Title: Inorganic N addition replaces N supplied to switchgrass (*Panicum virgatum*) by arbuscular mycorrhizal fungi

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Abstract

Arbuscular mycorrhizal fungi (AMF) provide many benefits in agroecosystems including improved soil tilth, carbon sequestration, and water and nutrient transfer to plants. AMF are known to affect plant nitrogen (N) dynamics and transfer N to plants, but there have been few studies addressing whether the amount of N transferred to plants by AMF is agronomically relevant. We used $\delta^{15}N$ natural abundance methods and $\delta^{15}N$ mass balance equations to estimate the amount of plant N derived from AMF transfer in perennial grasses managed for bioenergy production under different N addition treatments (0, 56 and 196 kg N ha^{-1}). Differentiation of $\delta^{15}N$ among plant, soil N, and AMF pools was higher than anticipated leading to calculations of 34 to 55% of plant N transferred by AMF in the treatments receiving no N addition to 6 to 22% of plant N transferred to plants in high-N addition treatments. AMF extra-radical hyphae biomass was significantly reduced in the high-N (196 kg N ha^{-1}) addition treatments, which was negatively correlated to enriched plant $\delta^{15}N$. Our results suggest that N addition decreases AMF N transfer to plants. When N was limiting to plant growth, AMF supplied agronomically significant amounts of plant N, and a higher proportion of overall plant N. Because differentiation between N pools was greater than expected, stable isotope measurements can be used to estimate N transfer to AMF plant hosts.

Key words: AMF; ^{15}N; isotopic abundance; perennial grasses; nitrogen transfer; nitrogen supply; extraradical mycelium; bioenergy crops; grasslands

Introduction

Arbuscular mycorrhizal fungi (AMF) have many beneficial qualities that can make our agricultural systems more sustainable (Gianinazzi et al. 2010), such as improving crop yields, increasing nutrient uptake efficiency, and reducing nutrient leaching (De Vries and Bardgett 2012, Hodge and Storer 2014, Bender and van der Heijden 2015). AMF play an important role in mineral scavenging and uptake by plants, but so far most research has demonstrated the role of AMF in plant phosphorus (P) rather than nitrogen (N) nutrition (Smith and Read 2008, Veresoglou et al. 2012). Similar to the balance of trade established for P uptake and transfer, AMF supply N to host plants in direct response to the carbon (C) supply provided from the host plant (Johnson et al. 2010, Fellbaum et al. 2012). Although it is established that AMF transfer N to plant hosts, it has not yet been determined if the amount of N uptake and subsequent transfer to plants is agronomically relevant (Smith and Smith 2011)(i.e. replacing the need for some or all of N fertilizer). Especially lacking is evidence that AMF N-transfer to plants occurs *in situ* (Hodge and Storer 2014) because most AMF studies are done in microcosms or greenhouses under highly controlled conditions. Understanding more about the contribution of AMF to N cycling will help determine if it is feasible to manage for beneficial mycorrhizal communities in agricultural systems.

Despite their inherent nutrient-conserving capabilities (Robertson et al. 2011), perennial C4 grasses managed for bioenergy typically are amended with N fertilizer with the intent of increasing biomass production (U.S. Department of Energy 2011). However, yield responses to N addition from fertilizer have been variable and idiosyncratic (Parrish and Fike 2005, Wullschleger et al. 2010, Jarchow and Liebman 2012, Jach-Smith and Jackson 2015). Given the negative consequences of excess N in the environment (Robertson and Vitousek 2009), finding ways to reduce or eliminate N addition is desirable.

55 Perennial warm-season grasses commonly form symbiotic relationships with AMF
56 (Treseder and Cross 2006, Miller et al. 2012) but N enrichment tends to reduce AMF abundance
57 (Johnson et al. 2003, Treseder 2004, Staddon et al. 2004). Nutrient limitation, and particularly N
58 limitation, can be a driving factor of whether AMF benefit their plant host (Johnson et al. 2003,
59 2010, Köhl et al. 2014). Therefore, altering soil N availability may have direct consequences on
60 AMF abundance and AMF-mediated N transfer to host plants (Johansen et al. 1994, van Diepen
61 et al. 2010, Schroeder-Moreno et al. 2011). One consequence could be a trade-off effect where
62 crops use fertilizer-N when available, or make use of AMF associations to supply the needed N
63 nutrition in circumstances of N limitation.
64 Natural abundance isotope measurements can be useful for evaluating plant N sources
65 without disturbing N cycling within the plant-soil system because they rely on stable isotope
66 fractionation patterns that occur naturally with N transformations in the soil or exchanges
67 between mycorrhizae and plants (Dawson et al. 2002). Studies that employ $\delta^{15}N$ natural
68 abundance measures have produced mechanistic confirmation of N cycling processes in plant-
69 mycorrhizal associations (Hobbie et al. 2000, Averill and Finzi 2011). Mycorrhizae transfer ^{15}N-
70 depleted forms of N to plants, because biochemical reactions occurring within the fungus
71 separate N into ^{15}N-enriched or ^{15}N-depleted pools, with the depleted pool being transferred to
72 plants and the enriched pool remaining in the fungal biomass (Hobbie and Högberg 2012). This
73 process enables use of the $^{15}N/^{14}N$ composition of the plant, fungus, and soil N pools to estimate
74 the proportion of plant N that is transferred or supplied by AMF.
75 AMF assimilate N as nitrate, ammonium, or amino acids (Hodge et al. 2010) and
76 transport N within the hyphae as arginine, which is four nitrogen atoms per molecule, thereby
77 transferring a more concentrated form of N from extra- to intra-radical hyphae. The hyphae then

