and plant mycorrhizal growth response where mycorrhizal associations do not increase plant growth if both the AMF and plant are N-limited because the AMF retain N. If N availability is high and plants are not N-limited, the mycorrhizal associations may not benefit plant growth because the plant is either C-limited or limited by another nutrient so the AMF becomes a C drain. Only if the plant is N-limited and the AMF are not N-limited, would AMF be beneficial to the plant for increasing plant growth and N uptake (Corrêa et al., 2015, 2014).

Plant-mycorrhizae relationships have been extensively studied, but many gaps remain in our understanding of mycorrhizal-mediated N cycling. This is often attributable to experimental limitations. Moreover, the majority of mycorrhizal nutrient uptake studies have focused on P uptake (Veresoglou et al., 2012). Most work that has confirmed N uptake via AMF for plant nutrition has also been in highly-controlled greenhouse or microcosm environments, which limits inference to *in situ* conditions (Graham, 2008). Without a field setting, plant competition, and plant density effects, which are known to have strong effects on mycorrhizal functions and interactions, are ignored (Facelli et al., 2009; Hetrick et al., 1994; Wilson et al., 2012). Veresoglou et al. (2012) suggested that more observational and correlative studies would produce results with more ecological relevance, albeit with less mechanistic detail. Previous studies have already indicated abundance, biomass and diversity decrease with N addition in perennial bioenergy crops (Emery et al., 2017; Oates et al., 2016), but more information on the effects of N addition on AMF function is still needed.

Our goal was to evaluate the benefit of AMF to plant yield and N-uptake along a gradient of soil N conditions. We assessed AMF abundance and function by measuring AMF root colonization and AMF fatty acid biomarkers. As an index of AMF function, we used an allometric ratio (Johnson et al., 2003) of AMF structures indicative of the investment of AMF nutrient uptake and transfer, relative to total abundance. Similarly, AMF fatty acid biomarkers 16:1ω5 (NLFA:PLFA) were used as another measure of AMF function and physiological fitness (Allison and Miller, 2004). We hypothesized that increasing N availability would decrease mycorrhizal abundance and function. We also hypothesized that the AMF allocation to nutrient transfer structures would be positively correlated to plant yield and N uptake in low N conditions, and that the strength of the correlation would decrease with increasing N availability.

2. Materials and methods

2.1. Site description and experimental design

We used both a manipulative experiment to modify soil N levels and an observational study to assess AMF relationships across different soil types and N availabilities. For the manipulative experiment, we chose cultivated sites where we added three levels of N fertilizer (0, 56 and 196 kg N ha^{-1}) applied as granules of ammonium-nitrate (ARL) or granular 46% urea or liquid 28% urea (KBS). No other fertilizer amendments were applied to these sites over the course of the study. Sites were located on established plots of switchgrass at the Arlington Agricultural Research Center (ARL) in Arlington, WI (silt loam Mollisols) and the W.K. Kellogg Biological Station (KBS) in Michigan in Hickory Corners, MI (sandy Alfisols) (Table 1). The switchgrass at KBS was established in 2008 and fertilizer rates applied since 2009. The switchgrass at ARL was also established in 2008, however the fertilizer treatments first began in 2013, therefore, KBS switchgrass had a five-year legacy of fertilization, whereas the ARL plots were treated with fertilizer in the first year of the experiment. All plots were established in a randomized complete block design, with four replicate blocks at KBS and five replicate blocks at ARL.

Extensive evidence indicates that hyphal networks can share resources belowground among individual plants (Fischer Walter et al., 1996; Leake et al., 2004; Pringle, 2009; van der Heijden and Horton, 2009), so we inserted physical barriers between N-fertilizer treatment plots to insure plot independence. Barriers were made of aluminum sheet metal (0.08 cm thick × 50 cm deep, Badger Diversified Metal) and installed in May 2013. Trenches were dug with a Vermeer RT-100 trencher to cut a 60 cm deep × 10 cm wide trench along plot borders. Aluminum sheets were installed and trenches refilled with original soil to stabilize the aluminum sheets, which remained in place for the duration of the study.

For the observational experiment, we chose eight restored grasslands (Extensive sites) across south central Wisconsin that varied in soil type and soil N availability (Table 1). All Extensive sites were unfertilized, with the exception of one agricultural site (Shinners) that had been fertilized with ~100 kg N ha^{-1} since a year after stand establishment in 2004 and subsequently applied on an annual basis. Four of

the sites were harvested for biomass in late fall or early spring (Becker, Becker Lake, Lund, and Shinners), whereas the other sites were not harvested (Manthey, Rowe, Fairfield, and Rocky Run). Mowing has been shown not to affect AMF root colonization (Eom et al., 1999).

2.2. Aboveground biomass and nutrient content

Semi-permanent sampling stations were located at each field site for each year of the study (2013 and 2014). At the Extensive sites, sampling locations consisted of 1-m^2 quadrats at six permanent sampling locations at each site. Sampling stations were spaced at ~10-m intervals along each transect. Transects were randomly located, but some attention was given to placing transects and quadrats in locations with significant C4 grass cover because many of the sites also included C3 forage grasses, and forbs. The permanent sampling locations at ARL and KBS were 0.5-m^2 quadrats placed in a systematically random fashion within each plot. Five quadrats were placed in each plot resulting in 60 experimental units at KBS and 75 at ARL.

