NITROGEN CONSERVATION IN PERENNIAL GRASSES MANAGED FOR BIOENERGY PRODUCTION

By
Laura C. Smith

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The dissertation is approved by the following members of the Final Oral Committee:
Randall R. Jackson, Professor, Agronomy
Erika Marín-Spiotta, Associate Professor, Geography
Julie C. Dawson, Assistant Professor, Horticulture
Brad L. Barham, Professor, Agriculture & Applied Economics
Stuart Grandy, Associate Professor, Natural Resources and the Environment

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Abstract

Reducing nitrogen (N) pollution from agriculture remains a major challenge. Using crops that are N-conservative and retains N in the plant-soil system is one method to help mitigate N that would otherwise be lost to the environment. Native warm-season grasses are inherently N-use efficient and could also be a source of cellulosic biofuel feedstocks for renewable energy. Management for perennial bioenergy crops often recommends N fertilizer to increase and maintain yields. However, N addition may actually compromise some of the biological mechanisms that help conserve N in the plant-soil system.

I investigated some of the mechanisms that conserve N resources in perennial grasses, with special attention to the effect of N addition on N conservation mechanisms and microbial associations, given the current N recommendations for perennial bioenergy grasses and the lack of regulation and policies associated with N management. My objectives were 1) to understand the genetic and environmental controls and variation in the perennial plant N conservation strategies of N resorption (internal plant N recycling) and nitrogen-use efficiency, 2) assess the importance of the arbuscular-mycorrhizal fungal (AMF) associations in perennial grass yield and N uptake under varying soil N conditions by measuring AMF abundance and function and 3) quantify the amount of AMF-supplied N to plants under varying soil N conditions using ^{15}N natural abundance techniques.

Results showed that the N conservation mechanisms of N resorption and nitrogen-use efficiency were highly variable and the dominant strategy used by plants varied by species, ecotype, and site. N addition had no effect on the plant N conservation strategies measured, but soil nutrient ratios affected plant N conservation across eight restored grassland sites. N addition significantly decreased AMF abundance and function (*i.e.* nutrient transfer with host plant) in

perennial grass systems, and in addition, plant N correlated with increased AMF allocation to nutrient transfer structures within host roots. Demonstrating these relationships *in situ* provides important evidence that AMF benefit perennial grasses by increasing N uptake. Differentiation of $\delta^{15}N$ among plant, soil N and AMF fungal pools was higher than anticipated, leading to estimates of 34 to 100% of plant N transferred from AMF in the treatments receiving no N addition and a significant reduction in plant N transferred in high N addition treatments. Our results suggest that N addition decreases AMF N-transfer to plants. When N is limited, AMF are able to supply N to plants in amounts comparable to recommended N fertilizer rates, highlighting that N fertilizer may be unnecessary in the management of perennial grasses for bioenergy production. A high degree of variation in N conservation parameters also suggested high potential for selection and management of C4 grasses for continued and improved N conservation.

Chapter 1: What are the best ways to incentivize conservative N use in perennial grasslands grown for bioenergy?

1.1 Introduction

Nitrogen (N) is one of the most common nutrients limiting plant productivity in ecosystems of temperate regions, and synthetic N fertilizer used to increase crop yields has been one of the most successful means to reduce N limitation in agroecosystems (Udvardi et al. 2015). Humans have benefited greatly from N fertilizer use, including improved health from increasing food and fiber production and reduced land clearing by intensifying agriculture with N fertilizer, however, these benefits come with significant costs to society (Galloway et al. 2008, Robertson and Vitousek 2009). Excess N fertilizer and poor management is responsible for N pollution that alters plant communities and degrades water, soil, and air quality (Galloway et al. 2003, Schlesinger 2009, Cameron et al. 2013). Nitrogen eutrophication has also been linked to increased human and wildlife pathogens (Johnson et al. 2010). Reducing N pollution remains a major challenge in agriculture (Robertson and Vitousek 2009, Ribaudo et al. 2011, Syswerda and Robertson 2014), so increasing N conservation in agroecosystems should be a primary goal to help restore N balance across ecosystems.

N loss from agroecosystems is often a direct result of improper application of N fertilizer. Excess N fertilizer in the plant-soil system then becomes subject to multiple loss pathways, including ammonia volatilization into the atmosphere that causes acid rain, nitrification that leads to nitrate leaching into lakes and rivers, and denitrification, which causes nitrous oxide emissions into the atmosphere that contributes to climate change. The amount (rate), timing, and method or placement of N fertilizer are all management factors that affect subsequent N loss or retention and dictate best management practices (Ribaudo et al. 2011, Cameron et al. 2013,

Nkebiwe et al. 2016). Corn is very inefficient in N-use, as it typically only takes up 40 to 60% of applied N (Simpson et al. 2008). Most of this N not taken up by plants is then subject to loss pathways and becomes an N pollutant (Cameron et al. 2013). Moreover, corn comprises half of the cropland that has been determined to be using improper N rates rather than following best management practices (Ribaudo et al. 2011).

Compared to corn, perennial warm-season (C4 photosynthesis) native grasses are inherently N-use efficient and N conservative (Dell et al. 2005, Jach-Smith and Jackson 2015). Growing perennial grasses for bioenergy is a hopeful alternative to other crops like corn for ethanol to reduce N pollution (Donner and Kucharik 2008, Porter et al. 2015). However, whether goals like increased N retention are realized will depend largely on how the bioenergy crops are managed (Robertson et al. 2008). Despite the N-use traits that make perennial grass crops productive even in low nutrient conditions, N fertilizer addition is generally recommended to maintain stand productivity (Walsh et al. 2003). However, yield responses to fertilizer are have been highly variable and idiosyncratic (Parrish and Fike 2005, Haque et al. 2009, Wullschleger et al. 2010, Bonin and Lal 2012).

Why are yield responses to N so variable? Perennial grasses are inherently N-conservative, so N requirements may be lower than projected, leaving excess N applied subject to loss pathways (Ruan et al. 2016, Duran et al. 2016). Perennial grasses have inherent N-conserving mechanisms, such as high N resorption, where perennial plants internally recycle N for use the following season (Heckathorn and Delucia 1996), and high N-use efficiency (NUE), where plants are highly productive for a unit of N uptake (Brown 1999). Plant-microbial interactions may also provide additional sources of N that have been unaccounted for to date (Parrish and Fike 2005). Symbiotic mycorrhizal fungal associations may benefit plants by increasing N

uptake and access to N resources (Smith and Read 2008) and N_2-fixers in the rhizosphere of perennial grasses may also supply additional N to perennial grasses (Bahulikar et al. 2014). Not only are perennial grasses inherently efficient and conservative with N resources, but N addition may actually compromise some of the biological mechanisms that help conserve N in the plant-soil system. N addition can have detrimental effects on N conservation mechanisms by decreasing N resorption rates (Norris and Reich 2008, Jach-Smith and Jackson 2015), decreasing NUE (Lambers et al. 2008), and decreasing mycorrhizal associations (Leff et al. 2015).

Perennial grass bioenergy systems are touted as being more sustainable and providing additional ecosystem services such as nutrient retention compared to conventional commodity crops (Porter et al. 2015). However, over-applying N fertilizer, particularly if there is little to no yield gain, will negate some of the promising attributes of bioenergy crops. This then begs the question: What should N recommendations be for perennial bioenergy grasses? Perhaps an equally important question is will N recommendations be followed and over-application of N prevented? Conserving N in agroecosystems and reducing N fertilizer to limit agricultural-associated pollution has been a focal point of conservation efforts in agriculture for decades. I discuss some of the history and the effectiveness of the N mitigation tactics currently used and policies that could help prevent overuse of N in bioenergy cropping systems. First, I discuss why N is often over-applied. Second, I explore existing and historical policy mechanisms for how to prevent excess N application and mitigate N pollution from agriculture.

1.2. Why is N over-applied?

Farmers often over-apply N by exceeding recommended N rates determined by best management practices, to avert risk (Lawley et al. 2009). Farmers are opposed to risk-taking when it comes to jeopardizing farm profits and yields, so adopting changes such as decreasing

fertilizer inputs or adopting other best management tactics may be perceived as too risky (Huang et al. 2001). Additionally, the cost of N fertilizer is low enough that the cost of over-applying is perceived to be less than the cost of potentially under-applying fertilizer. Therefore, farmers over-apply N for risk management and the associated low cost of N fertilizer. If fertilizer were more expensive, farmers may not use over-application of N fertilizer as a risk-aversion method (Williamson 2011).

Farmers try to achieve their highest yield potential, so management is often designed to achieve high yields with little regard to the fact that there is often large variability because of uncontrollable factors like seasonal weather fluctuations (Sheriff 2005). Nonetheless, fertilizer recommendations seem to assume a best-case scenario for growing conditions, with the hope that crops will need as much fertilizer as recommended for reaching yield potential (Ribaudo et al. 2011). Several other contributing factors cause over-application or less-than-ideal management of N fertilizer, such as rigid or limited schedules to apply N to the field and the cost of soil N tests to help estimate appropriate N rates (Lawley et al. 2009).

1.3. Policy to mitigate N use and pollution in agroecosystems

Compliance and insurance mechanisms

Historically, policy has used direct payments from conservation and commodity programs as a method for conservation compliance. To receive commodity program payments, farmers must comply with best management practices set to protect environmentally sensitive land. Federal conservation programs have primarily focused on erosion control and wetland protection, and there is some evidence that linking direct payments to conservation compliance has been successful for reaching conservation goals (Claassen 2012). A study by Ribaudo *et al.*

(2016) found that including N management in compliance program payments could be successful at reducing excess N application.

Provisions in the 2014 Farm Bill ended direct payment subsidies for commodity crops, which were often linked to conservation program compliance, and instead now rely on insurance programs for all financial support of farmers (Bruckner 2016). Linking insurance payments to conservation compliance may be beneficial for reaching conservation goals as well (Claassen 2012). A closer look at how insurance policy has so far influenced N use by farmers may provide some insight as to how a switch to insurance mechanisms could influence N use by farmers. Applying excess N fertilizer is itself a form of insurance (Babcock and Hennessy 1996), so we might expect that farmers who buy insurance policies would decrease fertilizer applications. However, a study by Horowitz & Lichtenberg (1993) found that crop insurance increased N inputs by 19% and pesticide use by 21%, likely because insured farmers undertake riskier production practices. Alternatively, others have found that crop insurance modestly decreases fertilizer applications per unit area on the farm (Smith and Goodwin 1996, Babcock and Hennessy 1996, Seo et al. 2005).

Although insurance policies may decrease N application on an individual farm, insurance policies can have differential effects over cropping patterns on a landscape scale. If insurance programs alter the prices that are paid for a crop's output, the incentives to plant particular crops may increase, which in the long-term can alter cropping patterns of what and where farmers decide to plant. Changes in cropping patterns often results in environmental problems, such as groundwater pollution and an increase in chemical inputs (Wu and Segerson 1995, Wu 1999) resulting from the disturbance of previously fallow land with tillage and planting. Wu and Adams (2001) also found that the counties that were most subject to changes in cropping patterns

were also the same counties that contained the most environmentally-sensitive areas. Since insurance decreases risk, farmers are willing to take on more risk by planting in areas that otherwise would have been avoided because of the inherent risk. Reduced risk from insurance can create some extra wealth, which in turn may create incentive to expand farm size and cropped acreage (Hennessy 1998). Generally, extra wealth causes farmers to be less risk-adverse; therefore, they may be more likely to take on more land to farm. Reducing farmers' risk may simply allow activities to bring them back up to the same level of risk at which they are comfortable.

Insurance policies have the potential to decrease N use by farmers on a per acre level, but can also change cropping patterns, which may increase overall N use by farmers if they are taking additional land into production. The net balance of these effects will dictate total N use by farmers. Although insurance policies have shown some positive results for reducing N use by farmers, the reduction is often relatively small in comparison to the N rates that are used. The potential for landscape-scale changes to cropping patterns and increases to farmed acreage may overwhelm any positive effects of insurance programs on the farm-level (Mitchell 2014).

Nutrient management planning

There are many management techniques, collectively called "best management practices" (BMPs) that are recommended to increase N conservation on farms and reduce non-point source pollution. BMPs include proper rate, timing, and application methods for N fertilizer, but also include attention to proper tillage, hydrologic management, fertilizer type, and crop rotations and crop choice (Snyder et al. 2009). One of the primary methods for implementing BMPs is creating on-farm Nutrient Management Plans (NMPs). NMPs document nutrient sources on the farm and then specify the amount of fertilizer and manure that should be applied to the field. The amount

of nutrient applied is determined by the calculated nutrients a crop needs for expected yields and also consider current soil N levels and any environmentally sensitive areas on the farm (Shepard 2005).

Federal and state agencies encourage NMPs, although most states allow farmers to adopt NMPs voluntarily. However, even with financial assistance, voluntary adoptions of NMPs are low because many farmers find them costly and challenging to implement, particularly when most benefits are realized down-stream of their farm (Ribaudo et al. 2016). Implementing NMPs is very time and resource intensive, and often cost the farmer more time than estimated (McCann 2009). The reasons why farmers adopt conservation measures like BMPs and NMPs are complex and variable. However, some common reasons given by farmers for not adopting NMPs include the high time requirement for design and implementation and concern that it will decrease farm profits and yields. That said, Osmond et al. (2012) found that NMPs do not negatively affect farmer yields or profit.

Maryland has recently adopted NMP requirements. While 99% of farmers comply with creating an NMP, only about 62% actually comply with plan implementation (Genskow 2012). Currently, NMPs in many states are voluntary, including Wisconsin where only 15% of harvested land is covered by NMPs (WDATCP, 2010). There are some requirements set forth by the Natural Resources Conservation Service 590 Nutrient Management Standard that require farmers to develop and implement NMPs as a prerequisite for enrollment in conservation programs that have cost-share incentives. However, there are often limited resources in county and state agencies for helping farmers to create NMPs (Genskow 2012). Farmers who make their own NMPs most often decreased their rates of fertilizer application suggesting win-win

situations where both the farmer and society benefit when farmers create their own NMPs (Lawley et al. 2009).

It is clear the NMPs are successful at reducing N applications (Shepard 2005), although there are several ways in which nutrient management planning can be improved. A study by Genskow (2012) reported that implementation of NMPs was significantly higher after farmers engaged in an educational workshop on NMPs. Most state agencies and extension agents are facing cutbacks, so there are an increasingly limited number of educators and outreach specialists to engage with farmers on creating NMPs and implementing best management practices. To make NMPs more successful, additional funding should be directed to public agencies to help farmers create and implement NMPs. Among activities that help ensure proper implementation of NMPs, personalized advice and one-on-one work with farmers has been found to be most effective (Osmond et al. 2012). If farmers are recognized as equal collaborators in the planning process, conservation programs are more likely to be successful (Nowak 2011).

Effectiveness of NMPs may also increase by creating incentive strategies for the land managers designing NMP plans. Rather than rewarding designers for the number of plans produced or the land area contracted under NMPs, land managers should be rewarded for producing plans that result in a disproportionately positive impact on the environment (Nowak and Cabot 2004). Often a relatively small percentage of cropland is responsible for a disproportionate amount of pollutants to the environment, particularly if mismanagement occurs on environmentally sensitive and vulnerable land (Nowak et al. 2006, Ribaudo et al. 2016). Policy and planning should focus efforts on ensuring that NMPs target cropland acreage that imparts the greatest benefit to the environment and society

1.4 Conclusions

Based on the assessment of insurance-type approaches compared to the historical direct payment from compliance programs in other cropping systems, it appears that insurance mechanisms may have only a modest, or even negative, effect on N conservation by farmers. Creating a compliance program for nutrient management, similar to current erosion and wetland compliance programs, likely would be more successful at reducing N pollution than relying on insurance mechanisms alone. NMPs have been shown to significantly reduce N applications, and with no effect on yields. However, adoption of NMPs remains a significant barrier to their utility in reducing N applications because farmers perceive BMP adoption as a risk. Increasing education and participation by farmers to create and implement NMPs would likely increase adoption of NMPs and their effectiveness at mitigating N pollution.

Policy strategies to mitigate N pollution have so far been relatively unsuccessful, as excess N in the environment from agriculture remains a major problem (Porter et al. 2015, Udvardi et al. 2015) and N continues to be one of the most unregulated nutrients in agriculture (Mclellan et al. 2015). Policy strategies that have not been discussed here, such as a direct tax on N fertilizer purchases, have had relatively little consideration as a viable policy option (Chamberlain and Miller 2012). Although some states have implemented an input tax, it has been at such low levels that it becomes a relatively ineffective tool for preventing overuse of N (Ribaudo et al. 2011).

Perennial grass bioenergy systems have yet to be planted on any significant amount of acreage on our landscape (Skevas et al. 2016). Landowner willingness to grow perennial bioenergy grasses remains low. Relatively low biomass prices, little to no incentive to switch from more profitable enterprises (such as dairy), and the current supply of biomass materials like corn stover relative to cellulosic material, all contribute to farmer reluctance to grow perennial

grasses for supplying bioenergy (Barham et al. 2016). However, thinking ahead to how nutrient additions would be managed in these systems, should they become part of our landscape, will help ensure that the expected environmental benefits of these crops are realized. Policies that encourage or enforce the adoption of nutrient management recommendations for perennial grass systems likely will be necessary to maintain the N retention services of perennial bioenergy landscapes as they are intended.

