SWITCHGRASS CULTIVAR AND INTRASPECIFIC DIVERSITY IMPACTS ON NITROGEN USE EFFICIENCY

by

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DEDICATION

I dedicate this thesis to my sweet boy Titan. I wish you could have been here buddy.

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ABSTRACT

Bioenergy feedstock production is an important component of the national renewable energy strategy, which is based on biomass supply. Biofuels for ethanol production may be produced in high-input crop production systems, but the efficacy of these systems for increasing net energy yields over its full life-cycle compared to traditional fuels is under debate, because it is now evident that the benefits of feedstock production are maximized only when biofuels are derived from feedstocks produced with much lower life-cycle greenhouse-gas emissions than traditional fossil fuels. To this end, the reduction of agricultural inputs is key to developing an effective biofuel feedstock crop. Native prairie grasses have low-input production requirements, and upon land conversion for biofuel production they have positive impacts on belowground carbon (C) sequestration, a measure of soil quality. Specifically, *Panicum virgatum* (hereafter switchgrass), a perennial C4 grass native to the mid-west of the United States, is a promising bioenergy crop. It has large root systems, which allow it to produce large amounts of biomass with less water and nutrient requirements than traditional bioenergy crops, such as corn.

To produce switchgrass feedstock in an environmentally sustainably manner (i.e., with the least amount of fertilizer inputs), we will need to adopt agricultural practices that promote N cycling efficiency in the system. Previous studies have found that different cultivars of switchgrass vary significantly in specific root length (SRL), and greater SRL may be linked to greater N acquisition owing to the root systems' greater surface area. In

addition, it has been found that growing switchgrass in genotypically diverse mixtures enhanced biomass production, which may result from belowground niche differentiation and complementarity effects that enhance N acquisition. With this study, I aimed to evaluate (1) whether differences in the architecture among root systems of switchgrass cultivars led to differences in the efficiency of nitrogen uptake, and (2) whether growing switchgrass cultivars in diverse mixtures would enhance the efficiency of nitrogen cycling though niche differentiation and complementarity effects.

Our experiment was conducted at the Sustainable Bioenergy Crop Research Facility at the Fermilab National Environmental Research Park, where experimental field plots consisted of seven switchgrass cultivars, planted either in monoculture or in diverse mixtures of 2, 4, or 6 randomly selected cultivars. To evaluate differences in nitrogen use efficiency (NUE) among cultivars in monocultures and among diversity treatments, I applied a stable isotope ^{15}N tracer at the beginning of the growing season. Following senescence, the switchgrass was harvested and the percent of ^{15}N recovered was measured in the aboveground biomass to determine NUE. I found that switchgrass cultivars differed in NUE and these differences could potentially be linked to germplasm origin in relation to the field site. I also found that NUE was not influenced by increases in cultivar diversity. Our results suggest that NUE is not the sole mechanism behind greater biomass production associated with enhanced diversity.

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LIST OF ABBREVIATIONS

GHG	Greenhouse Gas
MBC	Microbial Biomass Carbon
MBN	Microbial Biomass Nitrogen
N	Nitrogen
NUE	Nitrogen-Use Efficiency
C	Carbon
SRL	Specific Root Length

INTRODUCTION

Anthropogenic burning of fossil fuels has increased greenhouse gas (GHG) concentrations of carbon dioxide (CO_2) that contribute to climate change (IPCC 2007). These changes have inspired the need for greater energy independence and mandates for renewable fuel production in the United States by the Energy Independence and Security Act (EISA 2007). In order for a biofuel to be effective and reduce atmospheric CO_2 concentrations, the biofuel needs to be carbon (C)-negative, meaning that it takes up more CO_2 from the atmosphere than it emits. In addition, bioenergy production should not compete with food production (Tilman et al. 2006), as increasing population growth in combination with a changing climate requires food production to nearly double by 2050 (Godfray et al. 2010). For example, conventional corn-derived ethanol requires fertile land that could be used to grow food. Instead, land with undesirable edaphic conditions for food production could be managed to produce large amounts of biomass for energy use, but this requires agricultural intervention, and careful assessments of net energy gains.