Aboveground plant biomass was clipped from quadrats at peak-standing biomass – when plants had begun to recycle nutrients belowground (typically mid-August in southern WI), but timing varies from year to year (unpublished data). Biomass samples from the restored grasslands were sorted into the following three categories: C4 grass (*Panicum virgatum*, *Andropogon gerardii,* or *Sorghastrum nutans*), C3 plants, and litter. Biomass samples from KBS and ARL were almost exclusively switchgrass, therefore, no species sorting was done and all plant biomass was considered switchgrass. Biomass was dried to a constant weight at 65 °C and reported as oven-dry weights. Biomass samples were finely ground using a Wiley Mill to pass a 1-mm mesh screen and pulverized using stainless steel balls in 2-ml micro-centrifuge tubes to further homogenize. N concentrations were determined by combustion on a Carlo-Erba elemental analyzer (CE Elantech EA1112, Lakewood, N.J.). Plant N content was calculated as a proxy for plant N uptake, which assumed a negligible amount of the plant N taken up was lost to the atmosphere or leaching. We calculated plant N content by multiplying biomass dry weights by biomass N concentration.

To determine plant limiting nutrients, which may affect AMF- host interactions and responses to N enrichment, we assessed plant N:P ratios. Plant tissue N concentration was determined as above, and we followed a dry ashing method (Schulte et al., 1987) to determine plant P concentration. Plant tissue N:P ratios are reported as the total N concentration divided by the total P concentration. N limitation is indicated by a N:P ratio < 14, P limitation is indicated by a N:P ratio > 16, and co-limitation between 14 and 16 (Koerselman and Meuleman, 1996).

2.3. Soil chemistry

In 2014, we assayed soils for available inorganic N (as NO_3^- and NH_4^+) and available P (as PO_4^{3-}) by extracting fresh soils (Robertson et al., 1999). We composited five soil cores (2.5 cm diameter × 10 cm deep) from each plot at three timepoints: June, July and August/September. Samples were transported on ice and stored at 4 °C until soil processing and extraction, which occurred within 48 h of field collection and extracts were then frozen at −20 °C. Extracts were analyzed on a Flow Solution 3100 segmented flow injection analyzer (OI Analytical, College Station, TX). Total available inorganic N was calculated as the sum of nitrate and ammonium in mg kg^{-1} of dry soil, and available P as PO_4^{3-} in mg kg^{-1} dry soil. The soil N:P ratio was determined by dividing available inorganic N by available inorganic P. For soil metrics, we report the mean across the three collection timepoints.

To characterize soil N and organic matter at the Extensive sites, four soil cores (10 cm deep × 2.5 cm diameter) were composited from each quadrat at the Extensive sites or from each plot at the cultivated sites in June, July and August. Soils were homogenized and organic matter and rocks removed using a 2-mm sieve. Total C and N were determined by combustion on an elemental analyzer (CE Elantech Flash EA1112, Lakewood, N.J.).

2.4. Quantification of roots and AMF colonization

Four soil cores (2.5 cm diameter) were taken from the surface 10 cm in each quadrat. Each soil core was split longitudinally to form two subsamples. One subsample was used for AMF root colonization and root length and the other NLFA/PLFA analysis. Samples were stored in a portable cooler with ice until reaching the lab, where samples were moved to a 4 °C refrigerator until processing. Samples for fatty acid extraction and subsequent NLFA/PLFA analysis were frozen until sample processing.

To assess AMF root colonization, soil samples were sieved for roots within four weeks of field sampling. Soil was first passed through a 2-mm sieve and roots were handpicked, washed several times in distilled water, stored in 70% ethanol, and refrigerated until further analysis. Only roots ≤1 mm diameter were selected. This root diameter cutoff was chosen because fine roots are most actively colonized by AMF (Miller et al., 1995). Fresh and dry root weights were taken to obtain moisture content of roots.

Once roots had been rinsed several times with distilled water, subsamples were weighed, dispersed in a shallow pan of water, and scanned using an Epson v700 scanner and analyzed for root length using IJ Rhizo software (Pierret et al., 2013) and converted to fine root length (< 1 mm diameter) per square meter. Total root weights were converted to a dry weight basis, using moisture content determined from a separate subsample. Another subsample of roots was prepped for analyzing total AMF colonization. Rinsed roots were placed in labeled histo-prep tissue capsules. Roots were cleared and stained using a modified method of Vierheilig et al. (1998). Roots were soaked in 10% KOH followed by a wet autoclave cycle at 121 °C for 30 min. Roots from the ARL and several of the Extensive sites required further clearing, so these roots were first soaked in KOH overnight before the autoclave cycle. Roots were rinsed with distilled water and boiled in 2.5% ink-vinegar (Schaffer black ink) solution for 3 min. Roots were again rinsed in distilled water and stored in a weak vinegar-water solution to de-stain for one week. Roots were mounted in 1-cm segments onto glass slides with PVLG mountant and colonization (%) estimated by the gridline intersect method (McGonigle et al., 1990) using a compound microscope at 200x to 400x. A minimum of 75 root intersections were scored for each sample.

Root colonization by AMF represents abundance, but the presence of AMF does not necessarily relate to *function* (*i.e.* nutrient transfer with host plant). As an index of AMF function, we used an allometric ratio method (Johnson et al., 2003) that was calculated using percent AMF colonization of the root: (arbuscules + coils)/(intra-radical hyphae + vesicles + arbuscules + coils). The allometric equation originally used by Johnson et al. (2003) included a measure of extra-radical hyphae in the allometric ratio. Including extra-radical hyphae biomass in our calculation did not significantly change any of the results, so we used intra-radical structures only when calculating the allometric ratio for uniformity because we only had extra-radical hyphae data in 2014 for a subset of the treatments.