78 transfers the N to the plant cell as ammonium, transferring only nitrogen without carbon to the
79 host (Govindarajulu et al. 2005). These N transformation steps cause fractionation of ^{15}N-
80 enriched and ^{15}N-depleted pools as described above. As demonstrated by Hobbie and Colparert
81 (2003), the presence of high AMF extra-radical hyphal biomass was related to the $\delta^{15}N$ of the
82 plant biomass since the $\delta^{15}N$ of plants is related to the amount of N supplied by mycorrhizae
83 (^{15}N-depleted, assuming there is significant fractionation upon transfer of N molecules). While
84 the fractionation makes it useful to determine whether N is transferred by mycorrhizae, we are
85 not able to determine the source or form of N before fractionation occurs because of the multiple
86 N transformations within the hyphae (Emmerton et al. 2001). Hobbie and Hobbie (2006) found
87 that mycorrhizae contributed 61 to 86% of aboveground plant N in vegetation of the arctic
88 tundra. These plants are primarily ecto-mycorrhizal, grow in extremely N-limited conditions, and
89 ecto-mycorrhizal species are known to have significant soil organic matter-degrading capabilities
90 that theoretically would expose the mycorrhizae to additional N sources. Therefore, we expect
91 comparative estimates of AMF contributions to plants in in temperate grasslands to be
92 significantly less than that found by Hobbie and Hobbie (2006).
93 There is little research on $\delta^{15}N$ distribution in AMF and their plant hosts (Ouimette et al.
94 2012), as few studies use AMF in isotopic analyses, especially with regard to N cycling and
95 budgeting. There is currently little reference data on ^{15}N partitioning in AMF and their plant
96 hosts (Courty et al. 2015), although there are some examples demonstrating evidence of N
97 availability and AMF affecting plant $\delta^{15}N$. Craine et al. (2012) showed that foliar $\delta^{15}N$ values in
98 the tallgrass prairie were typically associated to soil N availability, with high foliar $\delta^{15}N$ relating
99 to high soil N availability. This trend was observed across many ecosystems and soil types
100 (Craine et al. 2009). In an experiment comparing non-mycorrhizal versus AMF-inoculated grey

alder, aboveground plant tissues were 1‰ depleted in $\delta^{15}N$ compared to non-mycorrhizal plants (Schweiger 2016), demonstrating that AMF are fractionating and transferring depleted $\delta^{15}N$ to plants.

Our objectives were to 1) provide quantitative, mechanistic evidence for the role of AMF in *Panicum virgatum L*. (switchgrass) N-supply and 2) determine whether AMF are capable of supplying agronomically relevant amounts of N to perennial grasses being harvested for bioenergy. We sampled plant tissue, AMF mycelium, and the available N pool for $\delta^{15}N$, searching for patterns indicative of N transfer from AMF to plants. We also estimated the proportion of plant N transferred by AMF using the model and mass balance equations proposed by Hobbie and Hobbie (2006). We hypothesized that AMF would transfer a higher proportion of plant N under conditions of lower soil N, with a decreasing proportion of plant N supplied by AMF under conditions of higher soil N (Figure 1). We also hypothesized that the $\delta^{15}N$ of switchgrass biomass would be negatively correlated with hyphal biomass reflecting greater transfer of N to the plant with higher AMF abundance.

Methods

Site description and experimental design

We used a manipulative experiment to modify soil N levels to examine $\delta^{15}N$ pools and estimate AMF N-transfer to plants. We chose established plots of switchgrass at two agricultural research stations where we modified soil N using three levels of N fertilizer (0, 56, and 196 kg N ha^{-1}). Sites were 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). Switchgrass was established in 2008 at both sites; however, the fertilizer

124 treatments first began at KBS in 2009, whereas the ARL plots were first treated with fertilizer
125 beginning 2013. Therefore, KBS switchgrass had a five-year legacy of fertilization, and ARL
126 had none when we began sampling in 2013 and ended sampling in 2014 for this experiment.
127 Plots were arrayed in a randomized complete block design, with three blocks at each site.
128 Fertilizer was broadcast on the soil surface annually in spring as ammonium-nitrate granules at
129 ARL and as granular or liquid urea ammonium nitrate at KBS. Because there is extensive
130 evidence of hyphal networks sharing resources belowground between individual plants (Fischer
131 Walter et al. 1996, Leake et al. 2004, Pringle 2009, van der Heijden and Horton 2009), we
132 inserted physical barriers between N fertilizer treatment plots to insure plot independence.
133 Barriers were made of aluminum sheet metal (0.08-cm thick × 50-cm deep, Badger Diversified
134 Metal) and installed May 2013. Trenches were dug with a Vermeer RT-100 trencher to cut a 60-
135 cm deep × 10-cm wide trench along plot borders. Aluminum sheets were installed and trenches
136 refilled with original soil to stabilize the aluminum sheets, which remained intact for the duration
137 of the study.

138 ***Sampling $\delta^{15}N$ pools***

139 Aboveground plant biomass. Semi-permanent sampling stations were located at each field site
140 for each year of the study (2013 and 2014). Sampling locations were 0.5-m^2 quadrats evenly
141 spaced within each N-addition plot. Three quadrats were placed in each plot, making 27
142 experimental units at KBS and ARL.

143 Aboveground plant biomass was clipped from quadrats at peak standing biomass, when
144 plants reached their peak growth for the season at anthesis, but before the plants began to senesce
145 and recycle nutrients belowground (typically around mid-August in southern WI, but timing
146 varies from year to year). Biomass samples were almost exclusively switchgrass, therefore, no

147 species sorting was done and all plant biomass assumed to be switchgrass. Biomass was dried to
148 a constant weight at 65 ºC before dry weights were recorded. Dried biomass samples were finely
149 ground using a Wiley Mill to pass a 1-mm mesh screen, then pulverized using stainless steel
150 balls in 2-ml micro-centrifuge tubes.
151 AMF hyphae biomass. In 2014, in-growth hyphal bags (~10 × 2.5 cm) were inserted in the
152 ground in early spring, extracted about three months later at plant maturity, and frozen until
153 further processing. Three in-growth hyphal bags were placed in the surface 10 cm of soil in each
154 quadrat, which were composited to one sample per quadrat. In-growth hyphal bags were
155 constructed of 50-µm nylon mesh, allowing fungal hyphae to grow through the mesh, but restrict
156 root growth. Mesh bags were filled with 100 g of ashed sand (#30-70, pure silica sand from
157 Ogleby Norton Industrial Sands) to minimize the growth of saprotrophic fungi (Wallander et al.
158 2001). Extraction of hyphae from these bags loosely followed methods described by van Diepen
159 *et al.* (2010). In-growth hyphal bags were cut open, inspected for root contamination (cores with
160 visible root infiltration were discarded), and dumped into a beaker of tap water making a
161 composite sample of three bags per quadrat. By flotation and agitation, hyphae were separated
162 from the sand and the hyphae/water mixture and decanted over a 50-µm nylon mesh filter. Sand
163 was again mixed with water, agitated and decanted; these steps were repeated until the water ran
164 clear. The sample on the mesh filter was then further cleaned and inspected in a petri dish viewed
165 under a dissecting microscope to remove grains of sand and debris. Subsets of samples were also
166 checked under a compound microscope to evaluate percentage of saprotrophic versus AMF
167 hyphae using a grid-intersect method. All samples contained >95% AMF hyphae upon visual
168 inspection (based on absence of cell septa in fungal hyphae), therefore all hyphal biomass
169 extracted from cores were considered to be AMF. Cleaned hyphae samples were frozen in petri