The following chapters explore biophysical mechanisms in the plant-soil system that conserves N resources in perennial grasses. The effect of N addition on N conservation mechanisms is of primary interest, given current N recommendations for perennial bioenergy grasses and the lack of regulation and policies associated with N management. Chapter 2 explores N conservation strategies in native C4 grasses. Mechanisms of how much N is internally recycled, the timing of resorption, the degree to which plants are able to withdraw N from tissues, and plant NUE are all strategies for conserving N that are affected by both genetic and environmental factors. Understanding the effect of plant ecotype (plants with different phenotypic and physiological differences) and N addition on the above N conservation strategies will help determine management decisions that can maintain the inherent N-conserving qualities of perennial bioenergy crops. Chapter 3 considers the yield and N nutrition benefit provided by the microbial-plant symbiosis of arbuscular-mycorrhizae fungi (AMF) and Chapter 4 explores the amount of N actually supplied to the plant by the AMF symbiosis. If AMF are able to provide a yield and N nutrition benefit to perennial grasses, AMF could potentially replace the need for N fertilizers. Policy could then focus on discouraging the use of N fertilizers and instead on incentivizing management that encourages healthy microbial communities and maintains plants' inherent N-conserving strategies in perennial bioenergy systems.

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Chapter 2: Nitrogen conservation strategies differ in the C4 grasses switchgrass and big bluestem

Abstract

Improving nitrogen (N) conservation in bioenergy crops is one approach to helping mitigate N imbalance in our environment. However, the genetic and environmental controls and variation in perennial plant N conservation strategies are difficult to improve because they are not well understood. We measured variation in the N conservation strategies of resorption efficiency (RE), resorption proficiency (RP), and nitrogen-use efficiency (NUE) of individual plants in populations of *Panicum virgatum* (switchgrass) and *Andropogon gerardii* (big bluestem) in their native habitat and compared upland and lowland genotypes of cultivated switchgrass with and without N addition. N conservation was highly variable and the dominant strategy varied by species, ecotype, and site. RP and NUE primarily were related to site × genetic factors, whereas the rate of RE was different each year. N addition had no effect on N conservation, but lower soil C:N was related to higher plant N conservation. Our study demonstrated a high degree of variation in N conservation parameters where RE was the most plastic and RP and NUE showed an integrated adaptation and response to site × genetic factors. This variation in N conservation suggests high potential for selection and management of C4 grasses for improved N conservation.

2.1 Introduction

One result of the Green Revolution has been a gross nitrogen (N) imbalance in our environment (Galloway et al. 2008, Schlesinger 2009, Robertson and Vitousek 2009). N imbalance is a local, regional, and global issue, with some areas facing N eutrophication and pollution (Galloway et al. 2003, Drinkwater and Snapp 2007, Johnson et al. 2010), while other areas struggle with devastating nutrient shortages (Vitousek et al. 2009). Understanding how to conserve N within a plant-soil system is part of ameliorating these N imbalances. While there are many approaches to increasing nutrient retention, such as cover cropping, proper timing of fertilizer application, and perennialization (Robertson and Vitousek 2009, McSwiney et al. 2010, Meehan et al. 2013), using crops that have inherent N-conserving strategies is a logical starting point. Perennial grasses are known to conserve N by resorbing it from shoots to crowns and roots. Moreover, they develop dense, fibrous root systems that promote nutrient uptake and inhibit soil erosion. However, our understanding of how plant genetic and environmental factors affect these N conservation strategies is lacking.

Conservation of N within plants involves efficient use of acquired N (N use efficiency [NUE]) and N retention via resorption to perennial plant tissues (Freschet et al. 2010). NUE is driven by a combination of ecological, physiological, and agronomic factors (Dawson et al. 2008). Ideally, measurements of NUE would encompass all the processes comprising plant N use: uptake, assimilation, and internal recycling (Masclaux-Daubresse et al. 2010), the latter determining the mean residence time of N in perennials. In its simplest definition, NUE is calculated as biomass produced per unit N taken up by the plant (Chapin, 1980).

Resorption is the remobilization of nutrients from live, but senescing, plant tissues to tissues associated with storage or active growth (Brant and Chen, 2015; Chapin, 1980). Retaining

N in plant tissues, rather than losing N to the litter pool, reduces the plant's need to take up additional N and thereby reduces competition with other plants and microbes for N. Resorption is an active process regulated by senescence-associated genes (Yang et al. 2016), in which enzymes break down N-containing structures, such as chloroplasts, and mobilize N in the phloem for transport to perennial tissues (Rejmánková 2005). Two separate metrics are used to evaluate plant N resorption: resorption *efficiency* (RE) and resorption *proficiency* (RP) (Killingbeck 1996). RE is the proportional withdrawal of N from the time of peak aboveground N content to the end of the senescence period. Higher values indicate a greater proportion of total plant N resorbed. RP is the N concentration of plant tissues at the end of the senescence period, indicating how completely the plant has resorbed N. Lower N concentrations indicate higher RP. Given the active nature of resorption, it is considered to be a plant adaptation, but our understanding about what affects N resorption and its variability is lacking (Freschet et al. 2010, Brant and Chen 2015).

Variation in N conservation appears to be influenced by genetic, climatic, and soil fertility factors (Brant and Chen 2015) and has been studied across many climatic and geographic ranges. Resorption varies considerably among perennial plants ranging from 0 to 90% of N in aboveground tissues. Globally, unfertilized perennial plants resorb an average of 62% of their original N stores, and grasses resorb significantly more N, with an average RE of 75% (Vergutz et al. 2012). Yang et al. (2009) examined 31 accessions of *Panicum virgatum* L. (switchgrass) and found their RE to range from 20 to 61% and their terminal tissue N concentration (RP) to range from 0.40 to 0.77%. Although the molecular pathways for resorption are not fully understood, a clear genetic component for these processes exists (Masclaux-Daubresse et al. 2010, Yang et al. 2016).

Throughout the lifespan of a plant, numerous processes can affect plant NUE such as extracting N from the soil, assimilating N into the plant, and retranslocating N within the plant. These are all potential areas that can be explored for variation (Masclaux-Daubresse et al. 2010). The variation in genetic mechanisms for NUE may depend on N availability to the plant (Dawson et al. 2008). Why some plants have a better capacity to take up N in low N conditions is currently unknown, but plant-microbial interactions may be a significant factor in plant N uptake in low-N environments (van der Heijden et al. 2008). Plants adapted to high N conditions typically exhibit lower NUE and likely, lower resorption (Lambers et al. 2008), but there is conflicting evidence for soil fertility effects on resorption (Aerts and Chapin 2000, Kobe et al. 2005, Norris and Reich 2008). Mounting evidence suggests that increased soil fertility decreases resorption (Brant and Chen 2015, Jach-Smith and Jackson 2015).

Resorption is of practical importance when perennial plants are being grown as crops. Examples of perennial crops include the C4 grasses switchgrass and big bluestem (*Andropogon gerardii* Vitman), which are native to the North American tallgrass prairie and are being proposed as feedstocks for bioenergy. Although cellulosic bioenergy crops are thought to help retain N in agricultural systems by being relatively conservative with N (Robertson et al. 2011), the management of these crops will have significant implications for their ability to improve or worsen N pollution (Robertson et al. 2008, Ruan and Robertson 2013, Duran et al. 2016). Despite variable yield responses to N fertilizer addition (Parrish and Fike 2005, Wullschleger et al. 2010, Jach-Smith and Jackson 2015), management recommendations for perennial bioenergy crops call for N addition to increase yields and maintain stand productivity (U.S. Department of Energy 2011), so understanding the consequences of N addition to plant N conservation strategies is a critical part of ensuring a more sustainable biomass feedstock supply.

The rate of N resorption also has important agronomic implications. If harvest is delayed to allow time for maximum resorption, yield losses can be significant (Adler et al. 2006, Jach-Smith and Jackson 2015). Since peak biomass occurs before senescence (Sarath et al. 2014), the ideal biomass crop would resorb resources earlier in the season and rapidly resorb N in order to conserve nutrients as completely as possible before harvest (Schwartz and Amasino 2013). Breeding efforts may be able to take advantage of variability in the onset of senescence, resorption, and NUE to develop a more N-conservative crop (Jakubowski 2013).

As a species, switchgrass has a high degree of genetic variation, but two groupings emerge from this variation that place the species into what are known as *upland* and *lowland* ecotypes (Casler 2005). These groups have many phenotypic and physiological differences (Lowry et al. 2014). Lowland ecotypes are adapted to southern latitudes (Casler and Boe 2003), where the growing season is longer. When grown in more northern latitudes, lowland ecotypes may not senesce early enough in the season to allow for maximal N resorption to occur before a killing frost. If late-flowering and senescence could coincide with similar or increased N resorption rates, yield gains could be made without sacrificing N conservation (Schwartz and Amasino 2013, Sarath et al. 2014). Previous research has shown that lowland ecotypes flower later in the growing season compared to uplands (Lowry et al. 2014), giving them time to produce more biomass before senescence and resorption is initiated. Lowland ecotypes are also known to produce significantly higher biomass yields than upland ecotypes (Tulbure et al. 2012). A focus of current breeding efforts is experimenting with lowland or hybrid switchgrass genotypes that are able to overwinter in northern latitudes.

To assess variability in N conservation strategies, we sampled native populations of switchgrass and big bluestem across a range of sites varying in productivity and soil type. To

help sort out environmental and genetic effects, we used a common garden of cultivated switchgrass to test how N addition and ecotype (upland and lowland) influenced the N-conservation strategies of RE, RP, and NUE. We hypothesized that lowland ecotypes would exhibit reduced N conservation compared to upland ecotypes and N addition would reduce N conservation overall compared to controls receiving no N. Further, we expected lowland ecotypes to resorb later and at a faster rate than upland ecotypes and plants receiving N to resorb at a slower rate than with N addition. Since N-amended plants should have access to more plant-available N, we expected that all three of our N conservation metrics would be lower in N-amended plants.

2.2 Methods

Study sites

We had two types of sites: 1) extant tallgrass prairie remnants and restorations (i.e., native sites) and 2) common garden experimental sites in which known accessions of lowland and upland switchgrass (*Panicum virgatum* L.) ecotypes were grown (i.e., cultivated sites). Native sites were selected from tallgrass prairies across south central Wisconsin for their range of soil type and inherent productivity (Table 1). Selected sites were either remnant prairies or prairie restorations of at least 20 years, so switchgrass and big bluestem (*Andropogon gerardii* Vitman) plants were assumed to be well established and adapted to site conditions. Cultivated sites had two genotypes of both upland and lowland switchgrass replicated in common gardens on very productive silt loam soil from Arlington Agricultural Research Station (Arlington, WI) and very unproductive sandy soil from Hancock Agricultural Research Station (Hancock, WI). Treatments included N addition (100 kg N ha^{-1}) and no N addition. Clones of the four switchgrass genotypes were taken from established plants at Arlington to be planted at Hancock

in 2011. At both sites, genotypes were replicated three times in a split-plot design with fertilizer level as the whole plot and genotypes randomized within each split-plot.

Plant sampling and analysis

Three different individuals of each species, in each year, were selected at native sites (or one individual genotype in each split-plot for cultivated sites) and were staked at the beginning of the growing season. Samples were collected from these selected individuals at each sampling time. A sample consisted of an individual tiller that was clipped to ground level, put on ice in a cooler, and transported back to the lab for measurement. Tillers were collected every two to three weeks from July (senescence begins) through November (senescence complete) in 2011 and 2012. The initiation of senescence for each site (native sites) or genotype (cultivated sites) was determined to be the collection period when tillers had reached maximum tiller N content, which often coincided with reproductive anthesis. The end point of senescence was determined to be two weeks after a killing frost (air temperature $\leq$ -2.2 °C for a minimum of 4 h for that specific site).

Tillers were processed by separating leaves from sheaths at the ligule and separating the inflorescence from the remaining tiller. Stem length and diameter was recorded. Leaf area was measured as the sum of all leaves from one tiller on a bench-top leaf area meter (LI-3100, Lincoln, NE). All tiller components were dried to a consistent weight and all tiller components were then summed for the total tiller dry weight. Tiller samples were then finely ground using a Wiley Mill to pass a 1-mm mesh screen, then pulverized using stainless steel balls in 2-ml micro-centrifuge tubes. Tissue N concentrations were determined by combustion on an elemental analyzer (CE Elantech EA1112, Lakewood, NJ).

For each collection time at a native site or for each genotype at a cultivated site, a mass-loss correction factor (MLCF) was calculated to prevent underestimating RE (van Heerwaarden et al. 2003) because graminoids (and other perennial species) have been found to lose significant mass during senescence (Gusewell 2005, Vernescu et al. 2005). To calculate the MLCF, tiller dry weights were standardized by their mean weight, which was then normalized by total leaf area and stem diameter (Vergutz et al. 2012). Plant samples were used to calculate the following metrics of N conservation:

RE (proportional reduction in plant N content relative to N content at anthesis)

$$RE = 1- ([N]_{senesced\ leaf}/[N]_{green\ leaf})* MLCF)*100.$$

RP (plant N concentration [N] after senescence) (Killingbeck 1996)

$$RP = g\ N\ g\ biomass^{-1}{}_{senesced\ tiller}$$

NUE (biomass/N)

$$NUE = g\ biomass\ g\ N^{-1}{}_{green\ tiller}$$

N loss from leaching was assumed to be negligible (Vergutz et al. 2012). We defined NUE as biomass produced in aboveground tissue per mass of N at peak aboveground N content. While this calculation of NUE ignores the N resorbed into roots and root N content and mean residence time of plant N (Aerts and Chapin 2000, Hirose 2011), it remains a useful index to evaluate N use in a perennial crop with aboveground biomass harvested as an agronomic crop.

Soil sampling and analysis

Total soil C and N were determined for each site in July and October in 2011 and 2012. Soil samples consisted of three composited soil cores (2.5 cm diameter × 15 cm deep) taken from within a ~30-cm radius of each individual plant. Samples were transported on ice and stored at 4 °C until processed, which was within 48 h of field collection. Soils were homogenized and

organic matter and rocks removed using a 2-mm sieve, followed by combustion on an elemental analyzer (CE Elantech EA1112, Lakewood, NJ). An average was calculated for each individual plant to correlate with plant N conservation metrics, and a site mean was calculated across sampling times and years to characterize sites. Inorganic soil N and potential net N mineralization rates were assayed for each site. Soil samples consisted of five composited soil cores (2.5 cm diameter × 15 cm depth) taken within the same area as the staked plants. Soils were homogenized and sieved as above, followed by extraction with 2M KCl. Extracts were filtered through Whatman A/E 0.2-µm glass fiber filters. A subsample of the sieved soils were incubated for 28 d in the lab before extraction to calculate net N mineralization rates (Robertson et al. 1999). All extracts were frozen (-20°C) until analysis on a Flow Solution 3100 segmented flow injection analyzer (OI Analytical, College Station, TX) for nitrate and ammonium determination. Total inorganic N and net N mineralization were calculated as the sum of nitrate and ammonium, calculated for the surface 15 cm of soil.

Data analysis

All statistical analyses were performed in R version 3.2.3 (R Core Team 2015). Variation in N conservation metrics for the native sites were evaluated by examining boxplots created in the R package *ggplot2* (Wickham 2009). Mixed effects or random effects models were used for all other statistical analyses using the R package *lme4* (Bates et al. 2015). To understand sources of variation in the N conservation metrics, we partitioned variance from mixed or random effects models. For RE through the senescence period, the start of senescence was a fixed effect and random effects were individual plant, nested within species, nested within site, and nested within year. For end-of-senescence final RE, final RP, and start-of-senescence NUE, we used the above random effects structure, except individual plant was not a term since we only had one time point

for each individual plant for these estimates. Variance was partitioned by dividing each factor's mixed model variance by the total variance of the mixed model and multiplying by 100. Coefficients of variation (CV) were calculated to compare relative amounts of variation between response variables by dividing the total standard deviation from the random effects parameters by the grand mean for each response variable (Jackson et al. 2007). Modeling N resorption efficiency through time was done using a mixed effects model using the days past the start of senescence and ecotype as fixed effects, and random effects of year, site, genotype and individual plant for cultivated sites. N level and its interaction with ecotype were then removed from the model because its effect was not significant. For the native sites, days past the start of senescence and species were fixed effects, while individual plant, site and year were random effects. For all models, fixed effects and their interactions were evaluated with an analysis of variance test, where significance was determined at $p < 0.05$. After interactions were determined not to be significant, mean comparisons were made using the R package *lsmeans* (Lenth 2015) for species as a fixed effect for the native sites, or ecotype, fertilizer level or site as the fixed effect at the cultivated sites. For the native sites, soil N, soil C, and soil C:N ratios were regressed against individual plant RP, RE, and NUE using least squares linear regression and significance determined at $p < 0.05$. We also regressed site means of inorganic soil N and potential net N mineralization rates against mean plant RP, RE, and NUE in addition to soil N, soil C, and soil C:N ratios.

2.3 Results

Native sites

Although there was high variation in all N conservation parameters, some were significantly more variable than others and the sources of variation differed widely. The

initiation of senescence across the sites and two species varied significantly, with the latest senescence start at 55 d past the earliest senescence start in 2011 at CP in big bluestem, and 64 d past the earliest start date in 2012 at RR in big bluestem (Figure 1). Site accounted for the majority of the variance in senescence start time, followed by species and year (Table 2). The rate of RE was most influenced by year and was also the most variable N conservation metric we observed with a CV of 2,581% (Table 2). A wide range of slopes also demonstrated the high variability in RE rate at the native sites (Figure 1), but most of this variation (57.7%) was unaccounted for in our model (Table 2).