2.5. Extra-radical hyphae biomass

In 2014, in-growth hyphal bags (~10 × 2.5 cm) were inserted in early spring and were extracted approximately three months later at plant maturity and frozen until further processing. Three in-growth hyphal bags were placed in the surface 10 cm of soil in four of the six quadrats at the Extensive sites (n = 12 per site), and in three of five quadrats for each N treatment plot at ARL and KBS (n = 12 per N treatment). In-growth hyphal bags were constructed of 50-μm nylon mesh, allowing fungal hyphae to grow through the mesh, but restricting

root growth. The mesh bags were filled with 100 g of ashed sand (#30–70, pure silica sand from Ogleby Norton Industrial Sands) to minimize the growth of saprotrophic fungi (Wallander et al., 2001). Extraction of hyphae from the bags loosely followed the methods described by van Diepen et al. (2010). In-growth hyphal bags were cut open, inspected for root contamination (cores with visible root infiltration were discarded), and emptied into a beaker of tap water making a composite sample of three cores per plot. By floatation and agitation, hyphae were separated from the sand and the hyphae/water mixture and decanted over a 50-μm nylon mesh filter. Sand was again mixed with water, agitated, and decanted; these steps were repeated until the water ran clear. The sample on the mesh filter was then further cleaned and inspected in a petri dish viewed under a dissecting microscope to remove grains of sand and debris. Subsets of samples were also checked under a compound microscope to evaluate the proportions of saprotrophic and AMF hyphae using a grid-intersect method. All samples contained > 95% AMF hyphae upon visual appearance (based on absence of cell septa in fungal hyphae), therefore all hyphal biomass extracted from cores were considered AMF. Cleaned hyphae samples were frozen in petri dishes and freeze-dried to a constant weight. A measure of total extra-radical AMF hyphae biomass was calculated as hyphal biomass per gram of sand.

2.6. Quantification of AMF biomass using fatty-acid extraction

Soil samples were taken in 2013 from the 0-N and 196-N addition treatments and analyzed for both the phospho-lipid fatty-acid (PLFA) biomarker 16:1ω5 (Balser et al., 2005; Zelles, 1999) and the neutral-lipid-fatty acid (NLFA) biomarker 16:1ω5 (Olsson, 1999). The NLFA biomarker is a measure of carbon resources that are stored in AMF, whereas the PLFA is a membrane lipid and therefore an index of AMF biomass. Studies have found that by varying nutrient availability, the plant host will adjust carbon allocated to the AMF, as reflected in the carbon storage in NLFAs of AMF (Olsson et al., 1997). Therefore, the NLFA:PLFA ratio provides some indication of AMF physiological state and nutrient status (Allison and Miller, 2004; Bååth, 2003). Additionally, using the NLFA as opposed to solely PLFA has been shown to be much more reflective of seasonal C exchange between plant and fungus (Lekberg et al., 2012). The neutral lipid marker has also been shown to be a better indicator for AMF biomass than the PLFA biomarker alone (Ngosong et al., 2012), as the 16:1ω5 PLFA is also shared with some bacteria which may create some misinterpretation of PLFA data. The turnover rate of PLFAs in soil is not always as rapid as previously assumed, which again warrants some caution in data interpretation. However, in our study, we are sampled the PLFA/NLFA biomass several months after treatment implementation (fertilizer application). Further, we are making comparisons between treatments, not interpreting absolute biomass of PLFAs, therefore making the interpretation of our NLFA/PLFA data less suspect (Frostegård et al., 2011).

Soil samples were freeze-dried, passed through a 2-mm sieve to remove roots and organic debris, and ground to further homogenize. Samples were frozen until lipid extraction using methods described by Allison et al. (2005). Lipid extraction followed a modified Bligh and Dyer (1959) method. The extracts were separated by silicic acid chromatography and the fractions bearing phospholipids and neutral lipids collected and dried in a rotary evaporator (Labconco). The acyl glycerides in the fractions were then converted to fatty acid methyl esters (FAMEs) by mild base methanolysis. The FAMEs were then extracted into hexane, dried, and then resuspended in hexane with an internal standard (19:0 ethyl ester) and transferred to a GC vial. Extracts were analyzed on a Hewlett-Packard 6890 gas chromatograph with a split/splitless inlet, a flame ionization detector and an Ultra 2 capillary column (Agilent Technologies, Santa Clara, CA). The lipid biomarkers 16:1ω5 for both neutral lipid fatty acids (NLFA) and phospholipid fatty acids (PLFA) were determined from MIDI peak identification software (Sherlock Microbial Identification System, MIDI Inc., Newark, DE) and converted to nmol lipid per gram of soil by comparing peak responses to those of the internal standard. Here, we report the NLFA:PLFA ratio of the 16:1ω5 biomarker, which we interpret as an index of AMF physiological fitness.

2.7. Data analysis

We used a linear mixed effects model for evaluating plant yield, N content, %N, soil parameters, root biomass and length, NLFA:PLFA, extra-radical hyphae biomass and plant allocation to AMF. We used a generalized linear mixed-effects model for analyzing AMF root colonization and the AMF allometric ratio, and the binomial family for a logit link function and weighted by the total number of root intersections counted on a slide for each sample. At ARL and KBS, we tested the fixed effect of N addition treatment using the random effects of plot nested within block. At the Extensive sites, we used site as the fixed effect and year as the random effect. For means comparisons, we used the *lsmeans* package (Lenth, 2015) with a cutoff of $p < 0.05$ to determine significance. For testing the effect of soil [N] on AMF colonization (using logit-transformed data), allometric ratio, and NLFA:PLFA ratio, we used least squares linear regression and significance determined at $p < 0.05$. To compare N addition and site effects on soil available N, P, soil N:P ratio, and plant tissue N:P ratio, we used an analysis of variance test and conducted mean comparisons again using the R package *lsmeans* (Lenth, 2015), with significance determined at $p \leq 0.05$.

All statistical analyses were performed in R version 3.2.3 (R Core Team, 2015). For ARL and KBS, a significant year × N addition interaction was observed, so we analyzed years separately for all response variables. At Extensive sites, year was not a significant fixed effect or interaction, so we analyzed all Extensive site parameters across both years. For analyzing all response variables, we used the *lme4* package (Bates et al., 2015). Data were tested for normality and we used a log transformation if assumptions of normality were not met.