dishes and freeze-dried to a constant weight. Samples were weighed and then placed in a 2-ml microcentrifuge tube for pulverization and subsequent analysis for $\delta^{15}N$. To account for soil contamination in the hyphae samples, we corrected for the soil $\delta^{15}N$ contributing to the proportion of the sample that was soil, similar to Nave et al., 2013, using the C:N ratio of the silt-clay fraction of the soil from each site. We assumed soil contamination was from the silt-clay fraction of soil given that it had to pass through a 50-µm nylon mesh. A measure of total extra-radical AMF hyphae biomass was calculated as grams hyphal biomass per gram sand.

Available N pool. To assess the plant- and microbially-available soil N pool, we used two different measurements: the $\delta^{15}N$ of buried cellulose filters and the $\delta^{15}N$ of plants growing near our plots from the Brassicaceae family, which are known not to form AMF symbioses (Rillig, 2004). The buried cellulose acts as an integrator of plant-available N under field conditions because the cellulose is a readily-available C source for microbes, who colonize the cellulose and assimilate any available N from the surrounding soil (Hendricks et al. 2004, 2006, Nave et al. 2013). This technique eliminates the challenges associated with extracting and analyzing the $\delta^{15}N$ signature of N pools from the soil, and further, allows for the $\delta^{15}N_{\text{available nitrogen}}$ pool to be an integration of all microbially-available N forms in the soil, as well as an integration of the time that the cellulose is left buried in the soil (Hobbie and Högberg 2012). We buried Whatman #4 filter papers, which were first encased in 50-µm nylon mesh to limit soil contamination. The filters were deployed by making a slit in the surface 10 cm of soil with a soil knife, inserting the filter paper, and closing the slit with a soil knife to ensure good soil-paper contact. Filters were buried in June or July and extracted four to six weeks later. Cellulose filters were removed from the nylon mesh bags, ground, and subsampled for $\delta^{15}N$ analysis. To account for soil contamination on the filters, a separate subsample was ashed at 475 °C and percent organic

193 versus inorganic material calculated using loss-on-ignition. Based on the inorganic and organic
194 proportions of the sample, we adjusted the final cellulose $\delta^{15}N$ value with site silt-clay fraction
195 soil $\delta^{15}N$.

196 Plants from the Brassicaceae family were also used as an integrated measure of plant-
197 available N. Since plants of the Brassicaceae family are known not to associate with mycorrhizae
198 (Rillig 2004), the $\delta^{15}N$ signature of the plants should be a direct measure of the $\delta^{15}N$ from the
199 plant-available N pool, assuming little or no fractionation on uptake (Marshall et al. 2007). Plant
200 tissue was sampled from naturally growing *Brassica* sp. in July 2013 within meters of the
201 sampling quadrat when possible. However, sometimes the brassica plants were ~10 m from
202 experimental quadrats, therefore we calculated a site and treatment mean for brassica $\delta^{15}N$. We
203 report the $\delta^{15}N_{\text{available nitrogen}}$ from the 2014 cellulose in Table 2, but we also considered an
204 alternative scenario for the mass balance equations where we calculated $\delta^{15}N_{\text{available nitrogen}}$ from
205 the 2013 and 2014 cellulose site means, and adjusted this value by the brassica $\delta^{15}N$ difference.
206 The $\delta^{15}N_{\text{available nitrogen}}$ from the cellulose and the brassica plants should be the same pool of N, but
207 the brassica site mean was on average 1.1‰ lower than the site $\delta^{15}N_{\text{available nitrogen}}$ measured by the
208 cellulose. Hence, we applied a -1.1‰ correction to the 2013 and 2014 cellulose $\delta^{15}N$ site and
209 treatment means to obtain an alternative $\delta^{15}N_{\text{available nitrogen}}$ estimate for the mass balance
210 calculations.

211 All samples were dried, ground, and analyzed on a PDZ-Europa model 20-20 isotope
212 ratio mass spectrometer with a PDZ-Europa ANCA-GSL elemental analyzer at the University of
213 Wisconsin-Madison. $\delta^{15}N$ abundances were reported as parts per mille (‰), which is
214 $(R_{\text{sample}}/R_{\text{standard}}-1)1000$, where R is the $^{15}N/^{14}N$ ratio of the sample to the reference standard

(atmospheric N_2). As a reference, the ammonium-nitrate fertilizer used at ARL and KBS had a $\delta^{15}N$ of -0.17‰ (± 0.01 S.E.).