Native high-yielding grasses have potential as a biofuel feedstock crop requiring less agricultural intervention (i.e. irrigation, fertilizers, and pesticide inputs) than traditional food crops such as corn and soybeans for ethanol and biodiesel production, respectively (McLaughlin and Adams Kszos 2005, Parrish and Fike 2005, Tilman et al. 2009). *Panicum virgatum* (switchgrass) is a fast-growing, warm-season perennial C_4 prairie grass that is native to the Midwestern United States but has a wide range spanning

from southern Canada to northern Mexico and east of the Rocky Mountains (McLaughlin and Adams Kszos 2005, Wullschleger et al. 2010). Perennial crops establish extensive root systems that may enhance soil organic C stocks and C accumulation rates, which improve soil quality and help mitigate increasing atmospheric CO_2 concentrations (McLaughlin and Adams Kszos 2005, Glover et al. 2010, Adkins et al. 2016), reduce the loss of water and nitrogen (N) from the soil (Scherer-Lorenzen et al. 2003), and improve nutrient cycling (Asbjornsen et al. 2013). The risk and severity of diseases caused by wind and rain-dispersed pathogens are also less for perennial plants compared to annual cropping systems (Knops et al. 1999). In summary, switchgrass is a viable candidate for biofuel production due to its low fertilizer, pesticide, and water input requirements, fast growth, and ability to grow on soils that are unsuitable for agriculture (McLaughlin and Adams Kszos 2005, Parrish and Fike 2005, Tilman et al. 2006). Conservation Reserve Program lands that currently support nonnative brome grass to reduce erosion have been proposed as a potential site for native switchgrass production (McLaughlin and Adams Kszos 2005). However, maintaining high yield of switchgrass feedstock on these marginal lands, while minimizing exogenous nitrogen inputs, is crucial for the efficacy of this bioenergy production system.

Obtaining high yields in conventional farming systems traditionally requires substantial fertilizer inputs, in particular nitrogen (N). Inorganic nitrogen (N) fertilizer can have negative environmental consequences if lost through leaching or N_2O emission, a GHG that is ~300 times more potent than CO_2 (Di and Cameron 2002, Cameron et al. 2013). Up to 6% of the earth's warming can be attributed to N_2O release from agricultural systems, and it adds to the depletion of the ozone layer (IPCC 2007).

Nitrogen can also be leached from fields into water systems, leading to eutrophication of aquatic ecosystems (Russell and Connell 2009). Eutrophication promotes algal blooms as a result of excessive nutrients and can have catastrophic consequences for aquatic life (Smith and Schindler 2009, Russell and Connell 2009, Cameron et al. 2013). Thus, to reduce the environmental impact of biofuel feedstock production, it is crucial to maximize yields while minimizing N inputs. Adopting agricultural practices that increase nitrogen use efficiency (NUE; dry mass productivity/ unit N available in the soil) in cropping systems aids in reducing the amount of N fertilizer inputs required to promote crop growth (Hirose 2011).

NUE can differ among cultivars in a single crop species. This has been observed for rice (Fageria and Baligar 2003), barley (Sinebo et al. 2004), potatoes (Ospina et al. 2014), and winter wheat (Le Gouis et al. 2000), and it has been postulated that these differences could be attributed to differences in root traits among cultivars (Le Gouis et al. 2000). Particularly, specific root length (SRL; cm root/ g root) is a trait that may affect NUE, since roots with a greater surface area should aid in the acquisition of N, thereby improving N use efficiency (Craine et al. 2002, Xu et al. 2012). Previous studies have found that SRL differs by threefold among a variety of switchgrass cultivars (de Graaff et al. 2013, 2014). In addition, these studies found that cultivars with a greater SRL had a greater relative abundance of first and second order roots compared to cultivars with a lower SRL (de Graaff et al. 2013). This result is important for NUE, because 1st and 2nd order roots are ephemeral roots that form associations with arbuscular mycorrhizal fungi and that regulate water and nutrient uptake (Eissenstat 1992, Ostonen and Lõhmus 2007). In contrast, 3rd and higher order roots function predominantly as structural roots

(Eissenstat 1992). In addition, a root system with a greater SRL may promote C input through exudation (Adkins et al. 2016), and microbial activity (Yin et al. 2014) which may improve internal N cycling. Thus, switchgrass cultivars with a greater SRL may promote NUE and lead to environmentally more sustainable bioenergy production systems.

NUE may also be improved in cropping systems by increasing plant species or cultivar diversity. Several studies have shown that increasing interspecific (i.e., between species) (Zak et al. 2003, Tilman et al. 2006, DeHaan et al. 2010), or intraspecific (i.e. within species, or genotypic) biodiversity can promote biomass yields (Ehrmann and Ritz 2014), which may result from diversity-induced increases in nutrient availability and nutrient uptake (Hooper and Vitousek 1998, Atwater and Callaway 2015 (Altieri 1999, Scherer-Lorenzen et al. 2003, Tilman et al. 2006, Loranger-Merciris et al. 2006, Dybzinski et al. 2008, Zilverberg et al. 2014)). In fact, those diversity-induced increases in yield in low-input, high-diversity grasslands, have reduced greenhouse gases 6-16 times more than traditional corn-derived ethanol and soybean biodiesel, in large part owing to a reduction in inputs such as fertilizers that are energetically costly to produce (Tilman et al. 2006). The positive impacts of species diversity can be in-part attributed to niche partitioning, allowing access to alternative nutrient pools in either time or space that otherwise may not be exploited in monoculture (Hooper and Vitousek 1998, Zak et al. 2003, Hooper et al. 2005, Dybzinski et al. 2008). In addition, diversity can influence nutrient cycling belowground by improving overall soil fertility through greater nutrient inputs from soil fauna and microbial diversity (Hooper and Vitousek 1998, Zak et al. 2003), increasing nitrogen mineralization rates (Dybzinski et al. 2008, Glover et al.