3. Results

3.1. Soil and plant nutrients

All sites and N treatments in our study appeared to have some degree of N limitation, as indicated by the plant tissue N:P ratios, which were all < 14 (Table 2). The high-N addition treatments at ARL and KBS had the highest plant tissue N:P ratios, indicating these treatments were the closest to co-limitation or P limitation. The soil N:P ratios also reflected greater N limitation in the 0-N and low-N addition treatments. In the Extensive sites, only Becker Lake showed less N limitation by the plant tissue N:P ratio, but Shinners, Becker Lake, and Rowe all had the highest soil N:P ratios. Available N was greatest in the high-N treatments at ARL and KBS, whereas there were no significant differences in soil available P. At the Extensive sites, Shinners had the greatest soil N availability. Manthey was the only site with significantly greater soil available P.

3.2. Plant biomass and N uptake

Peak standing biomass yields at ARL were not significantly affected by N addition in 2013 or 2014 (Table 3), however adding only 56 kg N ha^{-1} at KBS increased aboveground biomass yield significantly in both years. Plant N content, a corollary for plant N uptake, increased significantly by N addition at both sites and years, as did the aboveground plant tissue N concentration. Fine root biomass and length were not affected by N addition the first year of the study at ARL, however by 2014 and the second year of N addition treatments, the high-N addition treatment showed significantly lower fine root biomass and lengths than other treatment combinations. This trend was more apparent at KBS, where fine root mass and length generally decreased with N

Table 2
Mean soil inorganic N and available P, soil N:P ratio and plant tissue N:P ratio. Data are means with 1 S.E. in parenthesis. Letters within a column indicate mean differences between N addition treatments (ARL and KBS) or site (Extensive sites) at $p < 0.05$.

Site	N addition [kg N ha^{-1}]	Available N (mg kg^{-1})	Available P (mg kg^{-1})	soil N:P	plant tissue N:P
ARL	0	5 (1.6)^{b}	23 (3)	0.2 (0.0)^{c}	5.4 (0.5)
	56	17 (0.5)^{b}	18 (2)	1.1 (0.3)^{b}	5.5 (0.2)
	196	59 (14)^{a}	23 (2)	2.8 (0.6)^{a}	7.1 (0.8)
KBS	0	3.5 (0.2)^{b}	8 (1)	0.5 (0.1)^{b}	5.9 (0.4)^{b}
	56	4.4 (0.5)^{b}	8 (1)	0.8 (0.1)^{ab}	6.0 (0.3)^{b}
	196	32 (11)^{a}	11 (1)	3.1 (1.0)^{a}	10.3 (0.1)^{a}
Rocky Run	0	3.2 (0.2)^{c}	4 (1)^{b}	1.5 (0.4)^{ab}	3.5 (0.2)^{b}
Fairfield	0	3.8 (0.2)^{bc}	6 (1)^{b}	0.9 (0.2)^{ab}	5.2 (0.3)^{b}
Lund	0	3.1 (0.2)^{c}	4 (1)^{b}	1.3 (0.3)^{ab}	4.3 (0.3)^{b}
Manthey	0	4.0 (0.2)^{bc}	17 (2)^{a}	0.4 (0.2)^{c}	4.6 (0.2)^{b}
Rowe	0	3.1 (0.1)^{c}	3 (1)^{b}	1.7 (0.3)^{a}	4.4 (0.3)^{b}
Becker	0	3.4 (0.1)^{c}	7 (1)^{b}	0.5 (0.1)^{bc}	4.0 (0.3)^{b}
Becker Lake	0	5.0 (0.5)^{b}	5 (1)^{b}	2.8 (1.4)^{a}	7.4 (0.6)^{a}
Shinners	100	8.0 (1.1)^{a}	7 (1)^{b}	2.0 (0.7)^{a}	3.9 (0.2)^{b}

addition.

Aboveground biomass yield at the Extensive sites were overall much lower than ARL and KBS, with the exception of the Shinners site, which had remarkably high aboveground biomass production compared to the other sites (Table 4). A wide range of productivity was observed across sites, with Becker Lake being the least productive (3.9 Mg ha^{-1}) and Shinners the most productive (14.4 Mg ha^{-1}). Shinners was the least productive for fine root biomass and root length. Becker and Becker Lake were the least productive for aboveground biomass yield, but these sites had the greatest fine root mass and length (Table 4).

Aboveground plant N content followed a pattern similar to yield in Extensive sites. Plant N content varied considerably, with only 24 kg N ha^{-1} taken up by plants at Becker and 123 kg N ha^{-1} at Shinners. While Shinners produced much more biomass, the plants also had a much higher tissue N concentration compared to the other sites. Rocky Run had the lowest tissue N concentrations, which is also the site with the lowest soil N content. Rocky Run was the second most productive site when it came to aboveground biomass yield, indicating that the plants at Rocky Run were remarkably N-use efficient.

3.3. AMF root colonization and NFLA:PLFA

Stained roots revealed identifiable AMF structures, such as intraradical hyphae, coils, arbuscules, and spores. Total root colonization by AMF was relatively high across all sites and years. N addition treatments reduced colonization by AMF across both ARL and KBS sites, with the effect particularly strong at KBS (Fig. 1a). Any amount of N fertilizer addition caused a decrease in the AMF allometric ratio across both sites and years (Fig. 1b). The decrease in AMF function and physiological fitness was also demonstrated by the decrease in the NLFA:PLFA ratio with N addition, although this trend was only statistically significant at ARL (Fig. 1c).