Mass balance equations to estimate AMF N transfer to plants

To estimate plant N supplied by AMF, we used the $\delta^{15}N$ mass balance approach, as proposed by the model developed by Hobbie and Hobbie (2008, 2006). This method relies on using the $\delta^{15}N$ natural abundance signatures of the aboveground plant tissue ($\delta^{15}N_{plant}$), fungal tissue ($\delta^{15}N_{fungi}$) which we derived from the hyphal biomass, and the available nitrogen ($\delta^{15}N_{available\ nitrogen}$) pools using the following equations:

1) $\delta^{15}N_{available\ nitrogen} = (1\text{-}T) \times \delta^{15}N_{fungi} + T \times \delta^{15}N_{transfer}$

2) $\delta^{15}N_{plant} = f \times \delta^{15}N_{transfer} + (1\text{-}f) \times \delta^{15}N_{available\ nitrogen}$

3) $\Delta_f = \delta^{15}N_{available\ nitrogen} - \delta^{15}N_{transfer}$

where the following unknowns are calculated or defined as:

$\delta^{15}N_{transfer}$ = ^{15}N signature of N transferred from AMF to plant host

T = fraction of fungally-assimilated N transferred to plant host

f = proportion of plant N derived from fungal transfer

Δ_f = fractionation against ^{15}N during transfer from AMF to plant host

This series of three equations assumes that at least one of the four unknowns is specified. We calculated solutions to these equations for three different scenarios to understand the sensitivity of the overall N transfer estimates to the unknown fractionation value. A literature value of 2‰ was chosen as the starting discrimination factor by AMF upon N transfer to plants (Δ_f)(Craine et al. 2015) for calculating scenario 1. Since the mass balance equations produced solutions that were outside the range of biologically meaningful interpretations (i.e., over 100% plant N supplied), we considered alternative scenarios of 4‰ (scenario 2) and 8‰ (scenario 3).

While Δ_f > 2‰ for AMF transfer to plants is higher than indicated by Craine et al. (2015), values as high as 11‰ have been reported for ectomycorrhizae (Hobbie and Hobbie 2006).

Estimating plant and soil N and P

Soil inorganic N (as NO_3^- and NH_4^+) and available P (as PO_4^{3-}) were measured from 2-M KCl extracts of fresh soils (Robertson et al. 1999) sampled from plots in 2014. A composite of five soil cores (2.5 cm diameter × 10 cm deep) were sampled from each plot at three timepoints: early (June), mid (July) and late (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. Extracts were frozen at -20 °C until analysis on a Flow Solution 3100 segmented flow injection analyzer (OI Analytical, College Station, TX). Total inorganic N was calculated as the sum of nitrate and ammonium. Both total inorganic N and inorganic P (as PO_4^{3-}) were calculated for the surface 10 cm of soil.

Dried plant tissue samples were homogenized and subsampled for both total N and P analysis. To determine total N concentration tissue samples were combusted on an elemental analyzer (CE Elantech EA1112, Lakewood, NJ). To determine total P concentration we followed a dry ashing method (Schulte et al. 1987). Plant tissue N:P ratios are reported as the total N concentration divided by the total P concentration. Nutrient limitations for plant growth play a key role in mycorrhizal N uptake and responses to N enrichment (Johnson et al. 2003, 2010, van der Heijden et al. 2006). We determined limiting nutrients using plant N:P ratios, where N limitation is indicated by a N:P ratio < 14, P limitation is indicated by a N:P ratio > 16, and co-limitation is indicated by a N:P ratio between 14 and 16 (Koerselman and Meuleman 1996).

Data analysis

We used an analysis of variance test and conducted mean comparisons using the R package *lsmeans* (Lenth 2015), with significance determined at $p \leq 0.05$ and adjusted for multiple comparisons using the Tukey method. We used these methods for comparing N addition effects on the plant and soil N, P, and plant N:P ratios, as well as the differences between $\delta^{15}N$ pools (aboveground plant biomass, fungal tissue, and available N). We also used this method to evaluate the effect of extra-radical hyphae biomass on aboveground plant $\delta^{15}N$ for the combined ARL and KBS sites across both years. We used R version 3.2.3 (R Core Team 2015) for all statistical analyses.

Results

Soil and plant N and P composition

Total inorganic N levels in soil were significantly affected by N addition treatment at ARL and KBS: the high-N addition treatment (196 kg N ha^{-1}) was significantly greater than the lower N addition treatments ($p < 0.001$ and $p < 0.01$, respectively for ARL and KBS) (Table 1). Total orthophosphate P (PO_4^{-2}) levels did not differ between N addition treatments at either ARL or KBS ($p > 0.05$ for both sites) (Table 1).

Plant tissue N:P ratio, an indication of plant N or P limitation, showed that all sites and N addition treatments had N limitation rather than P limitation (Koerselman and Meuleman 1996), as all N:P values were < 14 (Table 1). The high-N addition treatments at both ARL and KBS were the closest treatments to achieve either no- or co-limitation (N:P ratios of 14), but still were well under the threshold N:P value of 14 indicating that even high levels of N addition do not completely alleviate N limitation in these soils (Koerselman and Meuleman, 1996). However, no

yield advantage was achieved with this high level of fertilization at ARL (Jach-Smith and Jackson 2018) which would indicate that plants were not N limited at the ARL site. Plant aboveground biomass and total N content was reported in Jach-Smith and Jackson (2018), where a yield gain was achieved only at KBS with the N addition treatments. N content increased with N application rate, but this is assumed to be N luxury consumption (an increase in plant N concentration that is not used for growth) since there was no yield gain.

δ^{15}N composition of plant tissue, AMF hyphae, and available N pools

The $\delta^{15}N_{plant}$ values spanned a high range within ARL and KBS with increasingly greater values with the N addition treatments (Table 2). The high-N addition treatment (196 kg N ha^{-1}) increased $\delta^{15}N_{plant}$ by 1.4‰ (± 0.7 S.E.) and 3.0‰ (± 0.4 S.E.) at ARL and KBS, respectively. The trend of greater $\delta^{15}N$ values with higher N addition was also apparent for the available N pool ($\delta^{15}N_{available\ nitrogen}$) and most dramatic in the fungal hyphae ($\delta^{15}N_{fungi}$), where $\delta^{15}N_{fungi}$ at KBS increased by 6.7‰ (± 0.6 S.E.) with the high-N addition treatment.

A useful comparison of $\delta^{15}N$ values is between pools, which gives insight into N uptake and N transfer pathways from the soil to AMF and plants. We expected $\delta^{15}N_{plant}$ to be significantly depleted, and the $\delta^{15}N_{fungi}$ to be enriched relative to the other pools if AMF were transferring N resources to the plant (Figure 1). There were significant differences between $\delta^{15}N_{plant}$ and $\delta^{15}N_{available\ nitrogen}$ pools in the 0 and 56 kg N ha^{-1} addition treatments and between the $\delta^{15}N_{plant}$ and $\delta^{15}N_{fungi}$ pools in the 56 and 196 kg N ha^{-1} addition treatments (Table 2).