The end-of-season proportion of N resorbed (final RE) was the least variable metric (Figure 2a,b), with species as the largest source of variation (Table 2). However, when subjected to a means comparison test, switchgrass (58%) and big bluestem (56%) were not statistically different in final RE ($p < 0.05$). For terminal N concentration of plant tissue, or N proficiency (final RP), site was the largest source of variation (Table 2). Final RP between years was relatively similar compared to differences between sites (Figure 2c,d). Although not explaining the majority of the total variation, species did have a significant effect on final RP ($p < 0.001$) with switchgrass at 0.44% N and big bluestem at 0.37% N. Plant NUE at the time of peak N content (peak NUE) followed a somewhat similar pattern to final RP (Figure 2e,f). Site accounted for the highest percentage of variation, followed by species ($p < 0.01$, Table 2) with NUE of 128 and 148 g N g biomass^{-1} for switchgrass and big bluestem, respectively.

Across all years, sites, and species, individual plants had a wide array of resorption characteristics (Figure 3). Individuals in the upper left-hand quadrant of Figure 3 exhibited exceptionally high resorption capabilities, with both high RE and RP. Conversely, highly efficient plants were not necessarily highly proficient, and vice versa. Some plants were grouped

by site, most notably the plants at SR, which had exceptionally high RE and RP. Other sites had individuals that spanned a broad range, such as RR and LM. It is noteworthy that plants from SR also had relatively high NUE (Figure 2), indicating that plants from these sites use multiple mechanisms to conserve N.

There was no relationship between any of the plant N conservation strategies to total soil N, total soil C, total inorganic soil N, or potential N mineralization rates (all p-values were >0.05). However, the soil C:N ratio had a weak, albeit significant positive relationship with plant RP, and a weak, but significant negative relationship with plant NUE (Figure 4). Therefore, soils with lower C:N ratios tended to have plants that were slightly more N conservative. There was also a significant negative relationship between potential N mineralization rates and soil C:N, such that sites with lower soil C:N had significantly higher N mineralization rates (p<0.01 and $R^2 = 0.71$).

Cultivated sites

Individuals in our cultivated sites showed significant variation in resorption through time, mostly because ecotype had a significant effect on the initiation of senescence ($p < 0.0001$). Upland ecotypes began the resorption process much earlier in the season than lowland ecotypes. In 2011, upland ecotypes began resorbing on average 8 d earlier than lowland ecotypes, while in 2012, upland ecotypes began resorbing an average of 25 d earlier. RE was also affected by ecotype (i.e., slopes in Figure 5, $p < 0.05$). In contrast to the native sites, there did not appear to be much variation in the rate of resorption between individuals within an ecotype (Figure 5). N addition had no significant effect on RE rates ($p > 0.05$).

Final RE again had a significant ecotype effect, with higher RE in upland ecotypes ($p <$ 0.01, Figure 6a). N addition had no significant effect on final RE (Figure 6b). In contrast to final

RE, lowland ecotypes had a higher peak NUE compared to the upland ecotypes ($p < 0.05$, Figure 6g) and N addition had no effect (Figure 6h). Neither ecotype nor N addition had an effect on final RP (Figure 6d,c). Site had a significant effect on all N conservation parameters, with Hancock plants displaying higher RE ($p < 0.001$), higher RP ($p < 0.01$), and lower NUE ($p < 0.05$) (Figure 6c,f,i).

2.4 Discussion

Our results suggest that site × genetic factors control plant NUE and RP, while local climate and environmental conditions control the start of senescence and plants adjust RE rate accordingly to minimize N remaining in aboveground tissues. Resorption is an active process during which the plant expends energy to resorb nutrients from aboveground tissues (Rejmánková 2005, Masclaux-Daubresse et al. 2010). There is always a tradeoff between the plant using energy to conserve resources that will be available for the subsequent year's growth and producing more biomass to be more competitive in the current year. By adjusting the rate of RE, plants can make the most use of growing season resources to produce biomass, while still conserving N for winter survival. A programmed NUE or RP, coupled with a plastic RE rate would seem to be the most advantageous combination of N conservation traits for competing and surviving interannual weather variability.

Final RE was relatively similar within a site and year, but genetic controls were apparent. At the cultivated sites, ecotypes differed while at the native sites, species differed. Differences between species or ecotypes, even within the same sites, suggests that genetic factors play a dominant role in controlling resorption compared to environmental factors alone. There may also be an interaction between plant phenology and climate that affects resorption processes. Differences in flowering time and the onset of dormancy are highly variable, but are affected by

temperature, precipitation, and photoperiod (Schwartz and Amasino 2013). At critical times of flowering, seed filling and senescence, weather conditions may play a larger role in the active process of resorption, depending on water availability or competition with other plants (Yuan et al. 2007) at the time of active resorption.

Significant differences between ecotypes in RE appeared partially attributable to variation in the initiation of senescence. As discussed earlier, lowland ecotypes flower later in the season, which is their cue to begin senescence. However, we did not expect that they would be able to "catch up" to the upland ecotypes by 1) having a slightly higher RE rate and 2) not needing to resorb as much labile N because they had less N per unit of biomass owing to their significantly higher NUE. In their study of 31 switchgrass accessions, Yang et al. (2009) found higher resorption efficiencies in lowland accessions and lower senesced tiller N concentrations, which had not differed among ecotypes before senescence.

Despite the clear genetic controls, we cannot ignore that the genetically identical plants at Arlington and Hancock had different end-of-season RE, RP and peak NUE values. Plants with identical genetics had significant responses to the change of site. Unfortunately, site differences here included the conflated factors of latitude, soil type, soil nutrient status, and local climate. Determining which of these environmental factors play a larger role was not possible here, but future common garden experiments could sort out the relative importance of these factors.

N conservation strategies were highly variable

The dominant N conservation strategy varied by site, but some degree of genetic control was evident. Both switchgrass ecotypes had similar RP at the end of the season, but upland ecotypes were more efficient at resorbing N (higher RE); lowlands were more N-use efficient (higher NUE); and despite these different mechanisms of N conservation, ecotypes had similar N

content in senesced biomass (similar RP) just before the time that these plants would be harvested in a biomass feedstock context. Although lowland ecotypes had lower RE, this did not prevent them from drawing N down to similar RP levels as the upland ecotypes.

Despite the generally inelastic nature of plant NUE and RP within a site or ecotype, there were some native populations that had remarkably high N conservation using all strategies to conserve N, with the plants at SR being the most noteworthy. These individuals had very high RE, RP, and NUE. Their efficient use of N throughout the growing season notwithstanding, the plants still resorbed significant amounts of N to reach very low terminal N concentrations. It should be noted that because these plants started with low N concentrations at the beginning of senescence (high NUE), they had less potential N to remobilize, so any N that was resorbed constitutes a higher proportion of its total N, hence the high RE. Since these genotypes also had very low N concentrations at the time of harvest, they would be exceptional bioenergy crops, because low N content is preferred for biomass quality (Tubeileh et al. 2016). Also, given their high N conservation, individuals from this native population would in theory require very little N fertilizer to maintain yields. Further study should explore how plants taken from their local climate and environment conserve N in a different climate and environment. Adaptation may be playing a large role in plant N conservation, so growing a plant in a new environment in which it is not adapted may result in less efficient execution of N conservation strategies.

Plant N-conservation strategies related to soil C:N but not affected by N addition

The high variability in plant N conservation parameters, the relatively weak relationship with soil C and N variates, and the lack of a response from N addition suggests factors other than N stress have a greater effect on N conservation strategies. The variation between all sites may stem from the genetics of the respective populations of switchgrass, or from some combination

of soil and environmental conditions. It was especially surprising that N-amended switchgrass at Hancock did not respond because this site has very sandy soils with low N. However, it is noteworthy that plants at Hancock had significantly higher RE, RP, and lower NUE, indicating that the clones grown in this environment were more N-conservative.

More soil N does not necessarily translate to a reduced need for conserving N, as it has been demonstrated that N conservation may actually increase if soil N pools are higher (McCulley et al. 2009). Low C:N ratios in soil generally translates to more mineralizable N (Booth et al. 2005, Gibson 2009), as was also seen in our soils. Higher amounts and rates of mineralized N without immobilization may mean that microbes are more C limited in these soils, which would mean more N has the potential to be lost through the soil profile via leaching. This would make N more limiting to the plant over the uptake period and plants would benefit from being more N conservative in soils with low C:N ratios.

The lack of a relationship between plant N conservation and soil N may be related to our method of assaying inorganic soil N and N mineralization, which may only shed light on a relatively small component of N availability for a given site. Native tallgrass prairie sites are often N-limited (Dell et al. 2005), and other N-cycling processes may be more important than inorganic N availability in these systems, or at the very least, not well-characterized by simple inorganic soil N assays. For example, organic N uptake may be a component of N nutrition (Schimel and Bennett 2004) and mycorrhizal associations likely play a significant role in plant nutrient uptake (Smith and Read 2008), in addition to the many rhizosphere and microbial processes occurring in a perennial grassland that affect soil N availability and actual plant N uptake (Frank and Groffman 2009).

The relationship between soil nutrient availability and resorption has been a long-standing debate in the study of N resorption. Numerous studies can be found to support both a lack of relationship to soil N availability, and also a negative relationship with increased soil N and lower N resorption (Kobe et al. 2005, Brant and Chen 2015, Yuan and Chen 2015). Many of these studies considered global datasets or plants of different life forms such as trees, shrubs, forbs or other graminoids, whereas our study included only two perennial grass species from sites of relatively similar latitude and climate. In addition, other plant nutrient economics, physiology, and environmental conditions may, in essence, trump the regulators of N resorption (Freschet et al. 2010). Since resorption is an active process that involves loading resorbed nutrients in the phloem, the low availability of water or the plant's water-use efficiency may have had more control over nutrient resorption than soil N availability itself.

Using N conservation strategies to guide agronomic decisions and breeding efforts

Our study highlighted that even within a single species, there is significant variation in not only N resorption metrics and NUE, but also in the dominant strategy used to conserve N. Plants with the ability to conserve N through multiple strategies may be ideal candidates for bioenergy crops because they have the capacity to conserve N under varying environmental conditions. To a significant degree, the likelihood of maintaining high N conservation depends on the site and conditions in which the crop is grown. Genotypes with multiple N-conservation strategies will be more resilient across a broad range of environmental conditions.

Across sites and years, big bluestem had higher RP and NUE than switchgrass growing at the same sites; therefore, big bluestem may be considered a more N-conservative species and should receive more attention as a candidate bioenergy feedstock crop. Big bluestem is commonly part of native grass or prairie mixtures being grown for bioenergy (Jarchow et al.

2012, Orr et al. 2015, Sanford et al. 2016) and there are currently some breeding efforts underway for developing cultivars of big bluestem as a bioenergy crop (Hong et al. 2013, Zhang et al. 2015), but most efforts are focused on switchgrass cultivar development (Anderson et al. 2016). While switchgrass may have a higher potential for biomass yields, the high yields realized from switchgrass are often from prime agricultural lands (Tulbure et al. 2012) and big bluestem may be a better candidate species for marginal lands given its remarkable ability for conservative N use.

While N conservation strategies were highly variable, it is important that breeders select for N-strategy traits in the "real world" environmental conditions that reflect likely production conditions. If selecting for high levels of a trait such as NUE, selection should occur in low-N conditions. As Anderson et al. (2016) observed, gains in yield will result in increased N uptake unless significant strides are made in reducing N uptake while increasing yields. Jakubowski, 2013, showed that by selecting for N conservation in addition to biomass yield, progress on yield increases likely will slow, but the tradeoff in reduced costs of N fertilizer make it a cost-effective proposition. It may be beneficial to also select from wild, or ancient germplasm since these plants are likely more adapted to low-N conditions and could be a source of genes that increase NUE in low N environments (Hirel et al. 2007, Dawson et al. 2008). Although N addition did not have significant effects on N utilization or N resorption in our study, others have found that uptake and utilization efficiencies can differ in low versus high N conditions, as discussed above. If breeding selections are made in fertilized, or high-N conditions, selections might be made for plants that are efficient at extracting, assimilating, and resorbing N only in high N conditions (Hirel et al. 2007).

2.5 Conclusions

Our study demonstrated that species and ecotypes of switchgrass and big bluestem used different mechanisms to conserve N, with RP and NUE under the main influence of site × genetic factors, whereas RE and the start of senescence was determined by local climate conditions. These grasses likely adapted over time to employ an integrated genetic × environmental response to local variation in climate and environment. Most of the variation in N conservation strategies we observed was between ecotypes or native populations and site. N addition had no effect on plant N conservation and only soil C:N was weakly related to plant N conservation strategies. The ideal bioenergy crop will combine all three mechanisms of whole-plant N conservation–RE, RP, and NUE–and breeders should make efforts to consider multiple traits of N conservation when making selections. Our work provides evidence that there is significant variation in N conservation traits even within a single species, which indicates that breeders and agronomists have the opportunity to work towards breeding and growing more N-conservative crops–a crucial step toward alleviating N imbalance in our environment.

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2.7 Tables and Figures

Table 1. Site characteristics and mean values for soil chemical properties.

Site type	Site name	Site code	Dominant soil series	Total N (%)	Total C (%)	C:N	Inorganic N (kg ha^{-1})	Mineralized N (kg ha^{-1} day^{-1})
Native	Chiwaukee Prairie	CP	Granby fine sandy loam	0.48	6.27	10.92	10.52	0.05
	Leopold Memorial Reserve	LM	Plainfield loamy sand	0.13	1.32	10.68	4.84	0.52
	Rocky Run State Natural Area	RR	Wyocena sandy loam	0.10	0.90	8.75	7.04	0.92
	Spring Green State Natural Area	SG	Sparta loamy sand	0.05	0.66	11.78	2.70	0.12
	Steege Reserve	SR	Edmond sandy loam	0.19	2.00	10.65	5.25	0.51
	Thompson Memorial Prairie	TP	Edmond silt loam	0.42	5.59	11.55	9.21	0.36
	Westport State Natural Area	WP	Griswold loam	0.25	3.20	10.40	10.99	0.29
	Young Prairie	YP	Pella silt loam	0.77	9.87	12.86	11.06	0.02
Cultivated	Arlington Ag Research Station	AARS	Plano silt loam	0.17	1.86	11.08	–	–
	Hancock Ag Research Station	HRS	Plainfield loamy sand	0.06	0.83	13.00	–	–

Table 2. The partitioning of variance calculated as the percentage of total variance from a random effects model using the random effects of individual, species (sp.), site, year and the residual variance, where a separate model was used for each response variable: resorption efficiency throughout the senescence period (RE through time), end-of-senescence resorption efficiency (final RE), resorption proficiency (final RP) and nitrogen-use efficiency at the start of senescence (peak NUE).

Response variable	Parameter	Percentage of total variance (%)					Coefficient of Variation (%)
		Individual	sp.	Site	Year	Residual	
RE through time	start time	5.27	20.10	58.93	5.41	10.29	75.9*
	rate	<0.1	<0.1	<0.1	32.24	67.71	2581.2†
final RE	mean	na	60.47	<0.1	9.50	30.03	40.0‡
final RP	mean	na	22.80	57.23	<0.1	19.97	64.8‡
peak NUE	mean	na	27.19	54.00	<0.1	18.81	59.7*

* calculated from tillers at the start of senescence (peak-N content), n= 81

† calculated from tillers sampled throughout the entire senescence period, n=322

‡ calculated from tillers at the end of senescence (final N content), n= 81

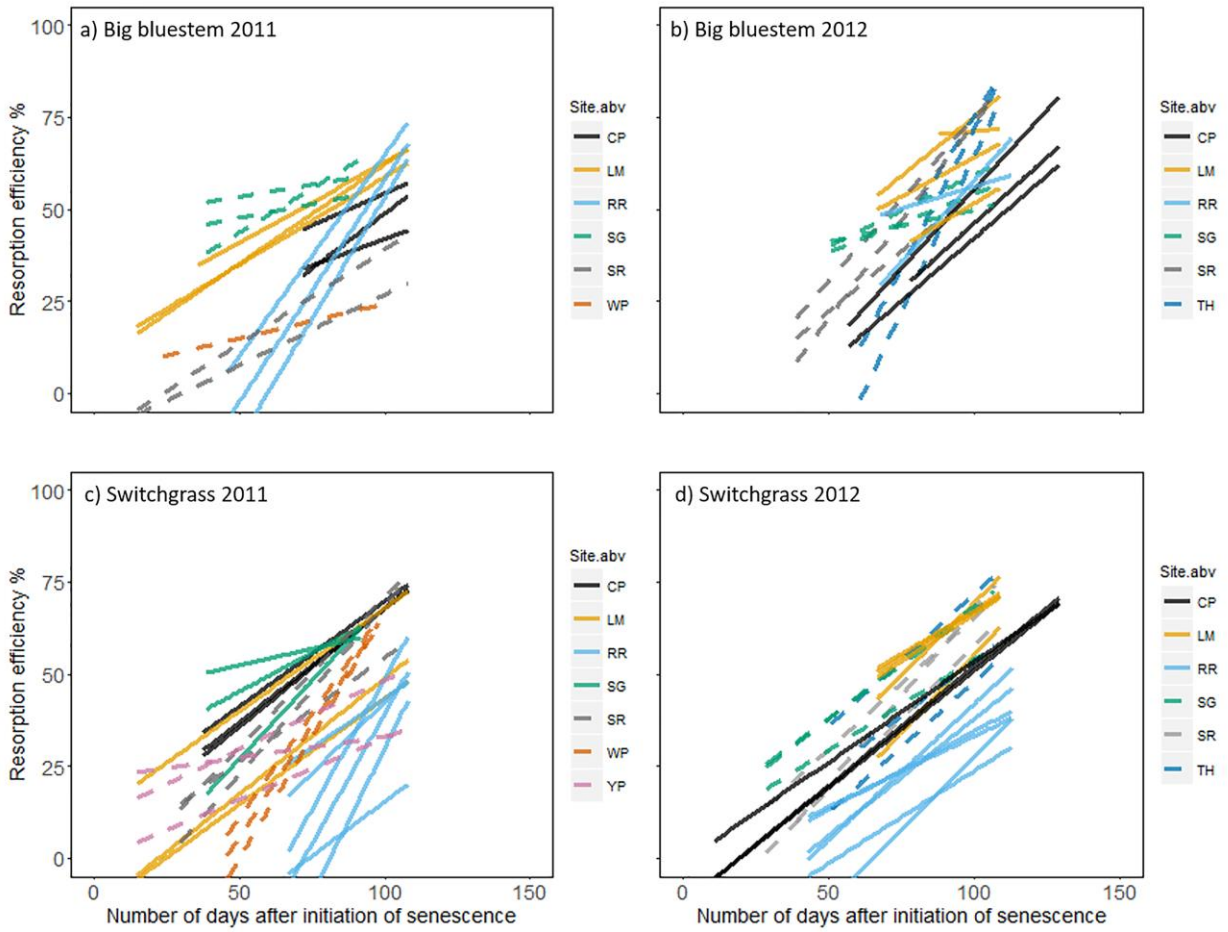

Figure 1. Resorption efficiency (RE) through time at the native sites for (a) big bluestem (*Andropogon gerardii*) in 2011, (b) big bluestem in 2012, (c) switchgrass (*Panicum virgatum*) in 2011, and (d) switchgrass in 2012.