At the Extensive sites, rather than comparing AMF parameters at the site level, we explored whether soil nutrients across sites had any correlation to AMF abundance and function. Among metrics that evaluated soil N and P availability (total soil [N], total inorganic N, inorganic P, soil N:P ratio, and plant tissue N: P ratios), total soil [N] was the only metric that was correlated to measured AMF parameters. Although the relationships were weak, there was a significant negative effect of total soil N on total AMF colonization ($R^2 = 0.01$, $p < 0.05$) and the AMF allometric ratio ($R^2 = 0.02$, $p < 0.05$) (Fig. 2a and b). There was however no effect of any soil N metric on the AMF NLFA:PLFA ratio across the Extensive sites ($R^2 = 0.04$, $p = 0.27$) (Fig. 2c).

3.4. AMF and plant allocation affected by N addition treatment

AMF allocation to nutrient transfer structures had no significant correlation to plant aboveground biomass or plant N content (data not shown). There was however a weak ($R^2 = 0.08$), but significant ($p < 0.05$) relationship with plant tissue [N] in the 0-N addition treatment (Fig. 3). Although a weak relationship, it is of some biological significance: back-transforming the data, an allometric ratio of 0.6 relates to plant [N] of 0.8 and an allometric ratio of 0.1 translates to a plant [N] of 0.6. While a difference of 0.2 may seem slight, this is the magnitude of difference between the average plant [N] in the 0-N addition treatment and the 56-N addition treatment. There was no correlation between the AMF allocation ratio and plant [N] in the 56 kg N ha^{-1} N treatment ($R^2 = 0.01$, $p = 0.33$) and the 196 kg N ha^{-1} N treatment ($R^2 = 0.02$, $p = 0.18$). AMF extra-radical hyphae biomass in 2014 was reduced in the high N-addition treatment at both ARL and

Table 3
ARL and KBS site yield, biomass N content, and biomass N from samples taken at peak-standing biomass at plant anthesis. Values are means with 1 S.E. in parentheses. Different letters within a column and within a site and year denote significant differences between N addition treatments at $p < 0.05$.

Site	Year	N addition [kg ha^{-1}]	Yield [Mg ha^{-1}]	N content [kg N ha^{-1}]	Plant tissue N [%]	Fine root biomass [g m^{-2}]	Fine root length [m m^{-2}]
ARL	2013	0	11.5 (1.0)	91.2 (6.9)^{b}	0.82 (0.03)^{c}	39.1 (3.4)	2305 (159)
		56	11.2 (1.1)	109 (10.1)^{b}	1.0 (0.03)^{b}	29.4 (2.1)	1941 (113)
		196	11.8 (1.0)	158 (12.1)^{a}	1.36 (0.04)^{a}	26.9 (2.1)	1796 (118)
	2014	0	10.2 (1.1)	74.9 (9.8)^{b}	0.71 (0.02)^{c}	32.0 (3.4)^{a}	2046 (242)^{a}
		56	10.1 (0.9)	80.5 (6.5)^{b}	0.81 (0.02)^{b}	20.8 (2.2)^{a}	1340 (125)^{a}
		196	10.7 (1.2)	134 (14.9)^{a}	1.27 (0.02)^{a}	14.6 (1.9)^{b}	976 (107)^{b}
KBS	2013	0	10.8 (0.9)^{b}	81.2 (7.6)^{c}	0.75 (0.02)^{c}	50.0 (5.4)^{a}	2108 (221)^{a}
		56	13.5 (0.8)^{a}	120 (9.1)^{b}	0.88 (0.03)^{b}	37.4 (3.3)^{ab}	1600 (139)^{ab}
		196	12 (0.9)^{ab}	191 (14.6)^{a}	1.62 (0.03)^{a}	19.4 (3)^{b}	970 (140)^{b}
	2014	0	9.5 (0.8)^{b}	64.9 (5.2)^{c}	0.7 (0.03)^{c}	65.9 (8.2)^{a}	3127 (429)
		56	12.6 (0.9)^{a}	105 (7.4)^{b}	0.84 (0.02)^{b}	57.8 (6.2)^{a}	3166 (355)
		196	13.2 (1.2)^{a}	173 (14.8)^{a}	1.34 (0.04)^{a}	35.6 (4.8)b	2566 (319)

Table 4
Extensive site yield, biomass N content, biomass N, fine root biomass and length from samples taken at peak-standing biomass. Values are means across both years 2013 and 2014 with 1 S.E. in parentheses. Different letters within a column denote significant differences between N addition treatments at $p < 0.05$.

Site	Yield [Mg ha^{-1}]	N content [kg N ha^{-1}]	Plant tissue N [%]	Fine root biomass [g m^{-2}]	Fine root length [m m^{-2}]
Rocky Run	7.02 (0.29) ^{b}	40.66 (4.29)^{bc}	0.57 (0.04) ^{c}	43.5 (3.9) ^{abc}	5021 (707) ^{bc}
Fairfield	5.27 (0.5) ^{bcd}	42.48 (1.97) ^{bc}	0.87 (0.07) ^{ab}	31.9 (4.2) ^{bc}	5151 (810) ^{bc}
Lund	5.04 (0.24) ^{cd}	33.59 (3.2) ^{bc}	0.66 (0.05) ^{abc}	44.1 (4) ^{abc}	5148 (569) ^{abc}
Manthey	4.1 (0.39) ^{d}	28.37 (2.24) ^{bcd}	0.71 (0.03) ^{abc}	48.1 (5.3) ^{ab}	6819 (1007) ^{abc}
Rowe	6.15 (0.7) ^{bcd}	43.82 (3.97) ^{b}	0.75 (0.05) ^{abc}	27.5 (4.4) ^{c}	3815 (840) ^{c}
Becker	4.05 (0.64) ^{d}	24.42 (3.99) ^{d}	0.65 (0.07) ^{bc}	81.8 (14) ^{a}	10,567 (1704) ^{a}
Becker Lake	3.89 (0.29) ^{d}	27.92 (2.72) ^{cd}	0.74 (0.07) ^{abc}	61.8 (4.5) ^{a}	7751 (507) ^{ab}
Shinners	14.42 (0.79) ^{a}	122.54 (6.01) ^{a}	0.86 (0.04) ^{a}	13.1 (2.1) ^{d}	808 (147) ^{d}