2010), and enhancing nutrient uptake by plants (Hooper and Vitousek 1998, Zak et al. 2003, Dybzinski et al. 2008). Recently, Morris et al. (2015) showed that enhancing switchgrass cultivar diversity increased yield, and given the variation in rooting structures among cultivars, this result may be attributed to greater NUE in switchgrass polycultures.

With this study, I aim to assess how (1) differences in SRL among switchgrass cultivars impact efficiency of N use, and (2) increasing intraspecific diversity of switchgrass impacts NUE in agricultural ecosystems. I hypothesized (1) that cultivars associated with finer root architectures would have greater NUE than coarse root cultivars when grown in monoculture. I also hypothesized that positive impacts of intraspecific diversity on yield could be due to increased NUE resulting from diverse root architectures (Dybzinski et al. 2008). To test my hypotheses, I set up a common garden experiment in Batavia, Illinois consisting of field plots of seven switchgrass cultivars, planted either in monoculture or in mixtures along a diversity gradient of 2, 4, or 6 randomly selected cultivars. To evaluate differences in NUE among cultivars in monocultures and among diversity treatments, a stable isotope ^{15}N tracer was applied at the beginning of the growing season. Following senescence, the switchgrass was harvested and the percent of ^{15}N recovered was measured in the aboveground biomass to determine NUE.

MATERIALS AND METHODS

Experimental Design and Sample Collection

Our experimental field site is located at the Sustainable Bioenergy Crop Research Facility at the Fermilab National Environmental Research Park in Batavia, IL (N 41.8414, W 88.2297). The soil is characterized as Grays silt loam (fine-silty, mixed, superactive, mesic Oxyaquick Hapludalf). Prior to the establishment of field plots, the field site was dominated for 36 years by cold season non-native C_3 grasses (primarily *Bromus inermis, Poa species*). In 2007, this vegetation was removed using the herbicides 2, 4-Dichlorophenoxyacetic in the spring, and glyphosphate in the fall. Any remaining vegetation was removed by burning in the spring of 2008. In spring of 2008, Switchgrass (*Panicum virgatum*), a warm-season native perennial C_4 grass, was seeded by hand in 20 cm rows to ~0.5 cm depth. Experimental plots (2X3 m) were planted with monocultures and polycultures of switchgrass cultivars Blackwell, Cave in Rock, Dacotah, Forestburg, Kanlow, Southlow, and Sunburst. These cultivars cover the large geographic area in which switchgrass is naturally found (Table 1). Plots consisted of each of the cultivars planted in monocultures (n=4, except Cave in Rock n=3), or diversity levels of 2 cultivars, 4 cultivars, or 6 cultivars that were randomly chosen (monoculture n=7, 2 cultivars n=11, 4 cultivars n=12, 6 cultivars n=12).

In spring 2013, we established 1 m^2 plots centered within the main 2X3 m experimental plots and applied 99 atom% $K^{15}NO_3$ by hand on the surface of the soil at the beginning of the growing season to determine NUE. In fall of 2013, the switchgrass from

the labeled 1 m^2 plot, within 2X3 m plot, was collected by clipping 0.5 m^2 circular quadrats within the plots down to 15 cm. The switchgrass biomass, as well as any weed biomass that was present in the plots, was dried at 65°C and weighed, ground in a Wiley mill using the 2 mm attachment, and pulverized to a fine powder using a ball mill. I analyzed C and N concentrations and ^{15}N using a Thermo Delta V Plus Isotope Ratio Mass Spectrometer (IRMS) coupled with a Costech Elemental Analyzer (Costech Analytical Technologies, Inc.) in continuous flow mode.

Following harvest, 8 soil cores (2 cm diameter to a depth of 15 cm) were taken from each plot directly next to the crown or in the interspace in a stratified random sampling design. The cores were then combined, sieved (4.5 mm), and stored at -20°C until further processing. The homogenized soil core samples were further homogenized through 2 mm sieve and then air-dried. The soil was then handpicked and fine roots (>2 mm in size) were removed from the sample. A subsample was pulverized using a ball mill and analyzed for C, N, and ^{15}N using a Thermo Delta V Plus isotope ratio mass spectrometer (IRMS) coupled with a Costech Elemental Analyzer in continuous flow mode.