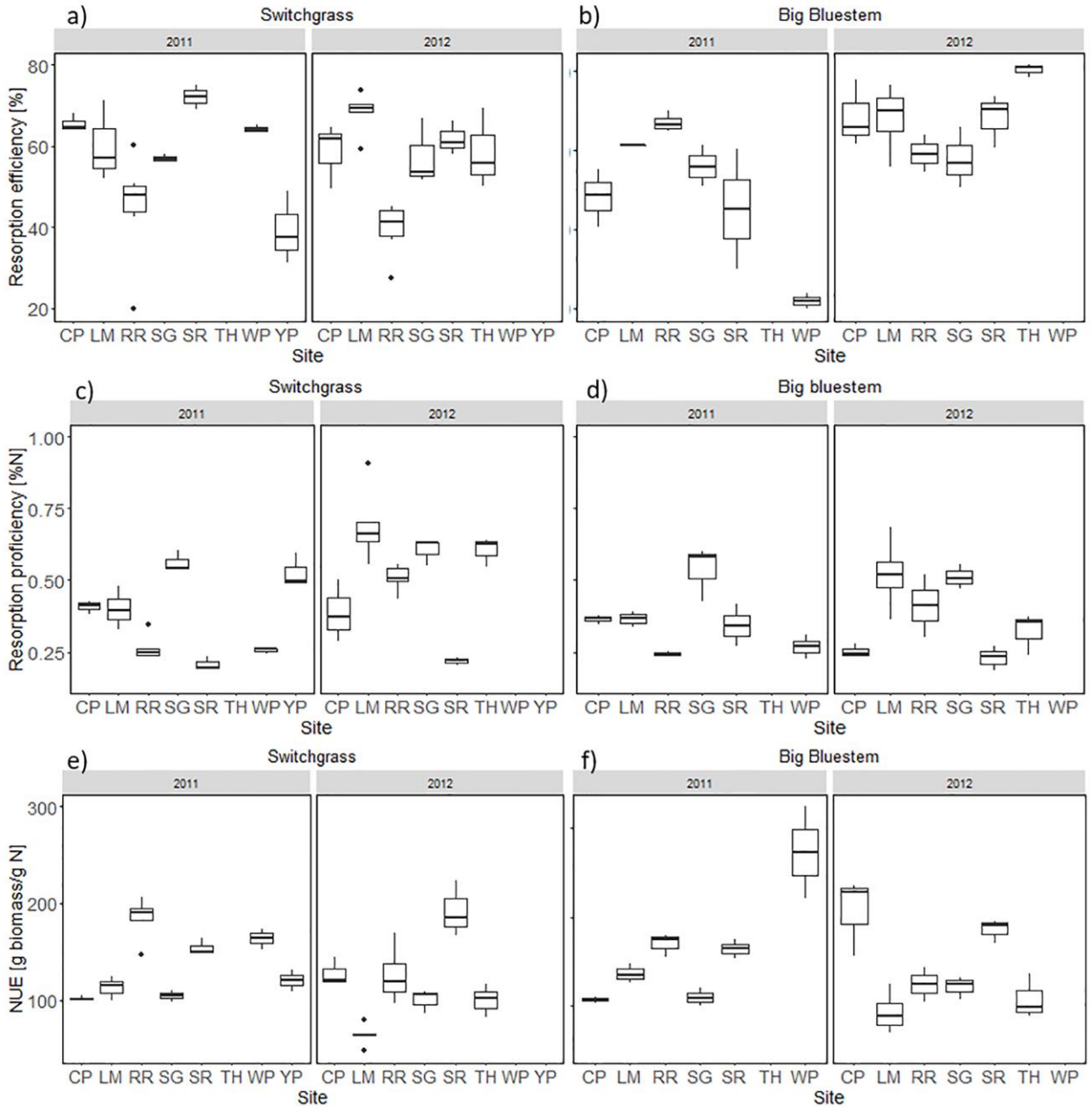

Figure 2. Boxplots of all native site populations illustrating (a) resorption efficiency (RE) in switchgrass (*Panicum virgatum*), (b) RE in big bluestem (*Andropogon gerardii*), (c) resorption proficiency (RP) in switchgrass, (d) RP in big bluestem, (e) nitrogen use efficiency (NUE) in switchgrass, and (f) NUE in big bluestem.

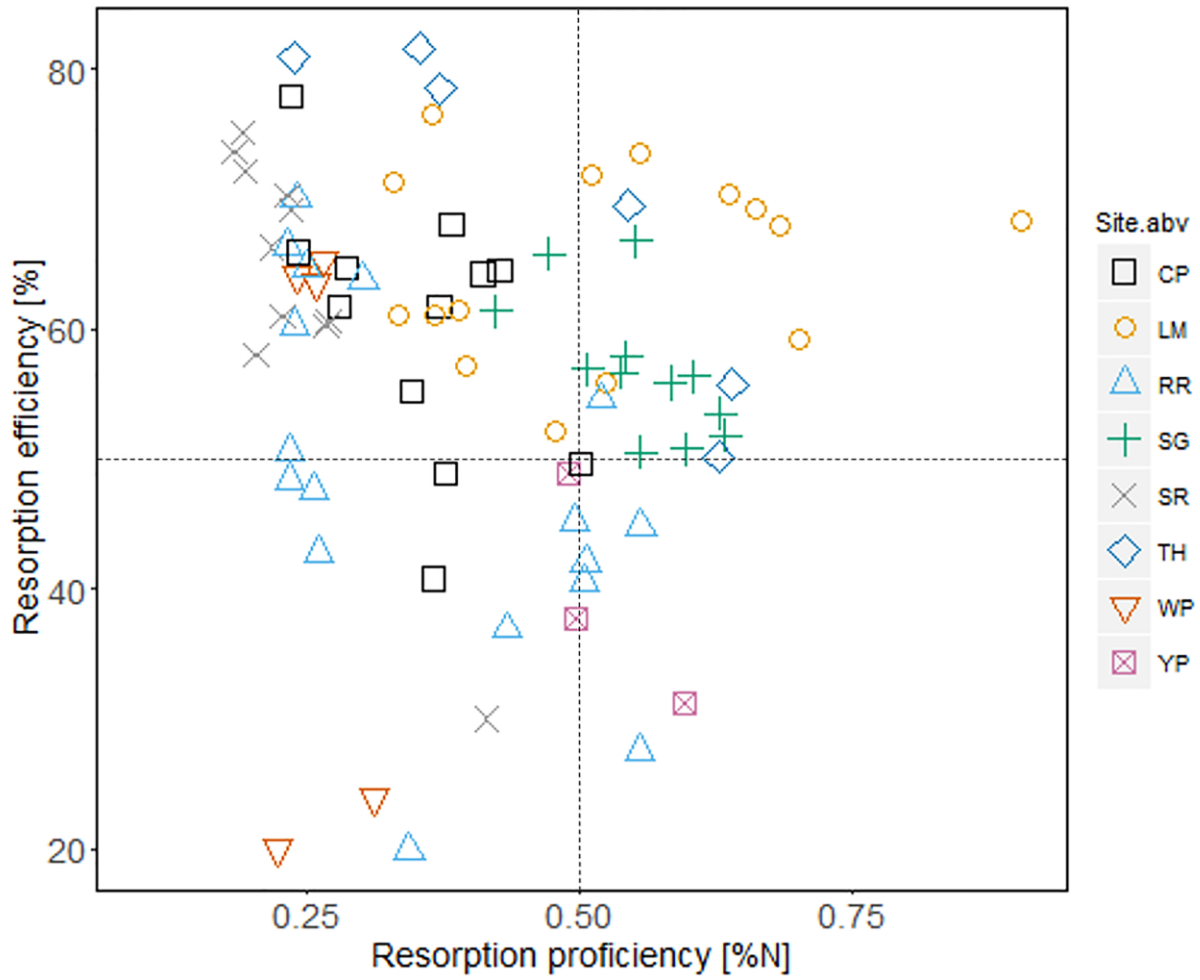

Figure 3. End-of-senescence resorption efficiency (final RE) and resorption proficiency (final RP) for each individual plant at native sites, based off the last collection timepoint for both *Panicum virgatum* and *Andropogon gerardii*.

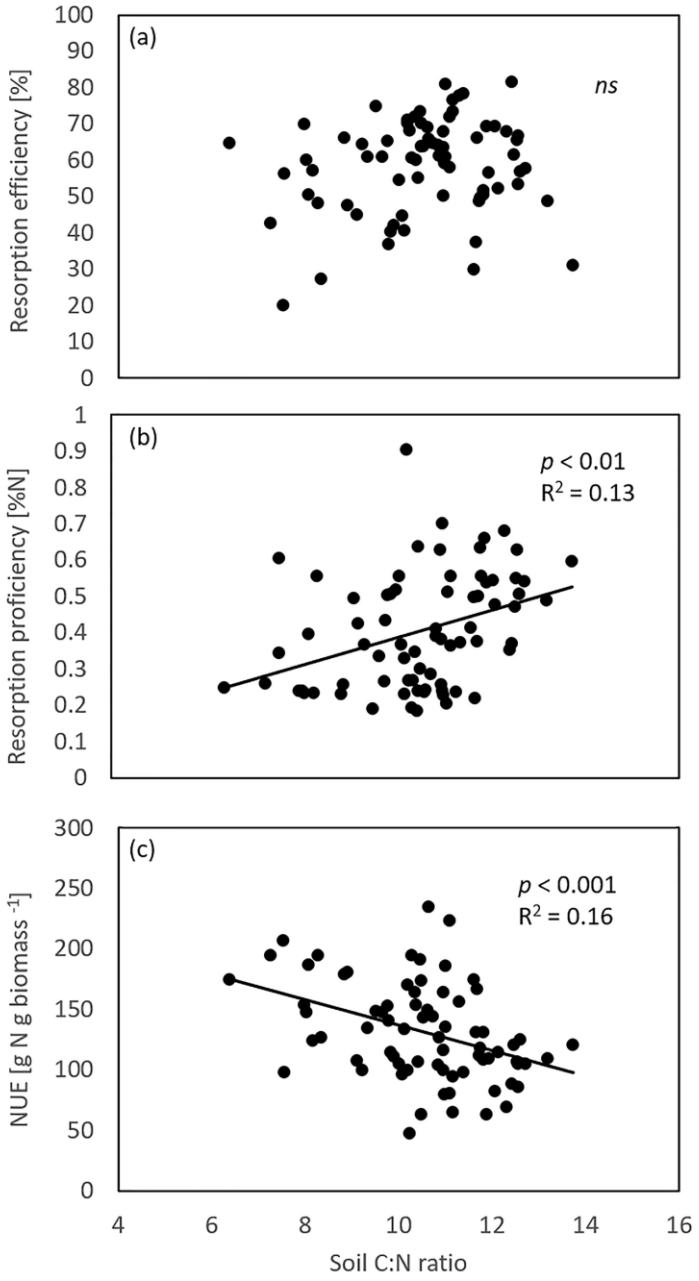

Figure 4. Linear regression of native site soil C:N ratio to individual plant (a) resorption efficiency (RE), (b) resorption proficiency (RP), and (c) nitrogen use efficiency (NUE) across all years and species *Panicum virgatum* and *Andropogon gerardii*.

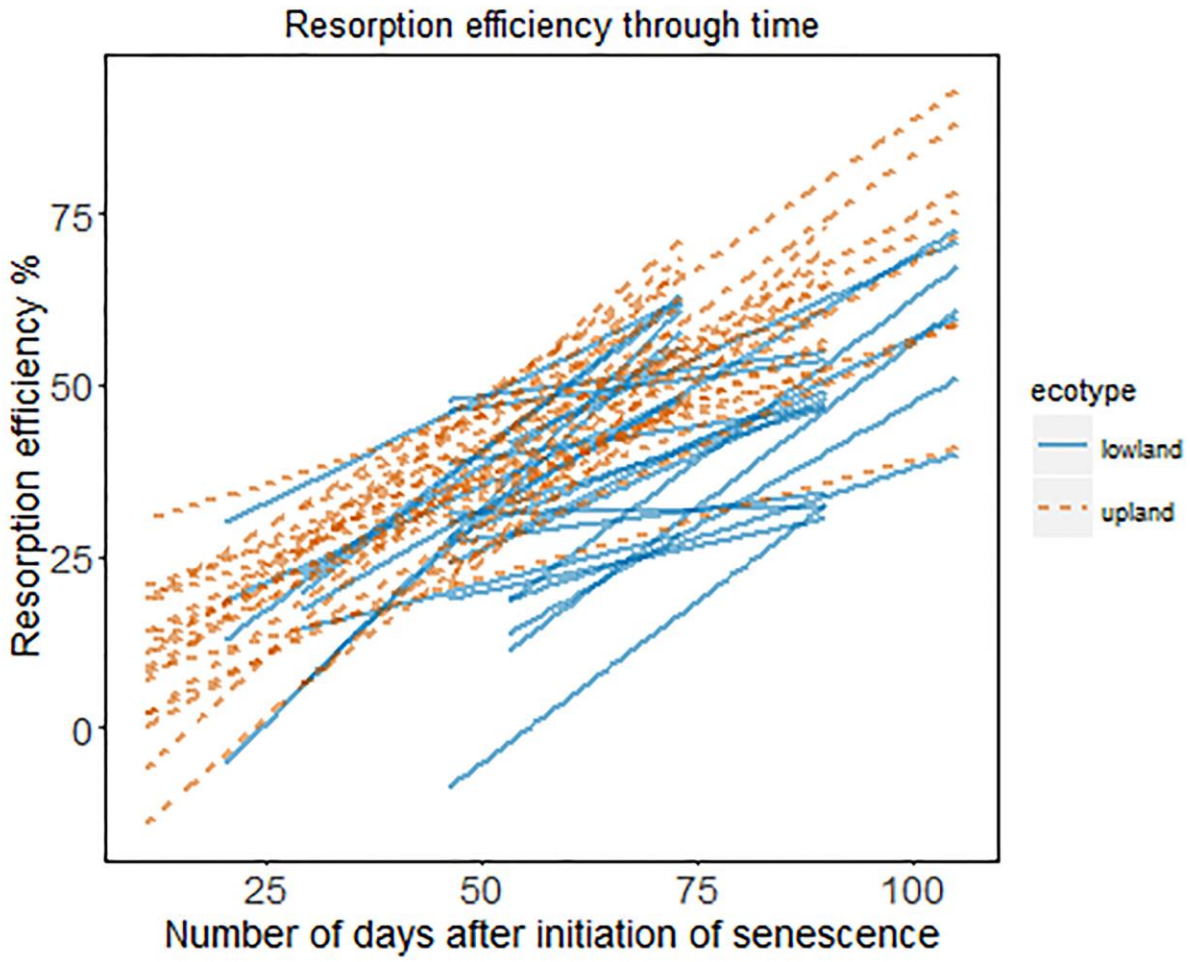

Figure 5. Nitrogen resorption efficiency RE (%) through time in *Panicum virgatum* at the cultivated sites (Arlington and Hancock). Each line represents one individual plant tracked through the entire senescence period.