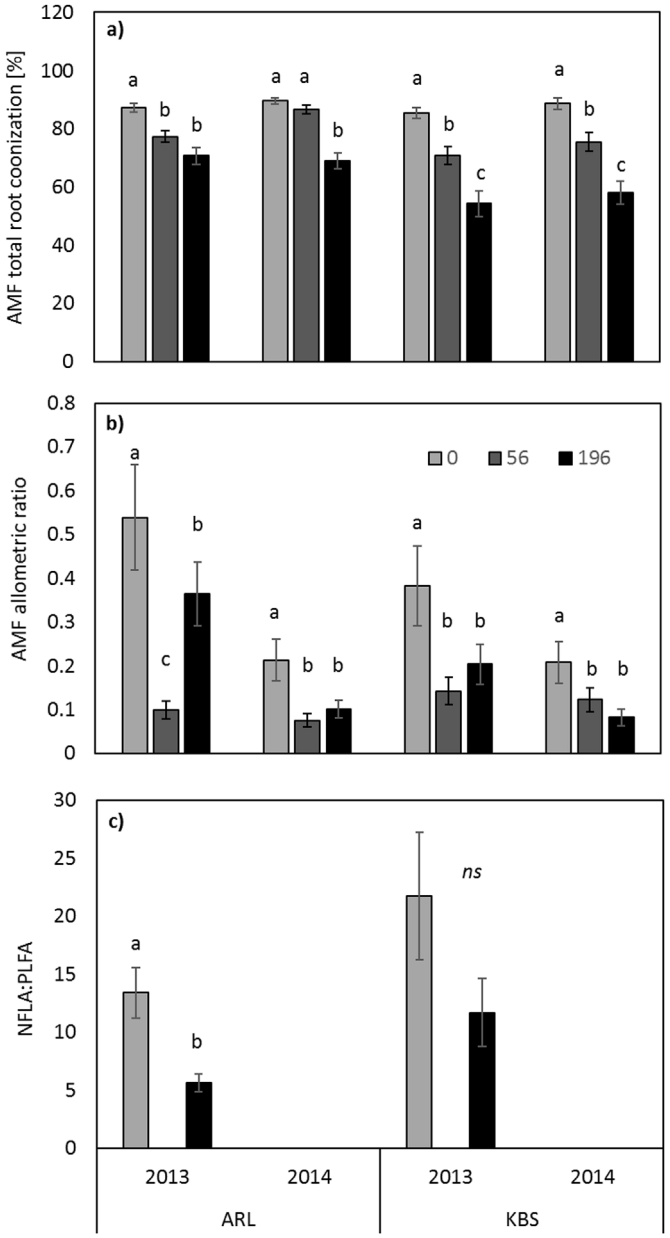

Fig. 1. Means for 0, 56, and 196 kg N ha^{-1} addition treatments for a) AMF root colonization [%], b) the AMF allometric ratio and c) the NLFA:PLFA ratio for 16:1ω5 for sites ARL and KBS in 2013 and 2014. Error bars are ± 1 S.E. and different letters above the bars represent means are significantly different at $p < 0.05$ across N addition treatments within each year by site combination.

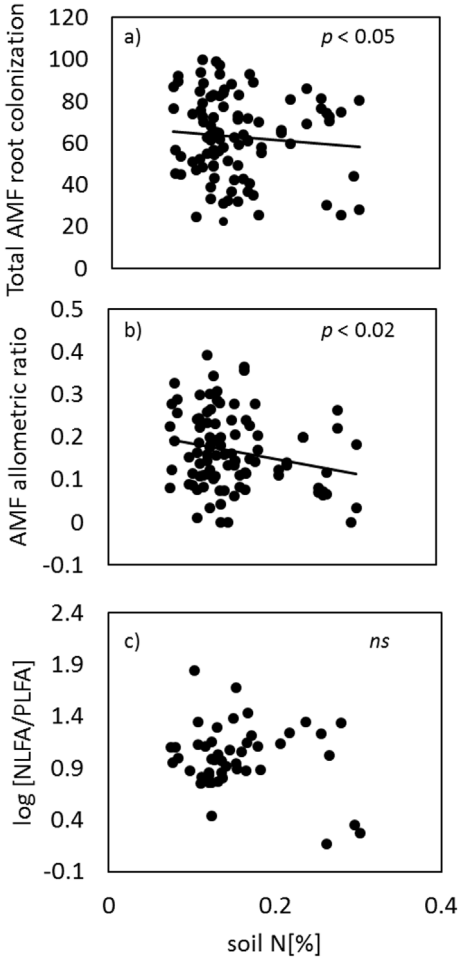

Fig. 2. Total soil N [%] across all Extensive site plots in 2013 and 2014 correlated with a) Total AMF root colonization [%] (displays untransformed data for ease of interpretation, but note that statistical testing used the logit transformation, see methods section), b) the AMF allometric ratio and c) AMF NLFA:PLFA ratio. Solid lines are significant linear regressions, with the indicated *p*-value.

KBS (Fig. 4a), but there was no difference between the zero- and low-N addition treatments. The proportional plant allocation to AMF mirrored the same pattern as extra-radical hyphal biomass where it was also less in the high N addition treatments for both ARL and KBS (Fig. 4b).