Mass balance equation solutions

The first set of solutions calculated using scenario 1 (using a Δ_f value of 2‰) produced some *T* and *f* values that represented biologically impossible situations (Table 3), because these numbers should fall between 0 and 100%. Solutions from scenario 2 (Δ_f value of 4‰), produced more meaningful values for *f*, however some *T* values calculated were still negative. Solutions from scenario 3 (a Δ_f value of 8‰) produced all *f* values and most *T* values within or closer to the constraints of biological possibility (0 to 100%). We considered scenario 2 most likely because a 4‰ fractionation is more plausible than 8‰ in scenario 3, when taking into account previous studies, as described above. Using results from scenarios 1 and 2, we estimated the proportion of plant N supplied by AMF (*f*) (values taken from scenario 1 and scenario 2, respectively) was in the range of 55 to 100% of plant N in 0-N addition treatments at ARL and 34 to 68% plant N at KBS. In contrast, high-N addition treatments showed 6 to 12% of plant N was supplied by AMF at ARL and 22 to 44% at KBS.

Extra-radical hyphae biomass

Plant $\delta^{15}N_{plant}$ at ARL and KBS was negatively correlated to the AMF extra-radical hyphal biomass as measured by the in-growth hyphal cores (Figure 2). There appeared to be some separation between the highest N addition treatment (196 kg N ha^{-1}) and the others (0- and 56 kg N ha^{-1})), indicating that the relationship between hyphal biomass and $\delta^{15}N_{plant}$ may only persist under high N availability conditions.

Discussion

AMF appeared to be supplying an agronomically significant amount of N to switchgrass, indicating that AMF may be transferring enough N to substitute for fertilizer N amendments in

some environments. Despite the uncertainty of the AMF fractionation value, results from our mass balance equations indicated that AMF were contributing agronomically meaningful amounts of N. Mass balance equations showed consistent patterns of AMF N-transfer to switchgrass.

Because AMF do not have the same SOM-degrading capabilities as other mycorrhizal species, and AMF are typically adapted to ecosystems that have higher rates of N cycling (i.e. grasslands rather than heathlands)(Hobbie and Högberg 2012), it has been assumed that AMF may not play a significant role in plant N nutrition. Hobbie and Hobbie (2006) found that mycorrhizae contributed 61 to 86% of aboveground plant N in the arctic tundra, which is comparable to the rates of N transfer we estimated in our unfertilized treatments. It was less clear why the hyphae were less enriched than expected in the 0-N addition treatments at ARL and KBS, and more enriched than expected in the 196-N addition treatments.

AMF hyphae biomass was negatively correlated to $\delta^{15}N_{plant}$, a trend also demonstrated by Hobbie and Colpaert (2003) in a study conducted under greenhouse conditions. We demonstrated this relationship under field conditions providing strong support for our hypothesis that increased abundance and functioning of AMF results in depleted $\delta^{15}N$ in aboveground plant tissues. In addition to the negative correlation we demonstrated here, the 196-N addition treatment at ARL and KBS had significantly less hyphae biomass when compared to the 0-N and 56-N addition treatments (Jach-Smith and Jackson 2018).

Assumptions and how they might affect interpretations

Our results support the assumption that there is *at least* a 2‰ fractionation of N in AMF transfer to plants, and our field evidence suggests it is closer to 4‰. Data from field studies have consistently demonstrated a depleted $\delta^{15}N$ signature of the plant host compared to the available

soil N pool (Hobbie and Hobbie 2008, Craine et al. 2015). It is plausible that there is some amount of fractionation from hyphae-to-plant transfer because of the arginine to ammonium transformation from hyphae to plant transfer (Govindarajulu et al. 2005). Assuming there is at least some fractionation upon transfer, our models and calculations should have been appropriate. Contrarily, our data would become more difficult to interpret if, hypothetically, there was no fractionation of N on transfer to plants. Scenarios 2 and 3, which considered higher fractionation values of 4‰ and 8‰, respectively, produced solutions to the mass balance equations that seem to be the closest to biologically plausible.

Hobbie and Hobbie (2006) assumed minimal fractionation results from uptake via plant roots, which assumed low N availability in the soil, or that N uptake closely matches N supply. This is often the case because N demand by plants is typically greater than N supply in natural systems (Marshall et al. 2007). As confirmed by numerous field and laboratory studies, there is no fractionation of N via root or hyphae uptake from the soil, as long as the system is N-limited (Hobbie and Hobbie 2006, Hobbie and Högberg 2012). If, hypothetically, our systems were not N-limited, plant roots would likely preferentially use the lighter ^{14}N isotope, which would accumulate in the soil. In that case, plant $\delta^{15}N$ would not be enriched irrespective of AMF N-transfer. Also, it is worth noting that the N-addition treatments produced biomass with greater N content overall (Jach-Smith and Jackson 2018), suggesting that the $\delta^{15}N$ in the biomass was a smaller portion of the overall plant N pool resulting in a dilution effect that we did not capture.

Other considerations for using $\delta^{15}N$ natural abundance in AMF systems

Using stable isotopes to estimate N transfer to plants is an important method, and our study shows great promise for helping to sort out plant-AMF symbioses. However, these methods make many assumptions that call for careful consideration in future studies. Relative to

370 $\delta^{15}N_{plant}$, the $\delta^{15}N_{available\ N}$ pools were more enriched than expected. Using the 2013 and 2014
371 $\delta^{15}N_{available\ N}$ mean, and adjusting with brassica $\delta^{15}N$, lowered the 2014 $\delta^{15}N_{available\ N}$ pools into a
372 more reasonable range. The cellulose filters and the brassica should both be reflecting the same
373 soil $\delta^{15}N$ pool, but it seems that the brassica may be more representative of the plant and hyphae
374 N source than the cellulose filters. Organic N sources typically have lower $\delta^{15}N$ than bulk soil
375 and inorganic N sources (Yano et al. 2009) and mycorrhizal fungi are known to use organic N
376 sources (Talbot and Treseder 2010). Plants and AMF may have used organic N sources more
377 than the microbial community that fed on the cellulose filters, which would explain why our
378 measurements of $\delta^{15}N_{available\ N}$ were higher than expected, relative to the other $\delta^{15}N$ pools. Future
379 studies should consider improved methods for estimating the available N pool, especially
380 considering that the plants, AMF, and microbial communities may be using different N sources
381 with differing $\delta^{15}N$ signatures.