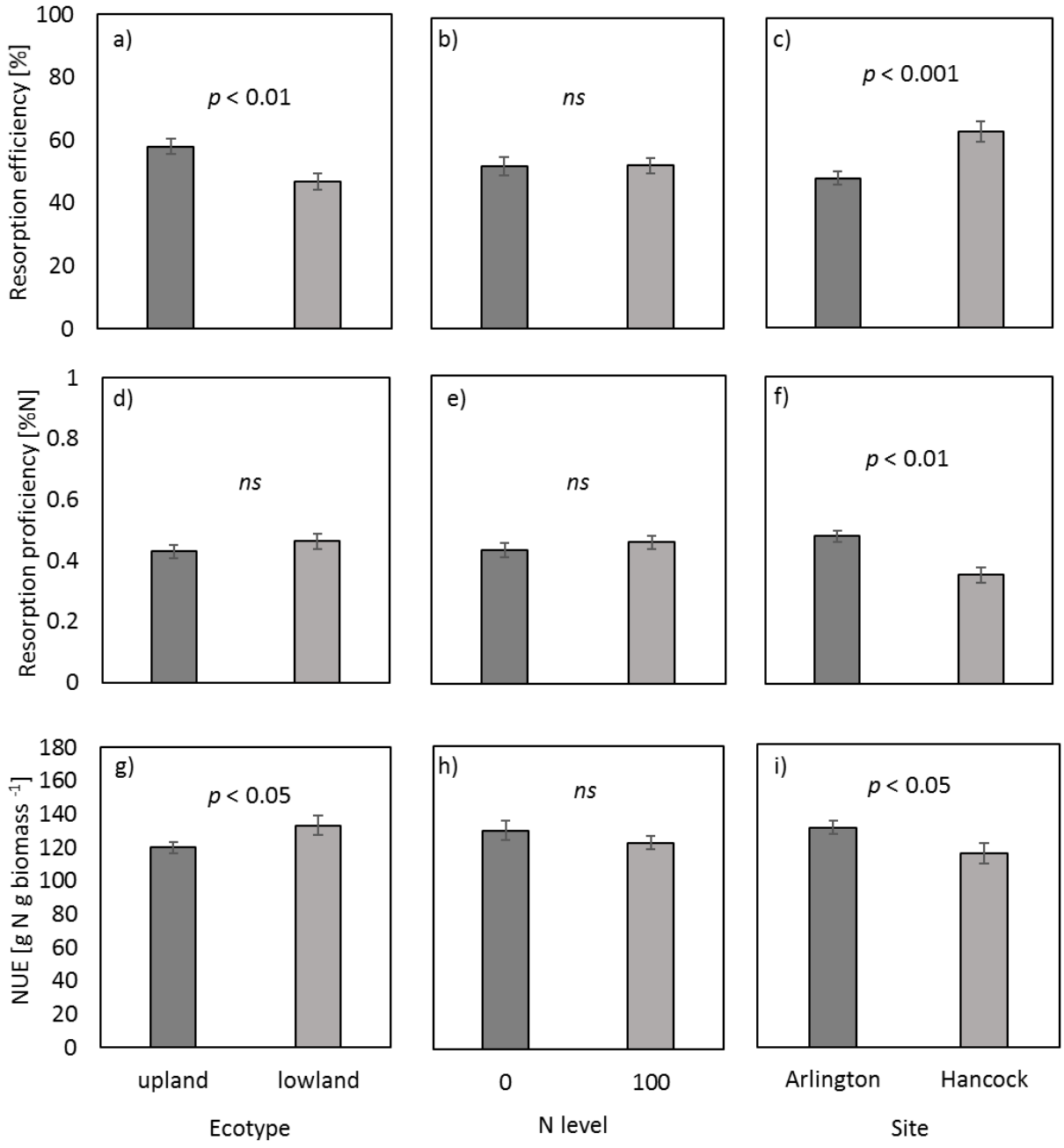

Figure 6. End-of-season resorption efficiency (RE), resorption proficiency (RP), and peak N-content nitrogen use efficiency (NUE) in *Panicum virgatum* by ecotype, N level (kg N ha^{-1}) and site ($p < 0.05$). Error bars represent ± 1 SE around the mean.

Chapter 3: N addition undermines N nutrition supplied by arbuscular mycorrhizal fungi to native perennial grasses

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 measure 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 and function. This pattern was supported by our survey study where increased soil N correlated with lower AMF abundance and function. In addition, plant N was positively related to increased AMF allocation to nutrient transfer structures within host roots. Demonstrating these relationships *in situ* across varying soil N levels at eight sites supported the hypothesis that AMF benefit perennial grasses by increasing N uptake. This is particularly notable for perennial grasses grown for bioenergy production because managing for higher AMF abundance and function may reduce or eliminate incentives for costly and environmentally problematic N addition to improve yields.

3.1 Introduction

One factor that may contribute to the conservative N-use of perennial warm-season grasses are symbiotic fungi – mycorrhizae – that increases 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 (Hodge et al. 2001, Govindarajulu et al. 2005, 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 (Correa 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 (Hodge et al. 2001, Atul-Nayyar et al. 2009, Barrett et al. 2011). AMF may also aid

in N mineralization through their relationship with plants, since plants can indirectly control N mineralization through priming (Hodge et al. 2010).

The role of AMF in plant N-uptake becomes even more uncertain in circumstances of increased N availability, which is typical of many agroecosystems receiving N amendments. Some studies show N addition decreases AMF abundance (Treseder 2004, Staddon et al. 2004, van Diepen et al. 2010, Leff et al. 2015), 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. These results demonstrate that increased AMF abundance does not necessarily translate to increased function. Additionally, 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 for increasing P-uptake (Johnson et al. 2003). In P-polluted soils, AMF colonization rates were higher in the 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. Correa 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. 2014, Correa et al. 2015).

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 (Hetrick et al. 1994, Facelli et al. 2009, 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.

Studies that explore mycorrhizae and N cycling relationships specific to bioenergy cropping systems are limited. AMF have been studied in perennial prairie grasses in the context of remnant (Egerton-Warburton et al., 2011) and restored prairies (Wilson et al. 2012), but there are few studies examining AMF-plant relationships in perennial grass bioenergy cropping systems. We are aware of mycorrhizal studies using bioenergy grasses only in highly controlled greenhouses or microcosms (Brejda et al. 1998, Wilson and Hartnett 1998, Ghimire et al. 2009, Ghimire and Craven 2011, Schroeder-Moreno et al. 2012), so studying AMF in perennial bioenergy crops *in situ* provides a novel perspective for understanding AMF-plant relationships.

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 that indicate 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.

3.2 Methods

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 applied as granules of ammonium-nitrate (0, 56 and 196 kg N ha^{-1}). Sites were located on established plots of switchgrass at the Arlington Agricultural Research Center (ARL) in Arlington, WI (silt loam soils) and the W.K. Kellogg Biological Station (KBS) in Michigan in Hickory Corners, MI (sandy soils) (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. 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).

Aboveground biomass and N 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, see Chapter 1). Biomass samples from the restored grasslands

were sorted into the following three categories: C4 grass (switchgrass, indiangrass, or big bluestem), 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 ºC 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.

Soil N and C

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 a Carlo-Erba elemental analyzer (CE Elantech EA1112, Lakewood, N.J.).

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-ºC 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, a subsample 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 ºC 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 100 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.

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.

Quantification of AMF biomass using fatty-acid extraction

Soil samples were analyzed for both the phospho-lipid fatty-acid (PLFA) biomarker 16:1ω5 (index for AMF biomass) (Zelles 1999, Balser et al. 2005) and the neutral-lipid-fatty acid (NLFA) biomarker 16:1ω5 (index for AMF storage lipids) (Olsson 1999). The NLFA:PLFA ratio provides some indication of AMF physiological state and nutrient status (Allison and Miller 2004). Using the NLFA (storage lipids) as opposed to solely PLFA (membrane lipids) has been shown to be much more reflective of seasonal C exchange between plant and fungus (Lekberg et al. 2012) and further, the neutral lipid marker has been shown to be a better indicator for AMF biomass than the PLFA biomarker alone (Ngosong et al. 2012). 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.

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 $p < 0.05$.

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.

3.3 Results

Plant biomass and N uptake

Peak standing biomass yields at ARL were not significantly affected by N addition in 2013 or 2014 (Table 2), 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 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 3). 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 3).

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.

AMF root colonization and NFLA:PLFA

Stained roots revealed identifiable AMF structures, such as intra-radical hyphae, coils, arbuscules, and spores (Figure 1). 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 (Figure 2a). Any amount of N fertilizer addition caused a decrease in the AMF allometric ratio across both sites and years (Figure 2b). 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 (Figure 2c).

At the extensive sites, rather than comparing AMF parameters at the site level, we explored whether soil N across sites had any correlation to AMF abundance and function. Among metrics that evaluated soil N availability (total soil [N], total inorganic N, 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 ($R^2 = 0.02$, $p < 0.05$) (Figure 3a, 3b). There was however no effect of any soil N metric on the AMF NLFA:PLFA ratio across the extensive sites ($R^2 = 0.04$, $p = 0.27$) (Figure 3c).

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 (Figure 4). This contrasted with findings of 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 KBS (Figure 5a), 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 (Figure 5b).

3.4 Discussion

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 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 P, light, or space that limited aboveground biomass growth. 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 to be the more unlikely case given that our results suggest 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. Although our study did not explicitly include any measure of system C allocation and balance, it would appear that the unfertilized plots at ARL produced more fixed C. 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. 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).

AMF abundance and function decrease 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 five 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 (Johnson et al. 2003, Treseder 2004, Grman and Robinson 2013, Correa et al. 2015). 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.

Linking AMF to agronomic management

Warm-season perennial grasses have inconsistent yield responses to N addition (Parrish and Fike 2005, Jach-Smith and Jackson 2015). 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 (Madakadze et al. 1999, Heggenstaller et al. 2009, Guretzky et al. 2010, Garten et al. 2011, Jung and Lal 2011, Jarchow and Liebman 2012, Jach-Smith and Jackson 2015). 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), among other ecosystem services that improve agroecosystem sustainability (van der Heijden et al. 2008, De Vries and Bardgett 2012, Bender

and van der Heijden 2015). 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.

3.5 Conclusions

AMF provide many benefits to plants, but so far, there has been little evidence of AMF contributing an agronomically-relevant amount of plant N. We provided evidence from remnant and restored grasslands 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 support of promoting AMF abundance and function, which in some cases, may essentially take the place of N fertilizer in providing adequate N nutrition to native C4 perennial grasses.

3.6 References

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3.7 Tables and Figures

Table 1. Site descriptions with soil properties.

Site				Soil properties						
Name	N addition [kg N ha^{-1}]	Latitude	Longitude	Texture	C:N	pH	Sand [%]	Silt [%]	Clay [%]	Bulk density [g cm^{-3}]
ARL	0	43° 17' 45" N	89° 22' 48" W	silt loam	8.24	6.6	9	66	25	1.30
	56									
	196									
KBS	0	42° 23' 47" N	85° 22' 26" W	sandy loam	7.82	6.1	63	31	6	1.60
	56									
	196									
Rocky Run	0	43° 27' 30" N	89° 19' 54" W	loamy sand	9.38	6.1	81	13	6	1.26
Fairfield	0	43° 28' 32" N	90° 21' 11" W	clay	7.14	6.5	19	20	61	1.10
Lund	0	42° 50' 40" N	90° 42' 19" W	sandy loam	8.76	6.4	54	28	18	1.45
Manthey	0	43° 26' 32" N	90° 48' 41"W	sandy loam	10.65	6.9	77	13	11	1.42
Rowe	0	43° 25' 24" N	90° 40' 44" W	sandy loam	10.21	6.4	58	25	17	1.46
Becker	0	43° 33' 44" N	90° 41' 32" W	sandy loam	9.35	5.7	77	9	14	1.57
Becker Lake	0	43° 36' 46" N	90° 44' 34" W	sandy loam	11.22	6.8	82	1	17	1.47
Shinners	100	43° 17' 58'' N	89° 21' 22'' W	silt loam	10.90	6.8	13	71	16	1.30

Table 2. 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)	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)	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 (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 (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 3. 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}	10567 (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}

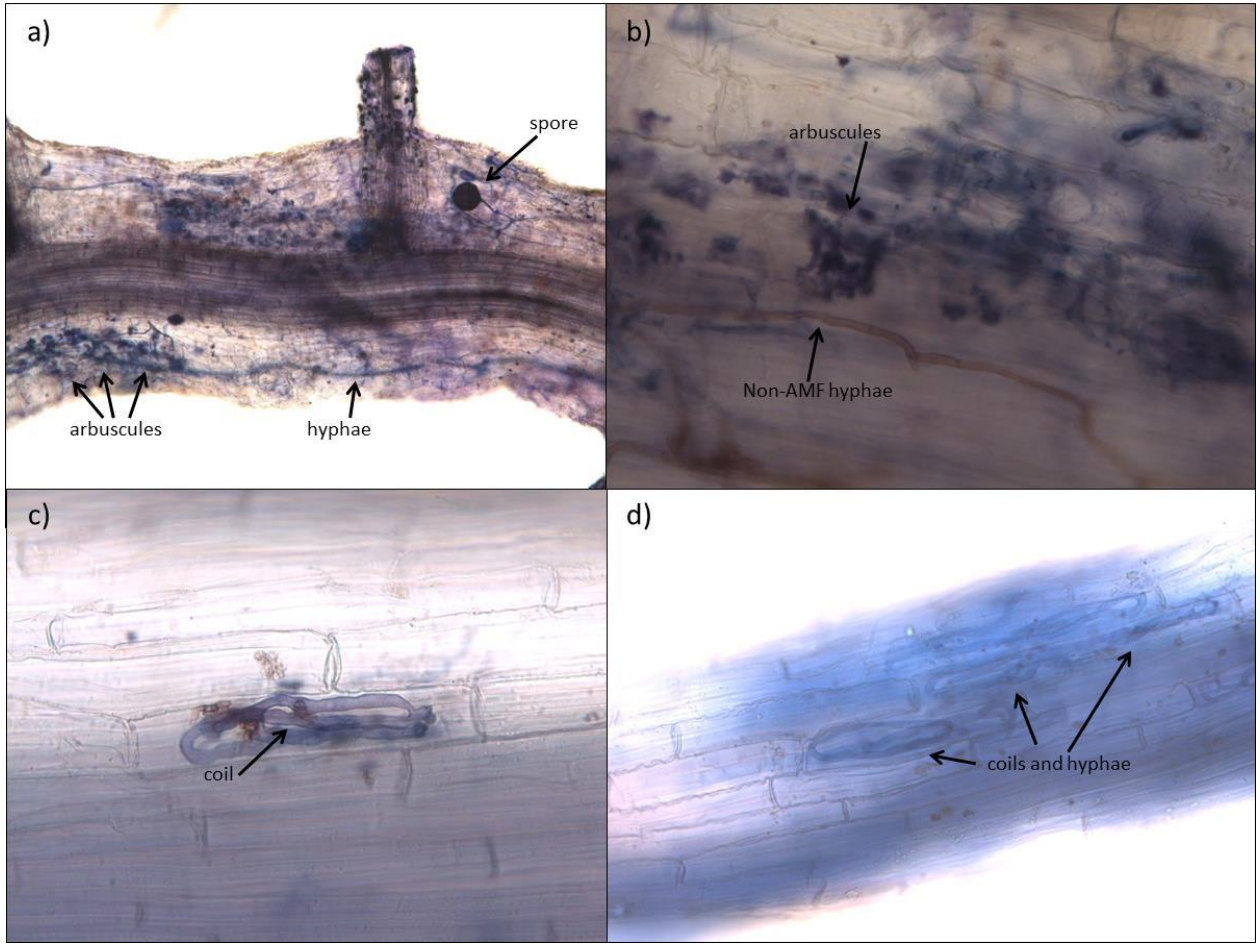

Figure 1. AMF structures present in stained roots, such as intra-radical hyphae, coils, arbuscules, spores, and non-AMF hyphae.

Pictures taken at 100x (a, d) and 400x (b, c).

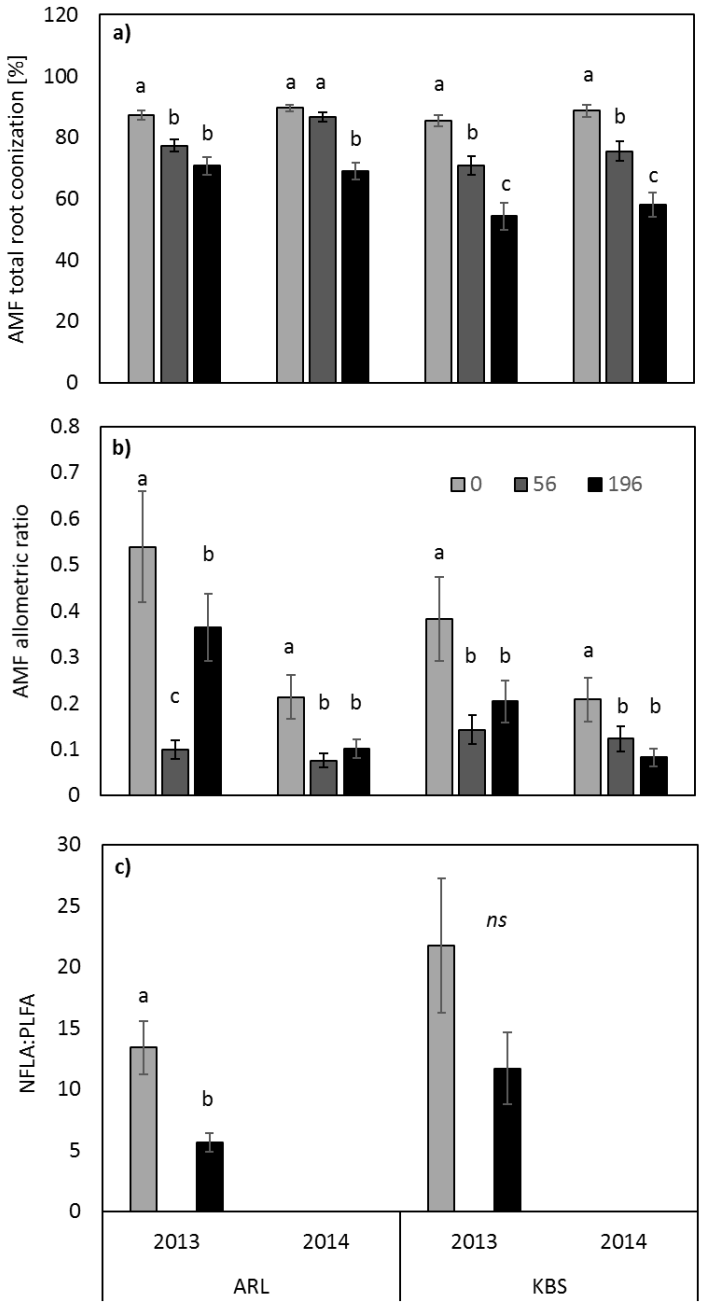

Figure 2. Means for 0, 56, and 196 kg N ha^{-1} addition treatments for a) AMF root colonization [%], b) the AMF allometric ratio and c) the NLFA:PLFA ratio for 16:1ω5 for sites ARL and KBS in 2013 and 2014. Error bars are ±1 S.E. and different letters above the bars represent means are significantly different at $p < 0.05$ across N addition treatments within each year by site combination.