Root ^{15}N

To assess differences in root ^{15}N among treatments, the roots were handpicked from the soil, rinsed with deionized water, freeze-dried for 24 hours using a FreezeZone Lyophilizer (Labconco), and weighed. A subsample of the dried roots was pulverized using a ball mill and analyzed for C, N, and ^{15}N using a Thermo Delta V Plus isotope ratio mass spectrometer (IRMS) coupled with a Costech Elemental Analyzer in continuous flow mode. In addition, the lyophilized samples were analyzed at Kansas

State University for genotype to verify that the genotypes in mixtures were represented in root samples.

Potential Denitrification

Soil samples were sieved (4.5 mm) to remove rocks and litter. A 250 mL mason jar was filled with 20 g of soil and soil moisture was maintained at field capacity with deionized (DI) water. Field capacity was determined by completely saturating soil in a funnel and allowing the water to gravimetrically drain for 48 hours at 6°C to minimize evaporation. The soil moisture was determined after drying soils at 100°C. The jars were flushed with argon gas to remove oxygen through a two-way septae on the lid. Then, 28 mL of argon was removed from each jar and replaced with 24 mL of acetylene gas to block the production of dinitrogen gas (N_2) that was synthesized in the lab using calcium carbide and DI water. Following a 24-hour incubation, 5 mL of gas was removed from each jar using a syringe after 1, 2, 4, and 6 hours. The samples were stored in an over-pressurized exetainer filled with argon gas and sealed with vacuum grease to prevent leaking. The samples were sent the Sustainable Agroecosystems Group (ETH, Zürich, Switzerland) where they were analyzed for N_2O using an Electron Capture Detector (ECD), separated with a 2 m x 1/8" Hayesep D packed column, after the dilution using a 5-mL glass syringe (CTC Analytics AG, Switzerland) on a Scion GC-456 (Bruker Daltronics GmbH, Germany) with a Combi-PAL autosampler (CTC Analytics AG, Switzerland).

Potential Nitrogen and ^{15}N-Nitrogen Mineralization

Subsamples (20 g) were used to assess potential N mineralization through a nitrate and ammonium extraction followed by a colorimetric assay. I added sieved (2

mm) subsamples of soil to a polypropylene specimen cup, and added water to achieve a soil moisture content of 60% water holding capacity (WHC). The samples were covered in plastic wrap, incubated for 7 days, and then extracted of N or immediately extracted of N by suspending the soil in a 2 M potassium chloride (KCl) solution and shaking on a reciprocal shaker for 45 minutes. The solution was filtered using Whatman #1 filter paper, and filtrates were kept at -20°C until further processing. To quantify potential N mineralization, I conducted colorimetric assays that utilized the manual vanadium (III) reduction and a Berthelot reaction of indophenol blue to determine the relative amount of NO_3^- and NH_4^+, respectively (Forster 1995, Miranda et al. 2001, Doane and Horwath 2003). The absorbance of each sample was read on a spectrophotometer (Shimadzu-UVmini1240) at 650 nm (NH_4^+) and 450 nm (NO_3^-) wavelengths. N mineralization was assessed by subtracting NO_3^- and NH_4^+ contents at day-0 from NO_3^- and NH_4^+ contents at day-7.

To quantify ^{15}N in the mineral N pool, I used the ^{15}N diffusion method using the KCl extractions from the N mineralization procedure. I added 100 mL to a specimen cup containing a 6 mm diameter filter disk suspended on a stainless steel wire. The filter disks were pre-treated with 10μL 2.5M $KHSO_4$ and dried at 70°C. Magnesium oxide was added to each sample solution in order to promote the volatization of NH_4^+ that would be captured on the filter disk. The specimen cup was sealed and incubated for 6 days in the dark after which the disks were dried and stored in a desiccator. To capture NO_3^-, Devarda's alloy and 30% Brij-27 were added to the sample solution. This solution underwent another 6-day incubation prior to drying the filter disks at 70°C for 24 hours. Samples were analyzed at the UC Davis Stable Isotope Facility for ^{15}N using a PDZ

Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

Microbial Biomass C and N

I used the chloroform fumigation-extraction (Brookes et al. 1985, Beck et al. 1997) to assess microbial biomass N and C. Two subsamples of soil (10 g each) were sieved (2 mm), after which one of the subsamples was immediately extracted of N using K_2SO_4. Another 10 g of soil was weighed into 50 mL beakers, water was added to achieve 60% WHC, and soils were placed in a vacuum desiccator containing a small beaker with ethanol-free chloroform and boiling chips. Air was removed from the desiccator until the chloroform boiled, after which the soils remained in the desiccator for another 72 hours. Following fumigation, the soluble N in the necromass was extracted using K_2SO_4. The soil slurry was shaken for an hour and then filtered through pre-leached filter paper. The N extract was frozen at -20°C until processed. Microbial biomass N (MBN) was assessed using a colorimetric assay to determine the concentration of NH_4^+ and NO_3, utilizing a manual vanadium (III) reduction and Berthelot reaction of indophenol blue. The difference between the N in the fumigated and non-fumigated samples is proportional to the MBN. Microbial biomass carbon was measured using a Total Organic Carbon Analyzer (TOC-L 4200, Shimadzu Corporation, Japan**)** and calculated using,

$$C = EC / kEC$$

where C is microbial biomass C, EC is chloroform-labile C pool kEC= 0.045 (Brookes et al. 1985)**.**

Nitrogen Use Efficiency

I calculated the % ^{15}N recovered in the aboveground switchgrass biomass to determine NUE using the formula below, where P is the total N in the plant, f is the rate of ^{15}N applied, and a, b, and c are the atom% ^{15}N in the labeled fertilizer, soil, and plant, respectively (Hauck and Bremner 1976). For % ^{15}N recovered, *P* is the total N in the aboveground biomass, *f* is the amount of ^{15}N applied to each plot, and a, b, and c are % ^{15}N concentration in the KNO_3 label, soil and aboveground biomass, respectively.

$$\%\ ^{15}N\ recovery = \frac{100P\ (c-b)}{f\ (a-b)}$$

Statistical Analysis

I compared cultivar means using a one-way ANOVA and upon significance I conducted a Tukey post-hoc test using the car package (Fox and Weisberg 2011) in R (R Core Team 2015). If the assumptions for normality or homogeneity of variances were not met, I performed a Kruskal-Wallis rank sum nonparametric test in R. To analyze the impact of genotypic diversity on various N pools, I conducted a regression analysis in R (R Core Team 2015). The mean values for the monocultures were consolidated such that n=7 for monocultures and n=12 for each subsequent diversity level. A soil sample was missing from the 2-cultivar diversity treatment and was therefore not included in any of the analysis. Likewise, a sample from the Cave in Rock monoculture was fertilized twice with 99 atom% ^{15}N tracer and was also not included in the analysis. I compared cultivars with finer or coarser SRLs with a Welch's t- test in R (R Core Team 2015).

RESULTS

Cultivar and Intraspecific Diversity Effects on Plant Biomass and Tissue N Concentration

Aboveground biomass in the 1 m^2 monoculture subplots was significantly different among cultivars ($p= 0.002$). Southlow produced the highest mean biomass 548.35 g m^{-2} ± 47.99 (SE) and Dacotah had ~77% lower biomass of 102.41 g m^{-2} ± 12.21 (SE) (Fig. 1a). Weed biomass significantly differed among cultivars, where Dacotah had the highest and Cave in Rock had the lowest weed biomass ($p=0.0178$, Fig. 7).

Dacotah had a 58% higher concentration of shoot N than Forestburg ($p=0.005$, Fig. 2a). In contrast, the N content in Dacotah was 80% lower than that in Southlow, and in general lower than in most cultivars ($p=0.003$, Fig. 1b). The concentration of N in roots also differed significantly among cultivars with Dacotah and Kanlow having a higher concentration of N in the root tissues than Blackwell and Cave in Rock ($p= 0.003$, Fig. 2b).

Greater intraspecific diversity did not affect aboveground biomass production in our 1 m^2 subplots (Fig. 5a). There were also no differences in shoot or root N concentrations at higher diversity levels when compared to monocultures (Fig. 4b and c). Furthermore, the total amount of N did not change with mixture of cultivars (Fig. 4a).

Cultivar and Intraspecific Diversity Effects on Soil C and N Pools and Fluxes

There were no differences in total C and N in the bulk soil among monocultures of cultivars (Table 2). The type of cultivar also did not appear to have an effect on soil

potential N mineralization (NO_3^- and NH_4^+) (Table 3), or microbial biomass C and N (Table 3). I found further that cultivar type did not significantly affect potential denitrification (Table 3). While there was no statistical difference among cultivars, Dacotah did have almost double the potential denitrification than the mean potential N_2O for monocultures (Table 3).

Soil C and N were not significantly impacted by increased diversity (Table 5). Similarly, diversity did not significantly impact potential mineral N, N denitrification, microbial biomass N, or microbial biomass C in the soil (Table 5).