382 Symbiotic N-fixation might have affected $\delta^{15}N$ signatures. Switchgrass associative N
383 fixation (ANF) in the rhizosphere and roots was estimated to be as much as 92% of unfertilized
384 switchgrass N demand (Roley et al., in press). If ANF were responsible for a significant amount
385 of plant N in our systems, plant $\delta^{15}N$ would be depleted relative to the $\delta^{15}N_{available\ N}$ pools
386 because fixed atmospheric N has a $\delta^{15}N$ near 0‰. However, only the nodules where fixation
387 takes place typically have depleted $\delta^{15}N$, thus overall plant $\delta^{15}N$ is generally not affected by N-
388 fixation (Craine et al. 2009, 2015). Roley et al. (in press) found that the majority of N was fixed
389 in the soil (not directly in the plant) post-senescence, perhaps when the microbial community had
390 a boost of carbon exudates from the senescing switchgrass plants. AMF hyphae are known to
391 help retain N pools and prevent N leaching (De Vries and Bardgett 2012), therefore the increased

392 extra-radical hyphae we found in the low-N addition treatments may play a primary role in
393 immobilizing and retaining N fixed from ANF.
394 Our measured $\delta^{15}N_{fungi}$ pools were lower than expected in the unfertilized treatments and
395 higher than expected in the high-N addition treatments. The similarity of the $\delta^{15}N_{plant}$ and
396 $\delta^{15}N_{availablleN}$ in the 196-N addition treatments suggested that there was little fungal N transfer
397 occurring, but why was the fungal tissue so enriched? Given the high N availability in the 196-N
398 addition treatment, the AMF may have been luxury-consuming N, in particular, the more easily
399 accessible mineralized N pools from the fertilizer. Although fertilizer N has a low $\delta^{15}N$, excess
400 fertilizer N likely has been subjected to N transformations that cause N losses, such as
401 nitrification and denitrification, which would leave the remaining soil N pool more enriched in
402 $\delta^{15}N$.
403 Our results may have been influenced by our use of fungal mycelium rather than
404 reproductive structures for measuring our $\delta^{15}N_{fungi}$ pools. Zeller *et al.* (2007) found that their
405 mycelium sample (n=1) had a $\delta^{15}N$ much lower than the sporophore stipes and gills of an
406 ectomycorrhizal fungus. Others have used spores and fungal fruiting bodies for measuring $\delta^{15}N$
407 of fungal tissue but there may be some isotopic fractionation during the formation of a fruiting
408 body (Hobbie et al. 2012, Courty et al. 2015), hence studies that have used sporocarps or spores
409 for isotopic analysis, rather than fungal mycelium, may see more enrichment. Some have used
410 plant roots as a proxy for AMF tissue (Stackpoole et al. 2008), but samples are diluted and
411 influenced by the plant root $\delta^{15}N$. AMF biomass makes up a relatively small portion of mass
412 compared to root tissue (Ouimette et al. 2012), consequently, using root tissue as a proxy for
413 AMF tissue may not be very reflective of AMF $\delta^{15}N$ values, especially when looking for
414 discrimination on the order of 2‰. Yet another factor to consider is that hyphae may have been

contaminated with organic compounds, as was the suspected case in an experiment using different methods to measure $\delta^{13}C$ of AMF tissues (hyphae, spores and biomarker FA C16:1u5) (Walder et al. 2013). We corrected our hyphae samples for soil contamination, but did not consider any other organic or biological contaminants. More data is needed on $\delta^{15}N$ partitioning within the AMF fungus to determine if using spores or mycelium is appropriate for measuring $\delta^{15}N$ patterns to interpret N cycling and transfer to plant hosts.

Conclusions

Our study provided estimates of plant N derived from AMF transfer *in situ.* Given the assumed small ^{15}N fractionation during AMF N-transfer to plants when compared to ectomycorrhizal systems, it is unprecedented that we were able to find differentiation between the AMF, plant, and available N pools. Using stable isotope measurements to estimate N transfer to plants has not been used in AMF systems, however, our study demonstrated that this can be a useful method and differentiation between pools is possible, and indeed, greater than expected. This work highlights the need for more research to determine AMF fractionation values under more controlled conditions, to converge on values that are more precise for AMF-derived N in plant hosts. Experiments could corroborate this study by using ^{15}N as a tracer instead of relying on the high variability of natural abundance $\delta^{15}N$.

Our results suggested that AMF supplied agronomically significant amounts of plant N, and a higher proportion of plant N when N was limiting. Assessing natural abundance of $\delta^{15}N$ can be a useful method for studying AMF function in perennial grass agroecosystems because there was more discrimination among ^{15}N pools than anticipated. Our results provided consistent evidence that N fertilizer addition reduces AMF N-transfer to plants. If AMF play a significant role in providing N nutrition to perennial grasses, there will undoubtedly be more questions

about how AMF diversity and composition affect AMF functioning and how these community effects relate to plant N uptake. Future research should consider how we might manipulate and promote the soil microbial community to increase AMF abundance and function. The reduction of AMF N-transfer under high N addition indicated that AMF symbioses may be a primary reason why perennial grasses require very little, if any, N addition when AMF symbioses are functioning.

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Table 1. Mean soil inorganic N and available P and plant tissue N:P ratio for *Panicum virgatum*. Data are means with 1 standard error of the mean in parenthesis. Means with different letters within a column indicate significant differences between N addition treatments within a site (ARL and KBS) at $p < 0.05$.