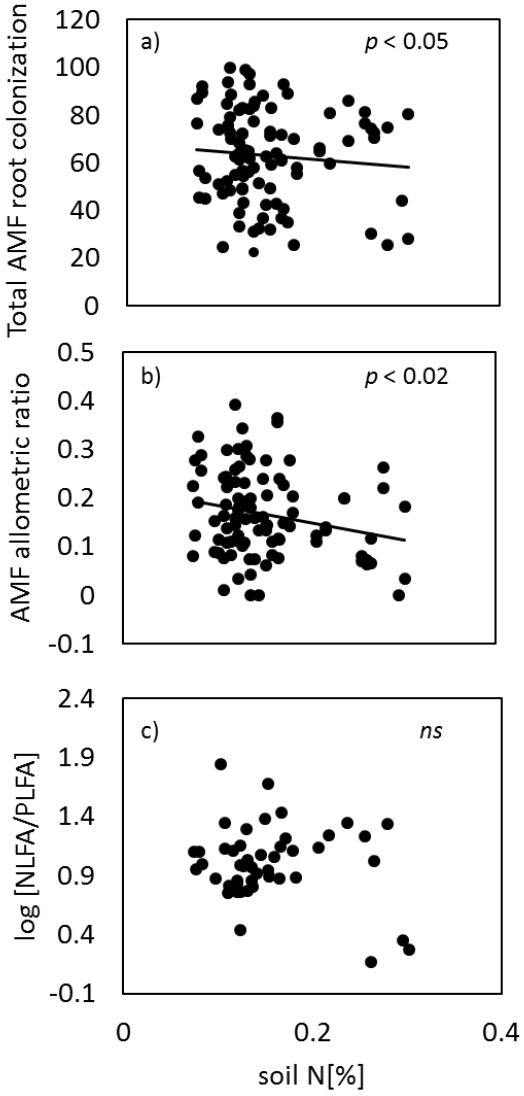

Figure 3. Total soil N [%] across all extensive site plots in 2013 and 2014 correlated with a) Total AMF root colonization [%] (displays untransformed data for ease of interpretation, but note that statistical testing used the logit transformation, see methods section), b) the AMF allometric ratio and c) AMF NLFA:PLFA ratio. Solid lines are significant linear regressions, with the indicated *p*-value.

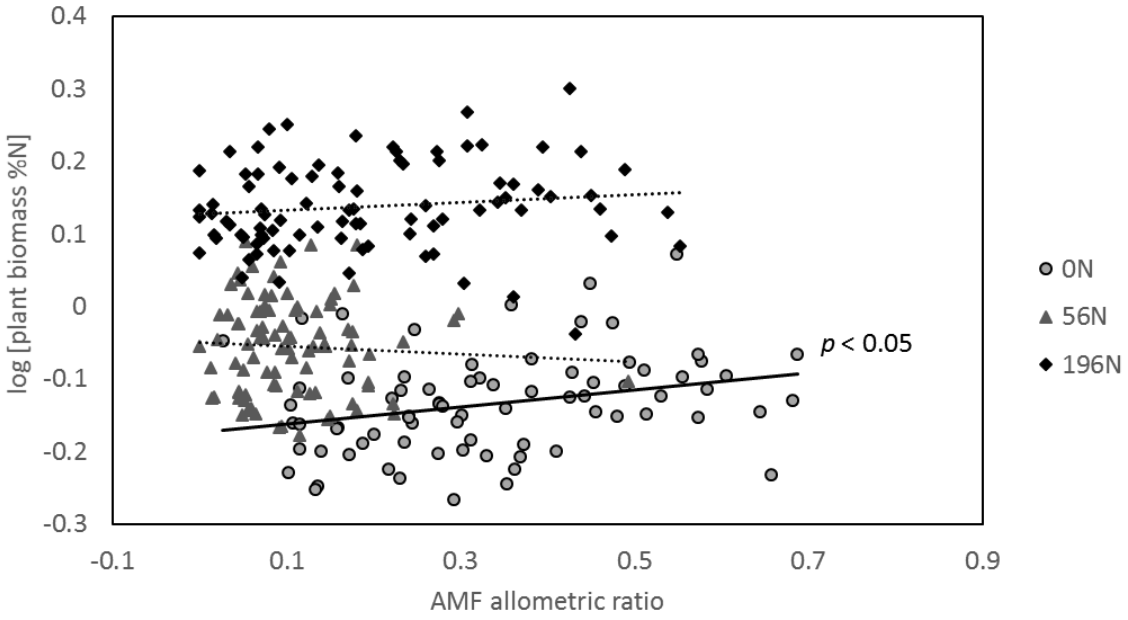

Figure 4. The AMF allometric ratio regressed against plant biomass [N] for all plots at ARL and KBS in 2013 and 2014 for each N addition treatment of 0, 56, or 196 kg N ha^{-1}. Solid line is the significant ($p < 0.05$) linear regression for the 0N treatment.

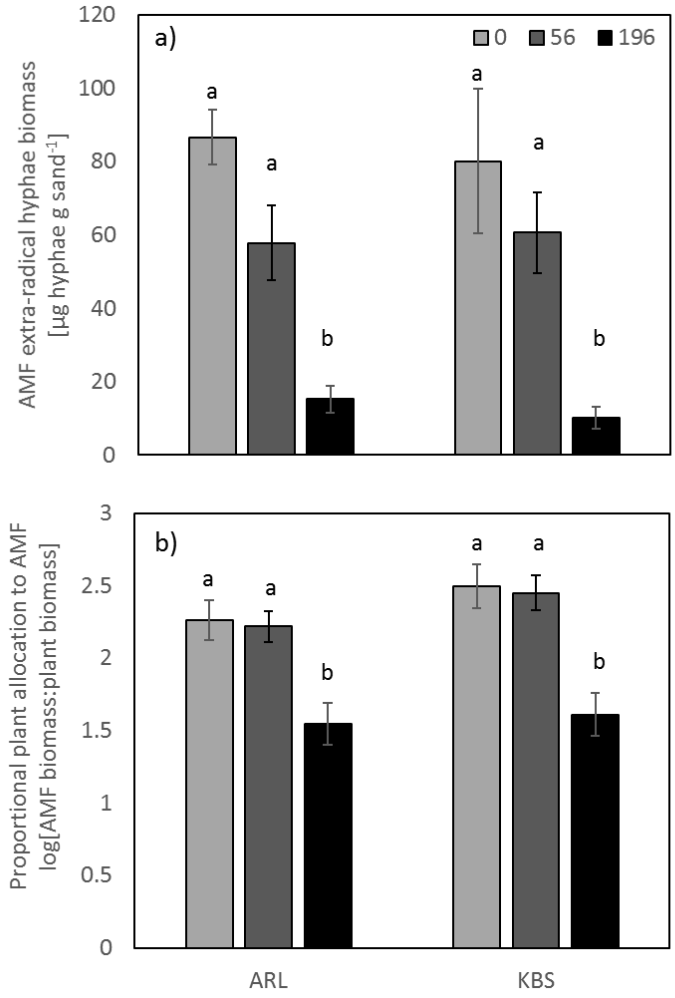

Figure 5. Mean AMF extra-radical hyphae biomass (a) and proportional plant allocation to AMF (b), measured as the ratio of AMF extra-radical hyphal biomass to plant aboveground biomass for both sites ARL and KBS in 2014. Error bars are ±1 S.E. Letters indicate significant differences between the means at $p < 0.05$ across all sites and N addition treatments (0, 56, and 196 kg N ha^{-1}).

Chapter 4: Evidence of arbuscular mycorrhizal fungi (AMF) supplying nitrogen to perennial grasses

Abstract

Arbuscular mycorrhizal fungi (AMF) symbioses 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 acquisition and transfer to plants by AMF is agronomically relevant. We used ^{15}N natural abundance methods and ^{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. Differentiation of $\delta^{15}N$ among plant, soil N, and AMF fungal pools was higher than anticipated, leading to estimates of 34 to 100% of plant N transferred from AMF in the treatments receiving no-N addition to 6 to 44% of plant N transferred to plants in high-N addition treatments. Additionally, AMF extra-radical hyphae biomass was significantly reduced in the high-N treatments, which was correlated with depleted plant $\delta^{15}N$, indicating that AMF function was reduced. Our results suggest that N addition decreases AMF N-transfer to plants. When N is limited, AMF are able to supply N to plants in amounts comparable to recommended N fertilizer rates, highlighting that N fertilizer may be unnecessary in the management of perennial grasses for bioenergy production.

4.1 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 P rather than N nutrition (Smith and Read 2008, Veresoglou et al. 2012). Similar to the balance of trade established for P uptake and transfer, AMF supply and transfer N to host plants in direct response to the 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). There is especially a lack of evidence supporting AMF N-transfer to plants *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 and N transfer to plants will help determine if it is feasible to manage for beneficial mycorrhizal communities in agricultural systems where plants are supplemented with N fertilizer for N nutrition.

Although dependent on soil-type and nutrient limitation, inoculation of field soils with native AMF communities has been successful for increasing plant productivity in grasslands (Köhl et al. 2016). Perennial C4 grasses managed for bioenergy are typically supplemented with N fertilizer, with the intent of increasing and sustaining 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, Jach-Smith and Jackson 2015). Given the negative consequences of excess N in the environment (Robertson and Vitousek 2009, Ruan et al. 2016), finding ways to reduce or eliminate N addition is desirable.

Perennial warm-season grasses commonly form symbiotic relationships with AMF (Treseder and Cross 2006, Miller et al. 2012), and AMF abundance is often reduced under N enrichment (Johnson et al. 2003, Treseder 2004, Staddon et al. 2004). Therefore, applying N fertilizer may inhibit one of the typical plant strategies for obtaining N, resulting in a trade-off effect of crops using fertilizer-N when available, or making use of symbiotic AMF associations to supply the needed N nutrition in circumstances of N limitation. Nutrient limitation (particularly N versus P limitation) has been shown to be a driving factor in determining AMF benefit to plants (Johnson et al. 2003, 2010); therefore, altering soil N availability may have direct consequences on AMF abundance and AMF-mediated N transfer to host plants (Johansen et al. 1994, van Diepen et al. 2010, Schroeder-Moreno et al. 2011).

Natural abundance measurements are a useful method to evaluate plant N sources without disturbing N cycling within the plant-soil system, because they rely on stable isotope fractionation patterns that occur naturally with N transformations in the soil or exchanges between mycorrhizae and plants (Dawson et al. 2002). Studies that employ ^{15}N natural abundance measures of stable isotopes have produced mechanistic confirmation of N cycling processes in plant-mycorrhizal associations (Hobbie et al. 2000, Averill and Finzi 2011). Mycorrhizae transfer ^{15}N -depleted forms of N to plants, because biochemical reactions occur within the fungus to separate N into ^{15}N-enriched or ^{15}N-depleted pools, with the ^{15}N-depleted pool being transferred to plants and the ^{15}N-enriched pool remaining in the fungal biomass (Hobbie and Högberg 2012). This process allows us to use the $^{15}N/^{14}N$ composition of the plant,

fungus, and soil N pools to estimate the proportion of plant N that is transferred or supplied by AMF.

AMF assimilate N as nitrate, ammonium, or amino acids (Hodge et al. 2010), and transport N as arginine to allow a more concentrated and non-toxic form of N to be moved within the hyphae. However, because of the uptake and metabolism of N by mycorrhizae, we are not able to determine the source of N before fractionation occurs (Emmerton et al. 2001). Hobbie and Hobbie (2006) found that mycorrhizae contributed 61 to 86% of aboveground plant N in the arctic tundra. Since tundra plants are primarily ecto-mycorrhizal, grow in extremely N-limited conditions, and ecto-mycorrhizal species are known to have significant SOM-degrading capabilities, it is likely that the range of mycorrhizal-derived plant N determined for their study is higher than AMF contributions to plant N nutrition.

There is currently scant evidence in the literature for $\delta^{15}N$ patterns between AMF fungi and their plant hosts (Ouimette et al. 2012). A study by Craine et al. (2012), showed that foliar $\delta^{15}N$ values in the tallgrass prairie were typically related to soil N availability, with high foliar $\delta^{15}N$ relating to high soil N availability. This trend was observed across ecosystems and soil types (Craine et al. 2009). In an experiment comparing non-mycorrhizal versus AMF-inoculated grey alder, the aboveground plant tissues were 1‰ depleted in $\delta^{15}N$ compared to the non-mycorrhizal plants (Schweiger 2016). Few studies use AMF in isotopic analyses, especially in regard to N cycling and budgeting, so there is currently little reference data on ^{15}N partitioning in AMF and their plant hosts (Courty et al. 2015).

Our objectives were 1) to provide quantitative, mechanistic evidence for the role of AMF in supplying N to perennial grasses and 2) determine whether AMF are capable of supplying agronomically relevant amounts of N to perennial bioenergy grasses. We sampled plant tissue,

AMF fungal mycelium, and the available N pool for $\delta^{15}N$ to look 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 & Hobbie, (2006). We hypothesized that AMF would transfer a higher proportion of plant N under conditions of low soil N or N limitation, with a decreasing proportion of plant N supplied by AMF under conditions of high soil N (see Figure 1 for hypothetical scenarios of AMF N transfer to plants). Since the $\delta^{15}N$ of plants is related to the amount of N supplied by mycorrhizae (^{15}N-depleted, assuming there is significant fractionation upon transfer of N molecules), the presence of high AMF extra-radical hyphal biomass should be related to the $\delta^{15}N$ of the plant biomass, as demonstrated by Hobbie and Colparert (2003). We therefore expected the $\delta^{15}N$ of grass biomass to be negatively correlated with hyphal biomass.

4.2 Methods

Site description and experimental design

We used both a manipulative experiment to modify soil N levels and a survey study to examine $\delta^{15}N$ pools and estimate AMF N transfer to plants. For the manipulative experiment, we chose cultivated sites at agricultural research stations where we modified soil N using three levels of N fertilizer (0, 56, and 196 kg N ha^{-1}). Sites were established plots of switchgrass at the Arlington Agricultural Research Center (ARL) in Arlington, WI (silt loam soils) and the W.K. Kellogg Biological Station (KBS) in Michigan in Hickory Corners, MI (sandy soils). The switchgrass at KBS was established in 2008 and fertilizer rates applied since 2009. The switchgrass at ARL was 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 beginning the same year as this experiment. All plots were in a

randomized complete blocks design, with four blocks at KBS and five blocks at ARL, although only three blocks were used at each site. Since there is extensive evidence of hyphal networks sharing resources belowground between individual plants (Fischer Walter et al. 1996, Leake et al. 2004, Pringle 2009, van der Heijden and Horton 2009), 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. Aluminum sheets were left intact for the remainder of the study.

For the observational experiment, we chose eight restored grasslands (hereafter Extensive sites) across south central Wisconsin that varied in soil type and soil N availability. All sites were unfertilized, with the exception of one agricultural site (Shinners), which was fertilized with ~100 kg N ha^{-1} every year after stand establishment in 2004. Four of the sites were harvested for biomass in late fall or early spring (Becker, Becker Lake, Lund, and Shinners), whereas the other sites are not harvested or burned (Manthey, Rowe, Fairfield, and Rocky Run). Mowing and burning has previously been shown to not affect AMF root colonization while N amendments do have a measurable effect (Eom et al. 1999), so we did not consider biomass harvest as a treatment variable of interest.

Sample collection for $\delta^{15}N$ pools

Aboveground plant biomass sampling. Semi-permanent sampling stations were located at each field site for each year of the study (2013 and 214). At the extensive sites, sampling locations were 1-m^2 quadrats, with six quadrats at permanent sampling locations within each site. Sampling stations were spaced approximately 10 m apart along a transect. Transects were

randomly placed, but some attention was given to place transects and quadrats in locations with significant C4 grass cover, as many of the sites included C3 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. Three quadrats were placed in each plot, making 27 experimental units at KBS and 24 experimental units at ARL.

Aboveground plant biomass was clipped from quadrats at peak-standing biomass, when plants reached their peak growth for the season at anthesis, but before the plants began to senesce and recycle nutrients belowground (typically around mid-August in southern WI, but timing varies from year to year). Biomass samples from the extensive sites were sorted into C4 grass species (switchgrass, indiangrass, or big bluestem), C3 grasses, forbs or "other", and litter. Biomass samples from KBS and ARL were almost exclusively switchgrass, therefore, no species sorting was done and all plant biomass assumed to be switchgrass. Biomass was dried to a constant weight at 65º C before biomass dry weights were recorded. For the extensive sites, only the dominant species (based on aboveground biomass weight) of each plot was selected for analysis. Dried biomass samples were finely ground using a Wiley Mill to pass a 1-mm mesh screen, then pulverized using stainless steel balls in 2-ml micro-centrifuge tubes.