Cultivar and Intraspecific Diversity Effects on ^{15}N Pools and Fluxes

Monocultures did not significantly differ in bulk soil ^{15}N concentration (Table 4). The potential mineral ^{15}N was not different among cultivars (Fig. 1c). There were also no differences in ^{15}N concentrations in either shoots or roots among different cultivars (Table 4). However, the amount of ^{15}N in the shoots was significantly different among cultivars ($p= 0.003$, Fig. 1a). Blackwell, Cave in Rock, and Southlow had on average 55% greater total ^{15}N content than Dacotah and Kanlow (Fig.1b).

Cultivar diversity did not affect bulk soil ^{15}N or potential mineral ^{15}N (Table 5), nor did it impact the ^{15}N concentration in roots (Fig. 4f) or the ^{15}N content in aboveground biomass (Fig. 4d). However, the concentration of ^{15}N in the aboveground biomass decreased significantly with increasing diversity ($p= 0.033$, Fig. 4e).

Overall the mean ^{15}N% recovered, a metric for NUE, in the aboveground biomass of the plants was low, ranging from 1.56% ± 0.63 (SE) to 5.35%. ± 0.79 (SE) across cultivars. Individual cultivars differed significantly in the ^{15}N% recovered ($p= 0.004$, Fig. 3), where ^{15}N% recovered from Blackwell, Cave in Rock, and Southlow was significantly

higher than that from Dacotah and Kanlow, with Blackwell being 2.8 times more efficient than Dacotah (Fig. 3). The $\%^{15}N$ recovered was not significantly different between fine and coarse rooted cultivars (Fig. 8). However, diversity level had no significant impact on $^{15}N\%$ recovered (Fig. 5b). Finally, I found that $^{15}N\%$ recovered significantly decreased in switchgrass biomass with greater weed biomass (Fig. 6).

DISCUSSION

Negative environmental impacts stemming from biofuel production can be mitigated by minimizing nitrogen (N) inputs. However, to promote biomass production in the absence of external N inputs, alternative management options, such as the use of germplasms that most efficiently utilize available N, and crop diversification to improve N use efficiency, need to be considered. With this study, I elucidated: (1) whether switchgrass cultivars that vary in SRL differ in their efficiency of N use (i.e., NUE [dry mass productivity/ unit N available in the soil), and (2) whether genetic diversification of switchgrass stands promoted NUE. The experiment returned two main results (1) individual cultivars differentially utilize N, and (2) increasing intraspecific diversity does not influence NUE, and therefore NUE is likely not the mechanism behind increases in yield from greater intraspecific diversity.

Individual Cultivar Impacts on N Cycling

It is well established that different cultivars of crops can significantly differ in anatomy and physiology, with consequences for their functioning in agro ecosystems (Le Gouis et al. 2000, Casler et al. 2007, Gesch and Johnson 2010, de Graaff et al. 2013). My results indicate that different switchgrass cultivars have divergent impacts on NUE. In previous studies, I observed a threefold difference in SRL among cultivars (de Graaff et al. 2013, 2014), and I hypothesized that the differences in NUE among cultivars may be driven by these differences in SRL. Namely, a greater SRL, which is associated with a more fibrous root system and thus a greater root surface area, should increase N

acquisition (Guo et al. 2008, Yin et al. 2014). However, although I found that N cycling differed among cultivars, these differences appeared unrelated to differences in SRL (Fig. 8). Instead, percent recovery of ^{15}N in shoot biomass, which I used to quantify NUE, was related to biomass production, where NUE increased in cultivars that achieved higher yields. Thus, in this system, it appears that factors other than SRL regulated differences in N cycling among cultivars.

Our experiment was designed as a common garden, thus differences in origin among cultivars, which may have influenced their performance in our common garden, may have impacted their efficiency of N use differently. I found that Cave in Rock, Southlow, and Blackwell had the highest NUE. In contrast, Dacotah and Kanlow had the lowest NUE. Cave in Rock is a local cultivar, which may have contributed to its high NUE, while Southlow and Blackwell are from southeastern Michigan and northern Oklahoma, respectively. Although Southlow and Blackwell are not local, their place of origin may be in close enough proximity to our field site that these cultivars were able to adapt to the local abiotic factors, which may have aided in their success in the common garden. In contrast, Dacotah, a drought and cold-adapted cultivar from northern North Dakota (Table 1), performed particularly poorly in terms of aboveground biomass and NUE (Fig. 5a and b). Dacotah had originated furthest, crossing several hardiness zones, from the field site, which may have contributed to such low yield and reduced weed resistance as the biomass of weeds matched that of switchgrass (Table 1). Indeed, it has been posited that cultivars can maintain optimal yield as long as they are planted within one hardiness zone of their origin (Casler et al. 2007)