		Soil		Plant
Site	N addition (kg N ha^{-1} yr^{-1})	Total inorganic N (kg N ha^{-1})	Available P (kg PO_4^{-2} ha^{-1})	N:P
ARL	0	9.8 (3.0)^{b}	3.34 (0.44)	5.4 (0.5)
	56	34.4 (9.7)^{b}	2.48 (0.27)	5.5 (0.2)
	196	114 (27)^{a}	3.08 (0.3)	7.1 (0.8)
KBS	0	8.3 (0.5)^{b}	1.29 (0.09)	5.9 (0.4)^{b}
	56	10.7 (1.3)^{b}	1.28 (0.04)	6.0 (0.3)^{b}
	196	77.2 (27)^{a}	1.67 (0.19)	10.3 (0.1)^{a}

629

Table 2. Mean $\delta^{15}N$ of the aboveground plant (*Panicum virgatum*), available soil N pool, and AMF hyphae. Data are means with 1 standard error in parentheses. Means with different letters within a row indicate significant differences between $\delta^{15}N$ pools at $p < 0.05$.

Site	N addition (kg N ha^{-1} yr^{-1})	Aboveground plant $\delta^{15}N$ ‰	Available N $\delta^{15}N$ ‰	AMF hyphae $\delta^{15}N$ ‰
ARL	0	0.8 (0.5)^{a}	4.1 (0.4)^{b}	2.3 (0.4)^{a}
	56	0.5 (0.2)^{a}	3.0 (0.6)^{b}	2.0 (0.2)^{b}
	196	2.2 (0.2)^{a}	3.7 (0.3)^{b}	4.5 (0.3)^{b}
KBS	0	1.0 (0.1)^{a}	3.0 (0.7)^{b}	2.3 (0.4)^{ab}
	56	1.9 (0.1)^{a}	4.1 (0.4)^{b}	3.5 (0.3)^{b}
	196	4.0 (0.3)^{a}	5.9 (0.8)^{a}	9.0 (0.2)^{b}

Table 3. Solutions to mass balance equations (scenarios 1 through 3), for Arlington (ARL) and Kellogg Biological Research Station (KBS) sites. Each scenario solves the mass balance equations assuming different, but possible AMF fractionation values (Δ). Numbers in parentheses are 1 standard error of the mean.

Scenarios	Site	N addition (kg N ha-1yr-1)	Δ (‰)	δ^{15}Ntransfer ‰	T (%)	f (%)
Scenario 1	ARL	0	2	1.0	-228 (131)	111 (27)
		56	2	-0.1	-7 (16)	72 (11)
		196	2	0.4	48 (4)	12 (12)
	KBS	0	2	0.4	-31 (19)	68 (7)
		56	2	1.0	10 (10)	57 (5)
		196	2	2.9	67 (1)	44 (13)
Scenario 2	ARL	0	4	-1.0	-32 (16)	55 (13)
		56	4	-2.1	0 (6)	36 (5)
		196	4	-1.6	33 (4)	6.0 (6)
	KBS	0	4	-1.6	-9 (8)	34 (3)
		56	4	-1.0	8 (6)	29 (2)
		196	4	0.9	50 (1)	22 (7)
Scenario 3	ARL	0	8	-5.0	-12 (-5)	28 (11)
		56	8	-6.1	1 (0.2)	18 (6)
		196	8	-5.6	20 (7)	3 (1)
	KBS	0	8	-5.6	-3 (-0.9)	17 (6)
		56	8	-5.0	5 (2)	14 (5)
		196	8	-3.1	34 (11)	11 (4)

Notes: Scenario 1 assumed an AMF fractionation value Δ of 2‰, as determined by literature values. Scenarios 2 and 3 used a higher AMF fractionation value Δ of 4‰ and 8‰, respectively, to explore hypothetical scenarios in which the AMF fractionation value for switchgrass is greater than previously thought, which brings T (%) (percentage of fungally-assimilated N transferred to plant host) and

f (%) (proportion of plant N derived from fungal transfer) solutions into more biologically-relevant ranges (ie., *f* (%) cannot be more than 100%).

Figure legends

Figure 1. Hypothetical scenarios for how plants obtain N, where T is the fraction of fungally-assimilated N transferred to plant hosts and *f* is the proportion of plant N derived from fungal transfer. Arrows illustrate the path of N uptake and resulting δ^{15}N pools. In the hypothetical **low N environment** (unfertilized or low native soil N), plants receive 50% of N via mycorrhizal transfer. This results in depleted δ^{15}N value in the aboveground plant biomass (**a**) relative to the enriched δ^{15}N value in mycorrhizal hyphae (**b**). This is the result of AMF hyphae taking up available soil N where the fractionation on transfer to the plant results in the heavier ^{15}N isotope remaining in the hyphae (**c**), and the lighter isotope is transferred to the plant and becomes incorporated into aboveground plant tissue with no further fractionation. In the hypothetical **high N environment** (fertilized or high native soil N), plants receive 25% of N via mycorrhizal transfer. This results in moderately depleted δ^{15}N value in aboveground plant biomass (**d**) relative to a slightly enriched δ^{15}N value in mycorrhizal hyphae (**e**) and available soil N pool (**f**). For reference, *Brassica* sp*.,* which form no AMF associations, receive no N via AMF transfer so the aboveground plant N of *Brassica* sp. (**g**) reflects the δ^{15}N value of the available soil N δ^{15}N pool (**h**), assuming negligible fractionation of δ^{15}N with direct plant root uptake. The thickness of arrows represents the amount of N supplied via mycorrhizal transfer (*dashed arrows*) or plant N uptake via roots (*solid arrows*).

Figure 2. AMF extra-radical hyphae biomass (log [μg hyphae g sand $^{-1}$]) is regressed with δ^{15}N ‰ of aboveground plant biomass at ARL and KBS. Each data point represents one plot, with colors in the legend indicating N fertilizer treatment (kg N ha^{-1} yr^{-1}).