4. Discussion

4.1. AMF provided more benefit to plants in low N conditions

Switchgrass plants appear to benefit more from associations with AMF in soils with lower N. The primary benefit to plants in this case was demonstrated by increased plant [N] at ARL and KBS in the zero-N

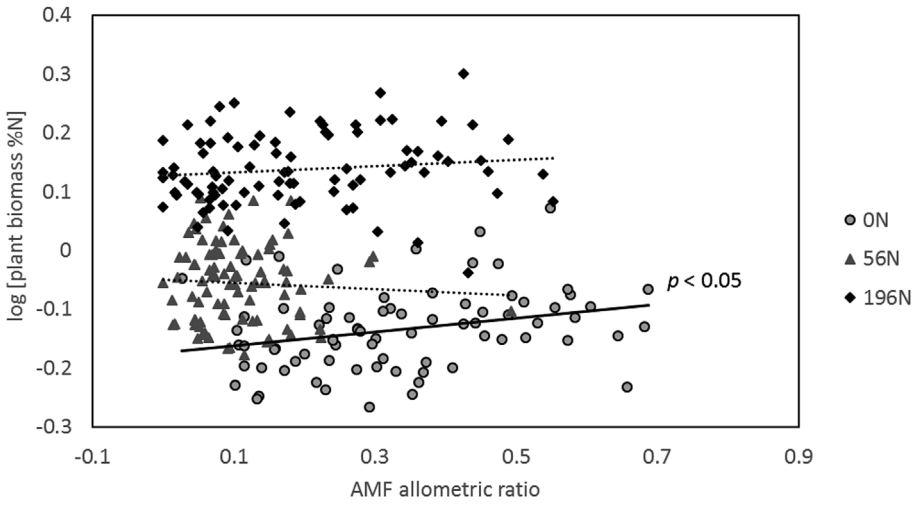

Fig. 3. The AMF allometric ratio regressed against plant biomass [N] for all plots at ARL and KBS in 2013 and 2014 for each N addition treatment of 0, 56, or 196 kg N ha^{-1}. Solid line ($y = 0.1184x - 0.1738$) is the significant ($p < 0.05$) linear regression for the 0 N treatment.

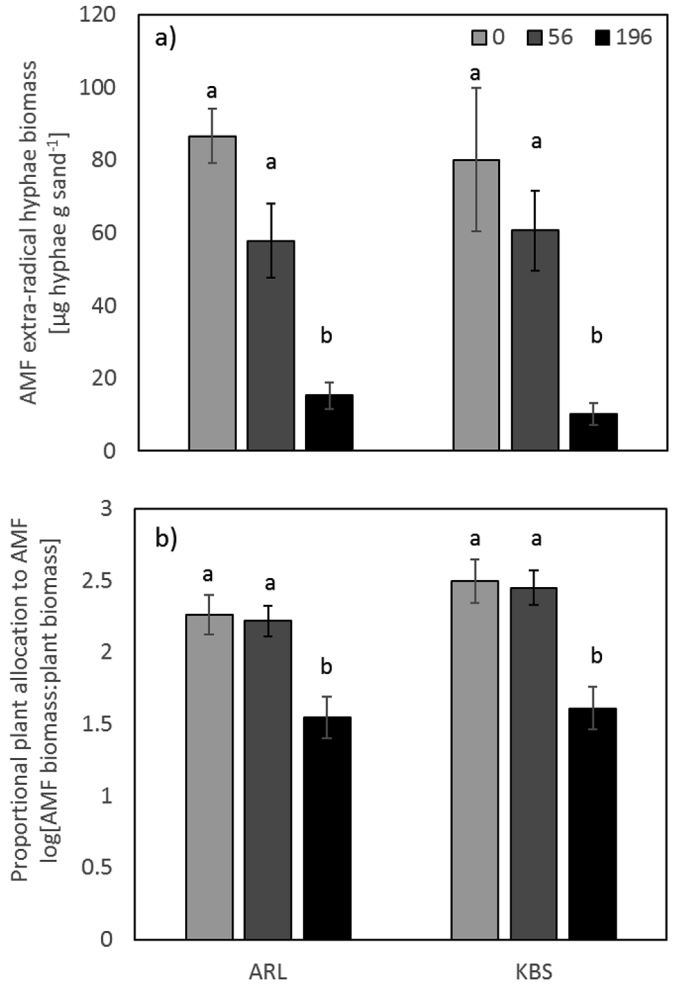

Fig. 4. Mean AMF extra-radical hyphae biomass (a) and proportional plant allocation to AMF (b), measured as the ratio of AMF extra-radical hyphal biomass to plant aboveground biomass for both sites ARL and KBS in 2014. Error bars are ± 1 S.E. Letters indicate significant differences between the means at $p < 0.05$ across all sites and N addition treatments (0, 56, and 196 kg N ha^{-1}).

treatment where AMF allocated more resources to nutrient transfer structures. These results support our hypothesis that the AMF allocation to nutrient transfer structures would be positively correlated to plant N uptake in low N conditions. Further, this relationship was not supported in soils with higher soil N. However, the additional increase in plant tissue N did not necessarily result in increased aboveground plant productivity as we had hypothesized. Perhaps because while plants were able to overcome some limitation to growth from N supplied by AMF, they also may have been faced with another limitation, such as light, water or space that limited aboveground biomass growth. It does not appear that these plants were limited by P, as both soil and plant nutrient analysis showed greater N over P limitation in all treatments. Conversely, if plants and AMF were under severe N-limitation, the AMF would likely retain any additional N resources for itself, and transfer little to no N to the plant (Püschel et al., 2016). This seems less likely given our results suggesting that with increased AMF allocation to nutrient transfer structures, plants were increasing in plant tissue [N].

Although our indicator of increased AMF function (allometric ratio) did not correlate to increased aboveground biomass yield, it was interesting that these unfertilized plants were able to produce more fine-root biomass and length, and support more extra-radical hyphal biomass with no cost to producing aboveground biomass. Aboveground biomass production did not differ across N-addition treatments, but unfertilized plots supported more fine root biomass and more AMF biomass. The mechanism for this increased biomass production would appear to be that the AMF were providing increased nutrient transfer to the plant hosts, as indicated by both the higher root colonization and in particular, the increased allocation of AMF nutrient transfer structures in the low-N conditions. In addition, the NLFA:PLFA ratio was greater in the zero N-addition treatment, suggesting that the AMF were more physiologically fit and likely receiving more C from their plant hosts than their fertilized counterparts, and in return were able to transfer more nutrients to the plant.