Differential performance of Switchgrass cultivars may also be related to their functional type. Switchgrass has two functional types, either lowland or upland, where lowland cultivars are more adapted to lower latitudes and upland cultivars are better adapted to higher latitudes (Casler et al. 2007). Kanlow, the only lowland cultivar, had a significantly lower NUE and considerably larger variation in yield among replicates than upland cultivars (excluding Dacotah). Lowland cultivars may be more prone to winter kill at northern latitudes, which could explain the variation of yield produced among replicates in Kanlow (Casler et al. 2007). In addition, its N translocation strategy at time of senescence may be different from the upland cultivars, possibly leading to the lower percent recovery of ^{15}N in its aboveground tissues. Yang et al. (2009) found lower N in the aboveground biomass of lowland relative to upland ecotypes after senescence despite similar N content at maturity. This effect was particularly pronounced for Kanlow, which appeared to have a high remobilization efficiency (Yang et al. 2009). My result, showing that Kanlow had a significantly greater root N concentration than upland cultivars at harvest supports this observation. Since N is inevitably removed during harvest every year (Reynolds et al. 2000), selecting cultivars with high remobilization efficiencies may reduce N requirements over the long term. A reciprocal transplant design where all cultivars are measured by their performance in their local versus non-local area may shed further light on the mechanisms that led to differences in NUE among cultivars.

Polyculture Effects on N Cycling

I found that NUE was not affected by intraspecific diversity. These results contradict findings of enhanced species diversity promoting greater N cycling (Loreau and Hector 2001, Cardinale et al. 2007, Fornara and Tilman 2008, Roscher et al. 2008,

Cook-Patton and McArt 2011, Kleinebecker et al. 2014). Positive impacts of increases in plant species diversity on ecosystem processes can often be attributed to niche differentiation or complementarity effects stemming from the increasing number of functional types in plant species-rich ecosystems (Craine et al. 2002, Gross et al. 2007, Eisenhauer 2012, Franco et al. 2015, Zuo et al. 2016). I may have not found an effect of increases in intraspecific diversity on NUE because the functional differences among our cultivars are not sufficiently distinct to lead to niche differentiation or complementarity effects. This assertion is supported by the findings of Kahmen et al. (2006), who found that absolute N acquisition is likely due to species identity and functional group rather than the effects of biodiversity. Similarly, Yang et al. (2015) found that accessions of *Pseudoroegneria spicata* from different ecotypes produced up to 30% more biomass than plantings from the same ecotype. It may be that diversity effects would increase with ecotypic diversity rather than randomly selected cultivars.

Similarly to NUE, yield was also not affected by enhanced cultivar diversity in our 1 m^2 subplots. However, Morris et al. (2015) did find differences in yield among treatments in the 2X3 meter experimental plots. This suggests that any niche differentiation or complementarity effects that could have impacted NUE in the larger plot was not detectable for us in the subplots. The lack of a response in both yield and NUE to increasing diversity may be related to the cultivars that were present in our subplots. Genotyping of cultivar roots in the soil samples suggested that not all of the cultivars in polycultures were present in our experimental subplots. First, this may be caused by a detection issue culminating from our sampling protocol. Eight soil cores (2 cm in diameter) were collected from each plot to avoid destructively harvesting our

experimental site. While we ascertained that we collected samples uniformly to increase our chance of collecting soil with roots from a number of cultivars that mix belowground, we may have inadvertently collected roots from fewer cultivars than were planted in the plots. Second, we subsampled the collected roots for genotyping analysis, which may have reduced the number of detectible cultivars. Third, it is possible that as diversity increased, some cultivars outcompeted others and therefore the likelihood of picking these up would be low.

It is also possible that NUE is not the mechanism behind overyielding (i.e., increased biomass production in plant mixtures when compared to monoculture) from intraspecific diversity. Atwater and Callaway (2015) found that increasing genotypic diversity of *Pseudoroegneria spicata* plantings led to overyielding, but they found no differences in resource depletion. Additionally, *Aridopsis thaliana* accession mixtures produced greater aboveground biomass than monocultures, but these differences could not be attributed to morphological traits above- or belowground (Bukowski and Petermann 2014). This further suggests that improved resource complementarity in space from root access to different nutrient sources may not be the driving factor for overyielding. However, I cannot discount that differences in NUE among cultivars may have canceled each other out. In our experiment, overyielding may have enhanced NUE for one dominant cultivar and suppressed NUE for other cultivars in mixture, which would dilute overall treatment effect on NUE (Roscher et al. 2008).