AMF hyphae biomass sampling. 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 the 0-, 56-, and 196-kg ha^{-1} N plots at KBS and ARL at 3 of the 5 quadrats in a treatment plot (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 restrict root growth. The mesh bags were filled with 100 g of ashed sand (#30-70, pure silica sand from Ogleby Norton Industrial Sands), thereby minimizing 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 dumped into a beaker of tap water making a composite sample of three cores per plot. By flotation 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 percentage of saprotrophic versus AMF hyphae using a grid-intersect method. All samples contained >95% AMF hyphae upon visual inspection (based on absence of cell septa in fungal hyphae), therefore all hyphal biomass extracted from cores were considered to be AMF. Numerous samples from the extensive sites were contaminated with what was believed to be bryophyte roots and impossible to separate from the fungal hyphae, therefore these samples were not used for δ^{15}N analysis. 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 δ^{15}N. To account for soil contamination in the hyphae samples, we corrected for the soil δ^{15}N contributing to the proportion of the sample that was soil, similar to Nave *et al.*, 2013. We assumed soil contamination was the silt-clay fraction of soil, given that it had to pass through a 50-µm nylon

mesh. We used the C:N ratio of the silt-clay fractions of the soils from each site. 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 δ^{15}N of buried cellulose filters and the δ^{15}N of plants from the Brassicaceae family, which are known not to form AMF symbioses (Rillig, 2004). The buried cellulose acts as in integrator of plant-available N under field conditions. Since the cellulose is a readily-available C source for microbes, the microbes 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 δ^{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 inserted in the surface 10 cm of the soil making a slit with a soil knife, followed by closing the slit with a soil knife to ensure good soil contact. Filters were buried in June or July and extracted approximately four to six weeks later. Cellulose filters were removed from the nylon mesh bags, ground, and subsampled for δ^{15}N analysis. To account for soil contamination on the filters, a separate subsample was ashed at 475 °C and percent organic versus inorganic material calculated based on loss-on-ignition. Based on percent of sample that was inorganic versus organic, we adjusted the final cellulose δ^{15}N value with site silt-clay fraction soil δ^{15}N.

Plants from the Brassicaceae family were also used as an integrated measure of plant-available N. Since plants of the Brassicaceae family are known to not associate with mycorrhizae

(Rillig 2004), the $\delta^{15}N$ signature of the plants should be a direct measure of the $\delta^{15}N$ from the plant-available N pool, assuming there is no or little fractionation on uptake (Marshall et al. 2007). Plant tissue was sampled from naturally growing *Brassica* sp. in July 2013. While effort was made to take samples nearby the quadrats when possible, sometimes the brassica plants were upwards of 50 m from the quadrats, therefore we calculated a site or treatment mean for brassica $\delta^{15}N$ rather than on an individual plot basis. We report the $\delta^{15}N_{available\ nitrogen}$ from the 2014 cellulose, but we also considered an alternative scenario for the mass balance equations where we calculated $\delta^{15}N_{available\ nitrogen}$ from the 2013 and 2014 cellulose site means, and adjusted this value by the brassica $\delta^{15}N$ difference. The $\delta^{15}N_{available\ nitrogen}$ from the cellulose and the brassica plants should in theory be the same pool of N, but the brassica site mean was on average 1.1‰ lower than the site $\delta^{15}N_{available\ nitrogen}$ measured by the cellulose. Because of this difference, and we only had a site mean for the brassica $\delta^{15}N_{available}$ nitrogen, we applied a -1.1‰ correction to the 2013 and 2014 cellulose $\delta^{15}N$ site and treatment means to obtain an alternative $\delta^{15}N_{available\ nitrogen}$ estimate for the mass balance calculations (scenarios 2 and 3 in Results).

All samples were dried, ground, and analyzed on a PDZ-Europa model 20-20 isotope ratio mass spectrometer with a PDZ-Europa ANCA-GSL elemental analyzer at the University of Wisconsin-Madison. $\delta^{15}N$ abundances were reported as parts per mille (‰), which is $(R_{sample}/R_{standard}-1)1000$, where R is the $^{15}N/^{14}N$ ratio of the sample to the reference standard (atmospheric N_2).

Mass balance equations to estimate AMF N transfer to plants

To estimate plant N supplied by AMF, we used the ^{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 plant tissue ($\delta^{15}N_{plant}$), fungal tissue ($\delta^{15}N_{fungi}$), and the available nitrogen ($\delta^{15}N_{available\ nitrogen}$) pools and 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:

$\delta^{15}N_{transfer}$ = ^{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 explore alternative explanations for our dataset. A literature value of 2‰ was chosen as the discrimination factor by AMF upon N transfer to plants (Δ_f)(Craine et al. 2015) for calculating scenarios 1 and 2. Because the mass balance equations produced solutions that were outside the ranges of biologically meaningful interpretations (i.e., over 100% plant N supplied), we also considered an alternative scenario of a 4‰ for a discrimination factor of AMF N transfer to plants (Δ_f) (scenario 3). While a discrimination factor of 4‰ for AMF transfer to plants is higher than indicated by Craine et al. (2015), discrimination values as high as 11‰ have been reported for ecto-mycorrhizae (Hobbie and Hobbie 2006), so it is not seem unrealistic for AMF.

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 15 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 has been found to 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, as followed by Koerselman and Meuleman (1996), where 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..

Data analysis

We used an analysis of variance test and conducted mean comparisons using the R package *lsmeans* (Lenth 2015), with significance determined at $p \leq 0.05$ for comparing N addition and site 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 used linear mixed effects models to evaluate the relationship between extra-radical hyphae biomass and

aboveground plant $\delta^{15}N$ using the R package *lme4* (Bates et al. 2015). One model was used for the combined ARL and KBS sites across both years, and a separate model was used for the extensive sites across both years, with individual site as the random effect. We used R version 3.2.3 (R Core Team 2015) for all statistical analyses.

4.3 Results

Soil and plant N and P composition

Total soil inorganic N levels were significantly affected by N addition treatment at ARL and KBS, with only the high-N addition treatment (196-N) being significantly greater than the lower N treatments ($p < 0.001$ and $p < 0.01$, respectively for ARL and KBS) (Table 1). Among the Extensive sites, site was a significant predictor of total inorganic N availability ($p < 0.001$). The Shinners site had far greater inorganic N availability compared to the other sites, followed by Becker Lake, Manthey and Becker. The remaining four sites did not differ in their total inorganic N availability. 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). For the extensive sites, Manthey was the only site with significantly higher P levels compared to the other extensive sites ($p < 0.001$).

Plant tissue N:P ratio, an indication of realized 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. The N addition treatment had a significant effect on plant N:P at KBS ($p < 0.001$), but not at ARL ($p = 0.08$). The extensive sites

were generally more N-limited than ARL and KBS and were surprisingly similar. Only the Becker Lake site had a significantly higher N:P ratio compared to the other extensive sites ($p < 0.001$).

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

The mean δ^{15}N of the aboveground plant tissue, $\delta^{15}N_{plant}$, ranged from -3.1 at Rocky Run to 3.2 at Shinners (Table 2). $\delta^{15}N_{plant}$ values spanned a high range even within ARL and KBS with increasingly greater values with the N addition treatments. The high-N addition treatment (196 kg N ha^{-1}) increased $\delta^{15}N_{plant}$ by 1.4‰ and 3.0‰ at ARL and KBS, respectively. The trend of greater δ^{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 6.3‰ with the high N addition treatment. The ammonium-nitrate fertilizer used at ARL and KBS had a δ^{15}N of -0.17‰ (±0.01 S.E.). δ^{15}N pools were generally much lower at the extensive sites, with the exception of Shinners, which was notably the only extensive site also fertilized with N.

The most interesting comparisons for δ^{15}N values were between pools within a site or N treatment, which would give some inference to N uptake and N transfer pathways from the soil to AMF and plants. We expected $\delta^{15}N_{plant}$ to be significantly depleted relative to the other pools, and the $\delta^{15}N_{fungi}$ to be enriched in δ^{15}N relative to the other pools if AMF were transferring N resources to the plant (Figure 1). If the pools were not significantly different from each other, it would indicate that plants were reliant on N uptake directly from the soil, assuming no fractionation with direct root uptake. At ARL and KBS, there were significant differences between pools in all of the N treatments, with significant differences between $\delta^{15}N_{plant}$ and $\delta^{15}N_{available\ nitrogen}$ pools in the 0-N treatments and between the $\delta^{15}N_{plant}$ and $\delta^{15}N_{fungi}$ pools in the

196-N treatments (Table 2). At the Extensive sites, there was a clear distinction between all of the $\delta^{15}N_{plant}$ and $\delta^{15}N_{available\ nitrogen}$ at all of the sites. Unfortunately, root and bryophyte contamination made the $\delta^{15}N_{fungi}$ data unusable except for Shinners, where the $\delta^{15}N_{fungi}$ showed intermediate $\delta^{15}N$ values between the $\delta^{15}N_{plant}$ and $\delta^{15}N_{available\ nitrogen}$ pools.

Mass balance equation solutions

The first set of solutions calculated using scenario 1 (using $\delta^{15}N_{availN}$ data from 2014 cellulose samples only and a Δ_f value of 2‰) produced *T* and *f* values that represented mostly biologically impossible situations (Table 3), because these numbers should fall between 0 and 100%. Solutions from scenario 2 (using $\delta^{15}N_{available\ N}$ data from the mean 2014 and 2013 cellulose samples, which was also adjusted by the *Brassica* sp. $\delta^{15}N$ and a Δ_f value of 2‰), produced more meaningful values for *f,* however some *T* values calculated were still negative. Solutions from scenario 3 (using $\delta^{15}N_{available\ N}$ data from the mean 2014 and 2013 cellulose samples, which was also adjusted by the *Brassica* sp. $\delta^{15}N$ and a Δ_f value of 4‰) produced all *f* values and most *T* values within the constraints of biological possibility (0 to 100%). Scenarios 2 and 3 had solutions that were mostly within biological constraints, and therefore the most likely solutions to estimate AMF N-transfer to plants. Using the results from scenarios 2 and 3, we estimated that the proportion of plant N supplied by AMF (*f*) was in the range of 55 to 100% of plant N in the 0-N addition treatments at ARL, and 34 to 68% plant N at KBS. In contrast were the high-N treatments, where approximately 6 to 12% of plant N was supplied by AMF at ARL, and 22 to 44% at KBS.

Extra-radical hyphae biomass

The plant $\delta^{15}N_{abv\ biomass}$ at ARL and KBS was negatively correlated to the AMF extra-radical hyphal biomass as measured by the in-growth hyphal cores (Figure 2a). There was clear

separation between the highest N treatment (196-N) and the others (0- and 56-N), indicating that the relationship between hyphal biomass $\delta^{15}N_{abv\ biomass}$ may only persist under high N availability conditions. The Extensive sites revealed no significant relationship between extra-radical hyphal biomass and plant $\delta^{15}N_{abv\ biomass}$ (Figure 2b). We attempted to improve the regression fit by standardizing the plant $\delta^{15}N_{abv\ biomass}$ values by site using z-score scaling, but standardizing only marginally improved the relationship (R^2 increased from 0.03 to 0.05).

4.4 Discussion

Given the differences between $\delta^{15}N$ pools and the solutions to mass balance equations (scenarios 2 and 3), our study indicated that AMF were supplying an agronomically significant amount of plant N to plant hosts, meaning AMF were transferring enough N to substitute for fertilizer N amendments. Our results supported the hypothesis that AMF transfer a greater proportion of plant N under low N conditions compared to high N conditions. Trends among $\delta^{15}N$ pools approximated what we expected regarding differences between the $\delta^{15}N_{plant}$ and $\delta^{15}N_{available\ nitrogen}$ pools, but the differences between the $\delta^{15}N_{available\ nitrogen}$ and $\delta^{15}N_{fungi}$ pools were much lower than expected for the 0-N treatments, and much higher than expected for the 196-N treatments. This was reflected in the solutions to the mass balance equations, where the solutions for *f* were mostly within expected ranges of biological constraints, whereas *T* was calculated to have more extreme values and not representative of a plausible scenario. This inconsistency in the mass balance of ^{15}N sheds some doubt of how well our models were representing our observed ^{15}N pools. Hence, we discuss alternative explanations of the $\delta^{15}N$ patterns in our study below to explore competing interpretations of the dataset. However, even if some of the solutions were not particularly precise, our results show consistent qualitative patterns of AMF N-transfer to switchgrass plants.

The estimated proportion of plant N supplied by AMF was much higher than anticipated. Since 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 (Hobbie and Högberg 2012), it has been assumed that AMF may not play a significant role in plant N nutrition, but our results suggest otherwise. 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. Not as clear was why the hyphae were not as enriched as expected at the 0-N treatments at ARL and KBS, and more enriched than expected in the 196-N treatments. Some of the assumptions used in our calculations, as discussed below, may help explain some of these inconsistencies.

Although exclusive to ARL and KBS, AMF hyphae biomass was negatively correlated to $\delta^{15}N_{abv\ biomass}$, a trend also demonstrated by Hobbie & Colpaert (2003) in a study conducted under greenhouse culture conditions. We were able to demonstrate this relationship under field conditions and provide strong support for our hypothesis that increased abundance and functioning of AMF results in depleted plant ^{15}N. The lack of a correlation between extra-radical hyphae biomass and plant $\delta^{15}N_{abv\ biomass}$ at the extensive sites may be in part because the range of sites sampled did not include enough samples with a high $\delta^{15}N_{abv\ biomass}$, as seen in the 196-N treatments at ARL and KBS. This indicates that site conditions may not be N limited enough or N-availability high enough to detect differences in AMF extra-radical hyphae growth. Since our extensive sites did span a range of soil N availability and soil types that are typical of agricultural lands and restored grasslands, the significance of soil N availability and soil N limitation on AMF growth and function may not be as influential as we previously hypothesized. The 196-N addition treatment at ARL and KBS had significantly less hyphae biomass, but this N treatment

represented a rather extreme treatment that likely will not reflect typical N management of perennial grasses for bioenergy or in the management of natural grasslands. Therefore, although we found significant N treatment effects on extra-radical hyphae biomass, the effect may not be apparent under typical N application in managed perennial grasslands.

Assumptions used and how they might affect interpretation of our results

Many assumptions went into the calculations of the mass balance equations, which may have affected our results and interpretation. The fractionation constant used for AMF fractionation upon N transfer to the plant host is one such assumption. While culture studies have conflicting results about whether AMF fractionate upon N transfer, data from field studies have consistently demonstrated a depleted δ^{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 (Govindarajulu et al. 2005). Assuming there is at least some fractionation upon transfer, our models and calculations should have been appropriate. However, our data become difficult to interpret in the scenario where no fractionation occurs upon N transfer to plants. In fact, scenario 3 that considered a higher fractionation value of 4‰ produced solutions to the mass balance equations that were more plausible and fell within biological constraints. 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‰.

The model of Hobbie and Hobbie (2006) assumed minimal fractionation on uptake from plant roots, which assumed low substrate concentrations in the soil, or that N uptake closely matches N supply. This is often the case, as N demand by plants is typically greater than N supply in most natural systems (Marshall et al. 2007). There is no fractionation of N compounds

from root or hyphae uptake from the soil, as confirmed by numerous field and laboratory studies, as long as the system is N-limited (Hobbie and Hobbie 2006, Hobbie and Högberg 2012). If our systems were not N-limited to prevent N fractionation on root uptake, plant $\delta^{15}N$ would be not be enriched irrespective of AMF N-transfer, assuming plant roots preferentially take up the lighter ^{14}N isotope and the ^{15}N isotope accumulates in the soil.

Other factors that may influence the $\delta^{15}N$ results and interpretation

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

We must also consider how symbiotic N-fixation might have affected $\delta^{15}N$ signatures. Switchgrass associative N fixation (ANF) in the rhizosphere and roots was estimated to be as much as ~92% of unfertilized switchgrass N demand (Roley *et al., unpublished data*). If ANF were responsible for a significant amount of plant N in our systems, plant $\delta^{15}N$ would be depleted relative to the $\delta^{15}N_{available\ N}$ pools because fixed atmospheric N has a $\delta^{15}N$ near 0‰.

However, only the nodules where fixation takes place typically have depleted $\delta^{15}N$, so overall plant $\delta^{15}N$ is generally not affected by N-fixation (Craine et al. 2009, 2015). Roley et al. found that the majority of N was fixed in the soil (not directly in the plant) post-senescence, perhaps when the microbial community had a boost of carbon exudates from the senescing switchgrass plants. AMF hyphae are known to help retain N pools and prevent N leaching (De Vries and Bardgett 2012), so the increased extra-radical hyphae we found in the low-N treatments may play a primary role in immobilizing and retaining N fixed from ANF.

Our measured $\delta^{15}N_{fungi}$ pools were lower than expected in the unfertilized treatments and higher than expected in the high-N addition treatments. The similarity of the $\delta^{15}N_{plant}$ and $\delta^{15}N_{availablleN}$ in the 196-N treatments suggested that there was little fungal N transfer occurring here, but it begs the question: Why was the fungal tissue so enriched? Given the high N availability in the 196-N treatment, the AMF may have been luxury-consuming N, in particular, the more easily accessible mineralized N pools from the fertilizer. Although fertilizer N has a low $\delta^{15}N$, excess fertilizer N has likely been subjected to N transformations that causes N losses, such as nitrification and denitrification, which would leave the remaining soil N pool more enriched in $\delta^{15}N$.

Our results may have been influenced by our use of fungal mycelium rather than reproductive structures for measuring our $\delta^{15}N_{fungi}$ pools. Zeller *et al.* (2007) found that their mycelium sample (n=1) had a $\delta^{15}N$ much lower than the sporophore stipes and gills of an ectomycorrhizal fungus. Others have used spores and fungal fruiting bodies for measuring $\delta^{15}N$ of fungal tissue but there may be some isotopic fractionation during the formation of a fruiting body (Hobbie et al. 2012, Courty et al. 2015), so studies that have used sporocarps or spores for isotopic analysis rather than fungal mycelium may see more enrichment. Some have used plant

roots as a proxy for AMF tissue (Stackpoole et al. 2008), but then samples are diluted and influenced by the plant root δ^{15}N. AMF biomass makes up a relatively small portion of mass compared to root tissue (Ouimette et al. 2012), so using root tissue as a proxy for AMF tissue may not be very reflective of AMF δ^{15}N values, especially when looking for 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 δ^{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 ^{15}N partitioning within the AMF fungus to determine if using spores or mycelium is appropriate for measuring ^{15}N patterns to interpret N cycling and transfer to plant hosts.