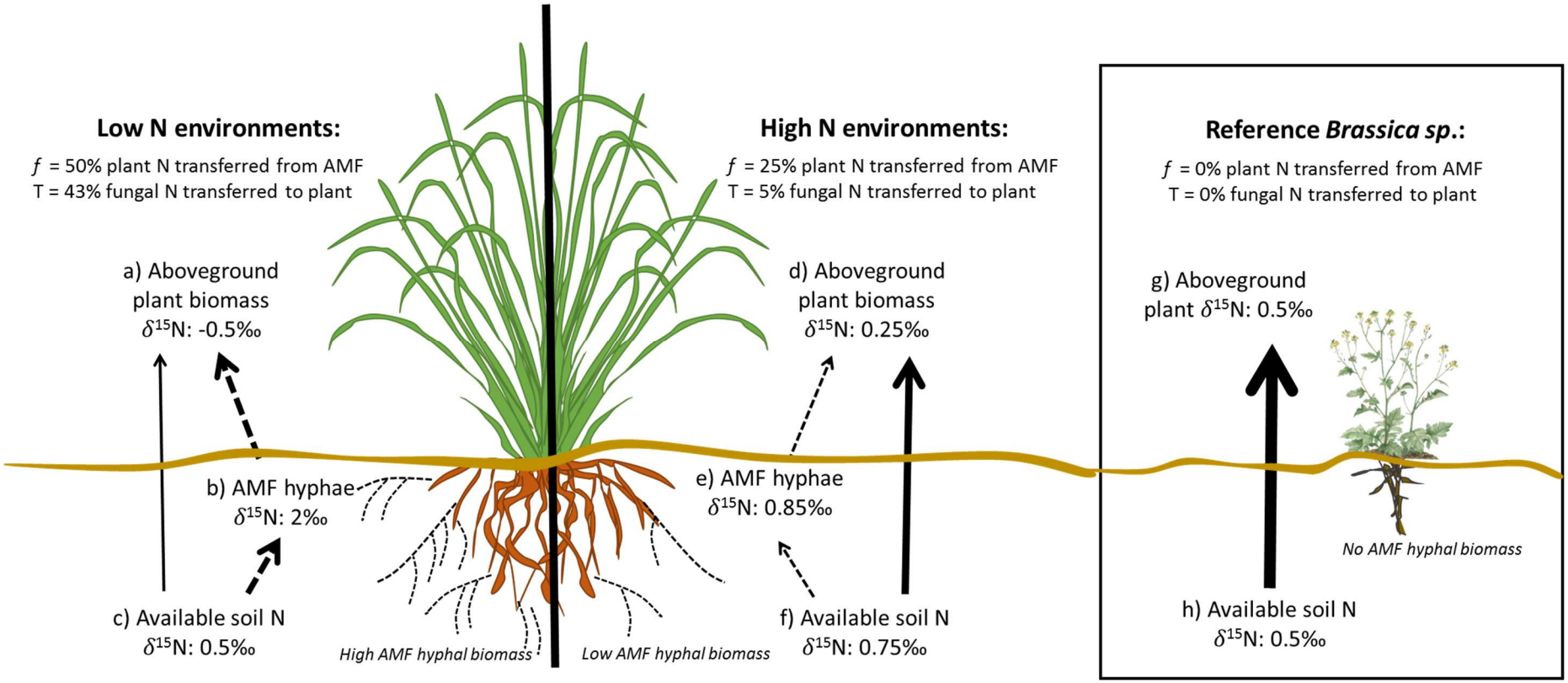
Low N environments:
f = 50% plant N transferred from AMF
T = 43% fungal N transferred to plant
a) Aboveground plant biomass
δ15N: -0.5‰
b) AMF hyphae
δ15N: 2‰
c) Available soil N
δ15N: 0.5‰
High AMF hyphal biomass
Low AMF hyphal biomass
High N environments:
f = 25% plant N transferred from AMF
T = 5% fungal N transferred to plant
d) Aboveground plant biomass
δ15N: 0.25‰
e) AMF hyphae
δ15N: 0.85‰
f) Available soil N
δ15N: 0.75‰
Reference Brassica sp.:
f = 0% plant N transferred from AMF
T = 0% fungal N transferred to plant
g) Aboveground plant δ15N: 0.5‰
No AMF hyphal biomass
h) Available soil N
δ15N: 0.5‰

Figure 1.

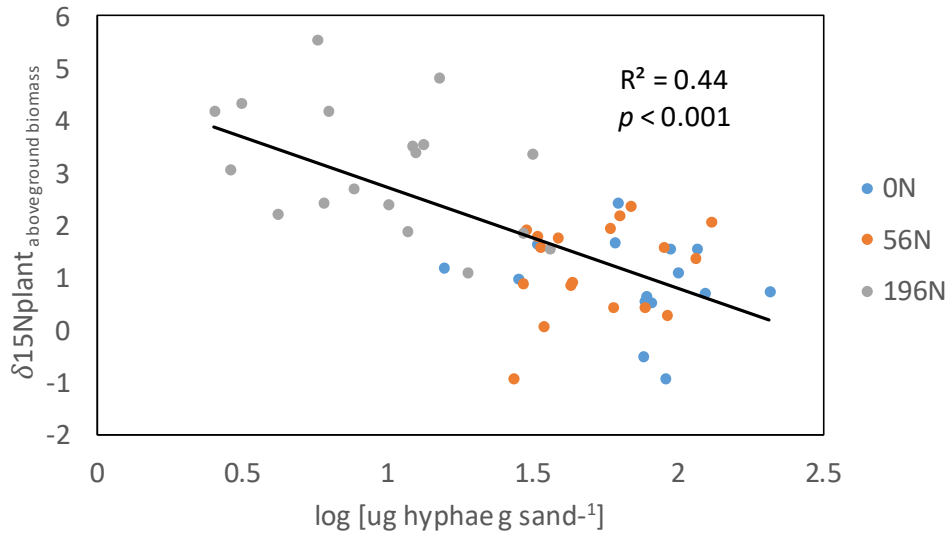
6
5
4
3
2
1
0
-1
-2
0
0.5
1
1.5
2
2.5
δ15Nplant aboveground biomass
log [ug hyphae g sand-1]
R² = 0.44
p < 0.001
0N
56N
196N

Figure 2.