Evidence for an AMF benefit to plants via N nutrition was not as compelling from the Extensive site results. However, the negative correlation of total soil [N] with AMF colonization and the AMF allocation suggested that AMF were not as abundant or functional in conditions of higher soil N. The lack of correlation to other soil nutrient parameters such as soil available N, P, and the N:P ratio, may be because these measures of soil nutrients reflect somewhat transient pools. Soil [N] may be more reflective of long-term soil N stocks by also including all forms of organic N, which may also be a component of N nutrition for plants (Schimel and Bennett, 2004) and AMF (Barrett et al., 2011). Additionally, the Extensive sites may not have spanned a great enough range in soil nutrient levels to detect relationships between nutrient availabilities and AMF presence and function, particularly if the relationship was curvilinear, as Corrêa et al. (2015) hypothesized.

Although the relationships we present show relatively small incremental losses in AMF abundance and function as soil [N] increased, it is compelling that we were able to detect a signal through the noise of all the environmental variation that accompanies *in situ* studies. As

Veresoglou et al. (2012) suggested, the field of mycorrhizal ecology needs more observational and correlative studies to demonstrate AMF's role in N cycling to provide more ecological relevance. Our study found support for AMF's role in N nutrition to plants across many different Extensive sites, which provides much-needed support of this hypothesis that so far has been demonstrated primarily in highly controlled microcosms or greenhouses (Graham, 2008).

4.2. AMF abundance and function decreased with N availability

N addition clearly decreased AMF root colonization and extra-radical hyphae biomass. This trend was most dramatic at KBS for root colonization, where the N addition treatments had been implemented for 5 years as opposed to the start of the study at ARL. This suggests that the longer the system is subjected to N addition, the more dramatic the effects on AMF relationships with plants. Multiple studies have demonstrated decreased AMF associations with plants under N addition (Corrêa et al., 2015; Grman and Robinson, 2013; Johnson et al., 2003; Treseder, 2004). So far, it has been relatively unclear if the decrease in AMF abundance is related to the AMF relationship with the plant, or a direct negative response of AMF to N addition. We provided evidence for decreased AMF function in addition to AMF abundance under increased N availability, indicating that decreased AMF abundance in high N conditions is driven by the lack of demand from the plant host (as measured by decrease in AMF function), rather than a direct effect of N addition decreasing AMF abundance.

The AMF allocation ratio was the most responsive metric to soil N conditions. We were able to measure an AMF allocation response across both ARL and KBS in both years, and across the varying soil N conditions of the Extensive sites. The changes driving the AMF allometric ratio were primarily the abundance of AMF coils and arbuscules relative to intra-radical hyphae and vesicles. Therefore, lower soil N conditions at the Extensive sites and the low N-addition treatments at ARL and KBS were related to an increase in AMF allocation to structures involved in nutrient transfer with the plant host. This suggests that AMF are investing more into plant nutrient exchange with the plant to maintain the symbiotic relationship.

4.3. Linking AMF to agronomic management

Warm-season perennial grasses have inconsistent yield responses to N addition (Duran et al., 2016; Hoagland et al., 2013; Jach-Smith and Jackson, 2015; Parrish and Fike, 2005). While N addition had no effect on plant yield at ARL, there was a positive response at KBS. However, the long-term trend of N fertilizer addition at KBS has consistently been an overall decrease in yield response to N fertilizer (Ruan et al., 2016). It appears that one explanation for inconsistent yield responses to N addition is that AMF are able to supply N to plants in low N conditions that otherwise would be provided by N fertilizer. We observed a clearly positive response to N addition for plant N content and plant tissue [N], which has been demonstrated by many others (Garten et al., 2011; Guretzky et al., 2010; Heggenstaller et al., 2009; Jach-Smith and Jackson, 2015; Jarchow and Liebman, 2012; Jung and Lal, 2011; Madakadze et al., 1999). While N addition may have a more dramatic effect on increasing plant N, we demonstrated that AMF abundance and function were also related to increased plant N. It may be that AMF were able to provide just the right amount of N to keep up with plant N demand and growth. Fertilizer N-addition often results in luxury-consumption of N and no additional biomass growth, resulting in very inefficient N-use (Jach-Smith and Jackson, 2015).

Reducing N addition also increased fine root growth and AMF extra-radical hyphae growth, both important sources of belowground C inputs. AMF are known for their ability to increase soil C storage (Wilson et al., 2009). While others have found that increased plant C inputs from N addition can decrease microbial biomass and activity (Geisseler et al., 2016; Riggs and Hobbie, 2016), it would appear our study demonstrates the opposite. We found that microbial biomass and plant C increase without N addition and could potentially improve soil C storage. Regardless, AMF communities in agroecosystems are increasingly recognized for improving agricultural sustainability (Rillig et al., 2016) and our study demonstrated that managing for AMF in agroecosystems is a worthwhile pursuit because they play an important role in plant N nutrition.

5. Conclusions

AMF provide many benefits to plants, but there has been scant evidence of AMF contributing an agronomically-relevant amount of plant N. In remnant and restored grasslands, we showed that under low-N conditions AMF were associated with higher plant N concentrations and AMF invested in more nutrient transfer structures. Increasing soil N was associated with a decrease in AMF abundance, biomass, function, physiological fitness, allocation to nutrient-transfer structures, and plant allocation, suggesting that the importance of AMF to plant N nutrition was reduced when N was more available. These findings suggest that the use of N fertilizer should be limited in switchgrass cropping systems to promote AMF abundance and function, which may supplant N fertilizer in providing adequate plant N.

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