4.5 Conclusions

Our study is one of the first to estimate plant N derived from AMF transfer *in situ.* Our results suggested that AMF supply agronomically significant amounts of plant N, and supply a higher proportion of plant N when N is limiting. Natural abundance of ^{15}N is 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 qualitative evidence that N addition as fertilizer decreases 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 fungal diversity and community composition affect AMF functioning, and subsequent benefits to bioenergy grasses. The reduction of AMF N-transfer under high N addition indicated that AMF symbioses may be a primary reason why perennial grasses requires very little, if any, fertilizer management when AMF symbioses are functioning.

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4.7 Tables and Figures

Table 1. Mean soil inorganic N and available P 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 $p < 0.05$.

		soil		plant
Site	N addition (kg N ha^{-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}
Rocky Run	0	6.0 (0.3)^{c}	0.47 (0.09)^{b}	3.5 (0.2)^{b}
Fairfield	0	6.3 (0.4)^{c}	0.71 (0.03)^{b}	5.2 (0.3)^{b}
Lund	0	6.7 (0.3)^{c}	0.58 (0.1)^{b}	4.3 (0.3)^{b}
Rowe	0	6.8 (0.3)^{c}	0.5 (0.09)^{b}	4.6 (0.2)^{b}
Becker	0	7.9 (0.3)^{bc}	1.12 (0.07)^{b}	4.4 (0.3)^{b}
Manthey	0	8.5 (0.5)^{bc}	2.23 (0.24)^{a}	4.0 (0.3)^{b}
Becker Lake	0	11.0 (1.1)^{b}	0.88 (0.04)^{b}	7.4 (0.6)^{a}
Shinners	100	15.6 (2.1)^{a}	0.87 (0.16)^{b}	3.9 (0.2)^{b}

Table 2. Mean $\delta^{15}N$ of the aboveground plant, available soil N pool, and AMF hyphae. Data are means with 1 S.E. in parenthesis. Letters within a row indicate mean differences between $\delta^{15}N$ pools at $p < 0.05$.

Site	N addition (kg N ha^{-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}
Rocky Run	0	-3.1 (0.1)^{a}	-0.6 (0.2)^{b}	-
Fairfield	0	0.8 (0.2)^{a}	3.6 (0.8)^{b}	-
Rowe	0	-1.8 (1.0)^{a}	1.1 (0.3)^{b}	-
Becker	0	-1.9 (0.2)^{a}	0.5 (0.3)^{b}	-
Becker Lake	0	-2.4 (0.5)^{a}	1.2 (0.6)^{b}	-
Lund	0	-0.5 (0.3)^{a}	2.0 (0.6)^{b}	-
Manthey	0	-1.6 (0.2)^{a}	0.0 (0.5)^{b}	-
Shinners	100	3.2 (1.3)^{a}	6.7 (0.4)^{b}	5.4 (0.2)^{ab}

Table 3. Solutions to mass balance equations (1-3), including the different scenarios for determining $\delta^{15}N_{availN}$ for Arlington (ARL) and Kellogg Biological Research Station (KBS) sites.

Scenarios	Site	N addition (kg N ha^{-1})	$\delta^{15}N_{availN}$ ‰	Δ (‰)	$\delta^{15}N_{transfer}$ ‰	*T (%)*	*f (%)*
Scenario 1	ARL	0	4.1	2	2.1	536 (535)	169 (29)
		56	3.0	2	1.0	0 (145)	127 (32)
		196	3.8	2	1.8	54 (32)	83 (24)
	KBS	0	3.6	2	1.6	347 (476)	129 (32)
		56	4.1	2	2.1	-198 (173)	112 (17)
		196	6.5	2	4.5	26 (22)	124 (36)
Scenario 2	ARL	0	3.0	2	1.0	-228 (131)	111 (27)
		56	1.9	2	-0.1	-7 (16)	72 (11)
		196	2.4	2	0.4	48 (4)	12 (12)
	KBS	0	2.4	2	0.4	-31 (19)	68 (7)
		56	3.0	2	1.0	10 (10)	57 (5)
		196	4.9	2	2.9	67 (1)	44 (13)
Scenario 3	ARL	0	3.0	4	-1.0	-32 (16)	55 (13)
		56	1.9	4	-2.1	0 (6)	36 (5)
		196	2.4	4	-1.6	33 (4)	6.0 (6)
	KBS	0	2.4	4	-1.6	-9 (8)	34 (3)
		56	3.0	4	-1.0	8 (6)	29 (2)
		196	4.9	4	0.9	50 (1)	22 (7)

Notes: Scenario 1 uses data collected in 2014 using cellulose filters only for $\delta^{15}N_{availN}$. Scenario 2 and 3 use the 2013 and 2014 mean cellulose filter $\delta^{15}N$ in addition to an adjustment data using the *Brassica sp.* $\delta^{15}N$ data collected in 2013. Scenarios 1 and 2 assume an AMF fractionation value Δ of 2‰. Scenario 3 uses a AMF fractionation value Δ of 4‰, to explore a hypothetical scenario, 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%).

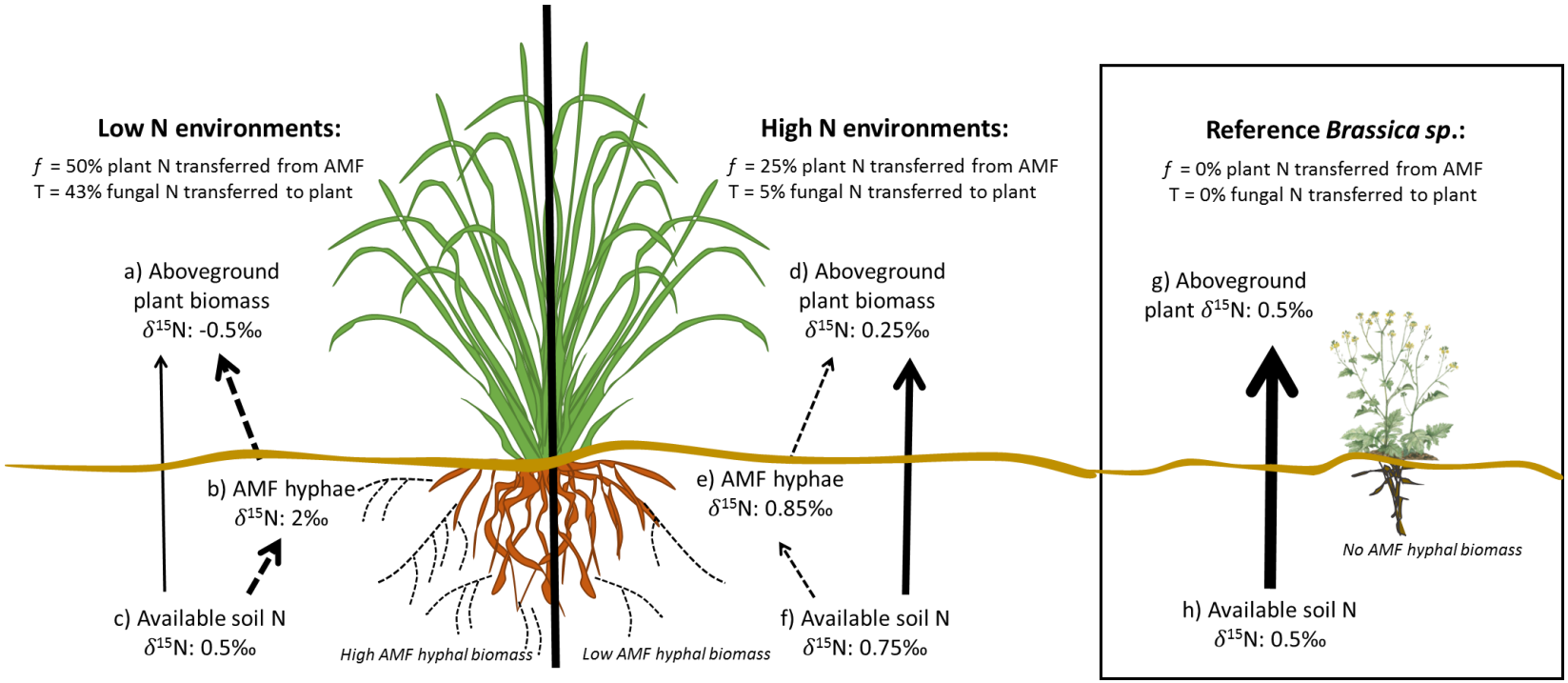

Figure 1. Diagram depicting hypothetical scenarios of how plants obtain N, with arrows illustrating the path of N uptake and resulting δ^{15}N pools. In the hypothetical **low N environment** (unfertilized or low native soil N), plants receive 50% of N via mycorrhizal transfer. This results in (**a**) depleted δ^{15}N value in the aboveground plant biomass relative to (**b**) the enriched δ^{15}N value in mycorrhizal hyphae. This is the result of AMF hyphae taking up (**c**) available soil N pool where the fractionation on transfer to the plant results in the heavier ^{15}N isotope remain in the hyphae, 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 (**d**) moderately depleted δ^{15}N value in aboveground plant biomass relative to (**e**) a slightly enriched δ^{15}N value in mycorrhizal hyphae relative to the (**f**) available soil N pool. For reference, *Brassica sp.*, which form no associations with AMF, receive no N via AMF transfer, therefore the (**g**) aboveground plant N of *Brassica sp.* reflects the δ^{15}N value of the (**h**) available soil N δ^{15}N pool, as we are assuming there is negligible fractionation of δ^{15}N with direct plant root uptake. The thickness of arrows is representative of the amount of N supplied via the mycorrhizal transfer (*dashed arrows*) or plant N uptake via roots (*solid arrows*).

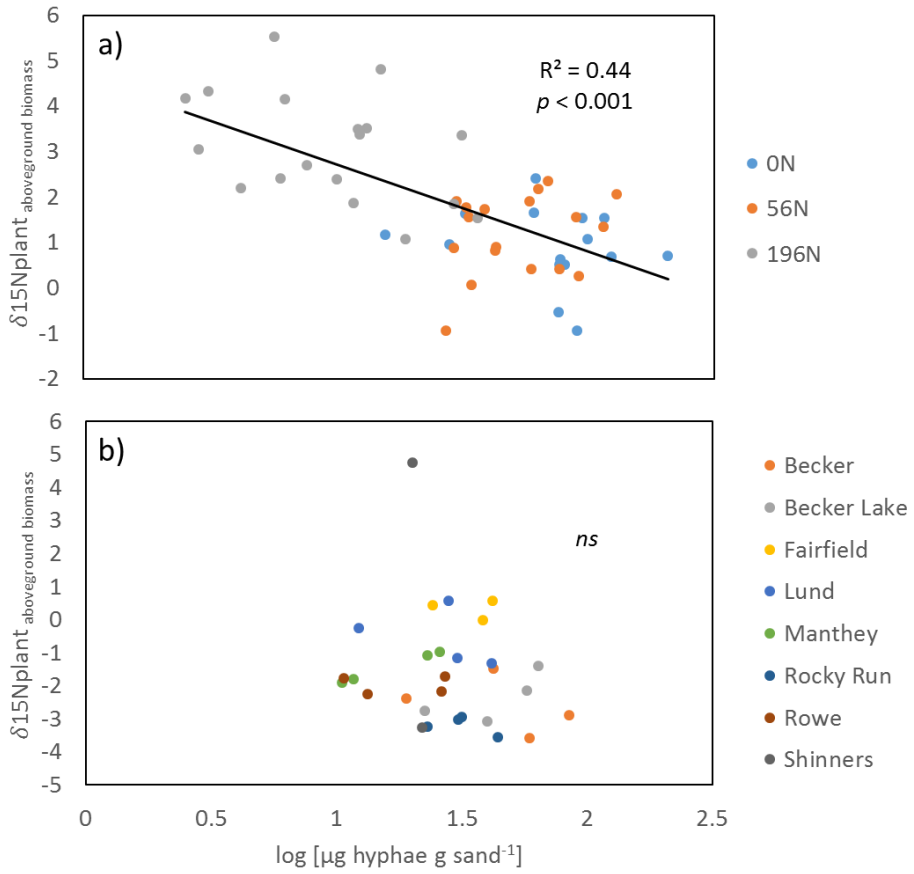

Figure 2. AMF extra-radical hyphae biomass (log [µg hyphae g sand $^{-1}$]) is regressed with δ^{15}N ‰ of aboveground plant biomass at a) ARL and KBS and b) extensive sites. Each data point represents one plot, with colors in the legend indicating N fertilizer treatment (ARL and KBS) or site (extensive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

Concluding remarks

In the planning stages of this study, perennial native bioenergy crops were anticipated to be a significant source of cellulosic biofuel feedstock for renewable energy. Although the outlook of native perennial grasses as a significant source of renewable fuel has become somewhat less probable (U.S. Department of Energy 2016), this cropping system still warrants consideration for improving N retention in agroecosystems as a crop managed for bioenergy production (Porter et al. 2015), or as a type of cover crop intended to help catch and retain N on the landscape (Zhou et al. 2014). Understanding more about how to improve the N conserving strategies of these crops and how to manage them for increased N retention is an important step in helping agriculture contribute to N mitigation rather than N pollution. Further, understanding N addition effects on plant N conservation strategies and arbuscular mycorrhizal fungi (AMF) can be extrapolated to other systems as well. Reduced nutrient inputs are a primary goal for new perennial food crops, such as intermediate wheat, and more information about N conservation strategies to increase N retention in the plant-soil system is in demand (Crews et al. 2016). Almost all food crops associate to some degree with AMF and tailoring management to increase AMF associations in crops will lead to increased sustainability in agroecosystems (Fester and Sawers 2011). However, our work focuses on N conservation strategies in perennial grasses, which has demonstrated important findings that relate to the management of perennial C4 grasses for bioenergy production, and has also demonstrated some findings that are of greater interest to plant-soil ecologists.

The variable and idiosyncratic yield responses to N fertilizer in perennial grasses were one of the primary motivators to our research regarding N conservation. Why are the grasses unresponsive to N addition, and in what circumstances? There are likely multiple reasons that

contribute to the variable N responses in perennial grasses: N2 fixation (Roley et al. n.d.), high NUE (Jakubowski 2013), luxury consumption of N (Jach-Smith and Jackson 2015), N losses (Ruan et al. 2016, Duran et al. 2016). Our hypotheses that plant N resorption, NUE, and AMF symbioses play important roles in plant N conservation, and consequently, cause plants to lack the demand for external N resources has proved to be at least part of the story of why perennial grasses can be unresponsive to N addition.

In chapter 2, we demonstrated that there is great variation in N conservation strategies among grass species, ecotypes, and site. It was somewhat unexpected that N addition at the cultivated sites did not have a greater impact on N conservation strategies. This suggests that N addition does not compromise N resorption and use efficiency. While we were able to demonstrate that soil nutrient status does have some effect on N conservation strategies, it may be that other factors, such as species, ecotype and climate can be more important in determining the final N economy of perennial grasses. This realization could be interpreted that N fertilizer addition is not as detrimental to N conservation as hypothesized, but it also suggests that these perennial grasses may cycle N dictated by genetic and other environmental factors *regardless* of N availability, so then why fertilize with N? Again, the use of policy and incentives will likely be necessary to ensure that N fertilizer is not used as an insurance policy for growing perennial grass crops.

Chapters 3 and 4 provide evidence that AMF do benefit perennial grass N nutrition, and likely at agronomically significant amounts, indicating that a functioning AMF community can replace the need for N fertilizer. In addition, we found that AMF function is compromised by N addition, which is likely another reason why perennial grass yields are so variable under N addition. This work is not only important information for managing perennial grasses for

bioenergy, but it also provides much-needed *in situ* evidence that AMF can and do play a significant role in N uptake and transfer to plants. This is an important finding for understanding more about how AMF function with plants in natural ecosystems (van der Heijden and Horton 2009), agroecosystems (van der Heijden and Wagg 2012, Rillig et al. 2016), and the basic science of understanding plant rhizosphere processes and microbial communities (Frank and Groffman 2009). Our work in Chapter 4 is also important for the application of using ^{15}N natural abundance techniques in ecology. So far, the use of these techniques in AMF-dominated systems is unprecedented, so this work provides a promising outlook for using ^{15}N natural abundance techniques and some reference values for working in grasslands and agroecosystems where AMF are the dominant mycorrhizal community.

If bioenergy crops expand onto the landscape, producers will make decisions about how to manage these new cropping systems, which will have environmental consequences. Given the current efforts to increase cellulosic bioenergy crops on the landscape, and considering the perceived need to fertilize biomass crops, there will likely be unnecessary applications of fertilizer in the coming decades, which could exacerbate N pollution. The sustainability of bioenergy crops and subsequent mitigation of N pollution hinges on growing crops that reduce our reliance on fertilizer inputs. These studies contributes to the mounting evidence that N conservation strategies and mycorrhizal associations play important roles in agroecosystems, and tailoring management recommendations to minimize N fertilizer additions will prevent the inherent N conserving mechanisms from being compromised. The perceived risk of not fertilizing may be difficult to overcome, but this study provides evidence that factors such as plant N conservation and AMF symbioses support productive perennial grass crops and may help to curtail fertilizer use.

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