Although I did find differences in ^{15}N recovery among cultivars and did not find differences in NUE with increasing diversity, I must address that our overall recovery of ^{15}N applied was low. I recovered ~1-6%, but other studies found ~30-40% in winter

wheat and ~40% recovery in perennial ryegrass (Kumar and Goh 2002, Ruisi et al. 2015). The main cause of this low recovery may be attributed to a wet spring. Within weeks of applying ^{15}N to the plots in the spring of 2013, there were some heavy precipitation events. The excessive rain likely caused leaching and runoff of the applied N before it was incorporated into the soil, leading to ^{15}N loss from our field plots. A lower recovery than expected could also be attributed to interspecific competition with weeds as weed biomass significantly correlated with decreasing nitrogen use efficiency, as weed biomass matched switchgrass biomass in the case of Dacotah, which also had the lowest NUE (Fig. 6). Weed biomass could impact switchgrass biomass production and therefore the ability to accumulate N in biomass tissues (Ruisi et al. 2015). Finally, I focused on ^{15}N recovery in aboveground biomass, but as switchgrass senesces, nutrients are translocated from the shoots of the plant to the roots, rhizomes, and stems to be utilized for the following season. The scope and design for this experiment did not allow for us to determine root biomass among cultivars. Furthermore, rhizomes were not included in the analysis for N and ^{15}N concentration and consequently the measurements are likely underestimated. Dohleman et al. (2012) found significant increases in rhizome N content and concentration from April to December, thus the dearth of ^{15}N recovery in our experiment could potentially be accounted for in these belowground tissues.

Conclusion

Our study demonstrates the importance of research involving ideal switchgrass cultivars for growing regions, as one cultivar is not going to be suitable for all. The use of intraspecific diversity did not negatively impact yield or NUE and therefore can be considered as an agricultural strategy to promote high yields in switchgrass biofuel

feedstock while reducing agricultural inputs. Aboveground variation also promotes community resilience by increasing resistance against pathogens and disease, pest infestation, or minimizing yield loss from erratic weather perturbations (Knops et al. 1999, Cox et al. 2005, Dybzinski et al. 2008, Tilman et al. 2009, DeHaan et al. 2010, Glover et al. 2010, Robertson et al. 2012). As a result, increasing biodiversity has the potential to reduce fertilizer, herbicide, and pesticide inputs, thus reducing contributions to GHG concentrations. Future research studies should explore these services both biologically and economically. Likely both ideal genotypic mixtures and environmental interactions are needed to minimize agriculture intervention and maximize switchgrass yield. Further research should include strategic combinations of rooting systems (i.e., coarse and fine) and ecopool variety (i.e., lowland and upland) to determine if NUE is improved. Other mechanisms that contribute to diversity-dependent overyielding apart from NUE should also be explored, as the mechanism that leads to overyielding may be found above- rather than belowground.

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TABLES

Table 1. Switchgrass cultivar information on germplasm origin, total annual precipitation (TAP), specific root length (SRL), ecotype and hardiness zone.

Cultivar	Origin*	TAP (mm)†	SRL‡	Ecotype§	Hardiness zone ‖
Blackwell	Northern Oklahoma	584	coarser	upland	6b
Cave in Rock	Southern Illinois	920	coarser	upland	6b
Dacotah	Northern North Dakotah	380	finer	upland	3b
Forestburg	Eastern South Dakotah	584	finer	upland	4b
Kanlow	Central Oklahoma	686	coarser	lowland	7a
Southlow	Southern Michigan	889	finer	upland	6a
Sunburst	Southeastern South Dakota	584	finer	upland	5a

* (Morris et al. 2015)

† NOAA

‡ (de Graaff et al. 2013)

§ (Zegada-Lizarazu et al. 2012, Morris et al. 2015)

‖ (Agricultural Research Service 2012)

Table 2. The means ± standard error (SE) of bulk soil N and C among switchgrass cultivars. There were no significant differences in soil N or C among cultivars ($p > 0.05$, n=4, Cave in Rock n=3).

Cultivar	Soil N (mg/m^2)	Soil C (mg/m^2)	Soil C/N
Blackwell	2.36 ± 0.06	25.53 ± 1.18	10.81 ± 0.26
Cave in Rock	2.26 ± 0.05	24.25 ± 0.68	10.73 ± 0.13
Dacotah	2.28 ± 0.06	24.61 ± 0.29	10.79 ± 0.19
Forestburg	2.24 ± 0.04	24.59 ± 0.89	10.97 ± 0.37
Kanlow	2.29 ± 0.05	24.48 ± 0.57	10.68 ± 0.07
Southlow	2.27 ± 0.05	24.34 ± 0.41	10.72 ± 0.26
Sunburst	2.33 ± 0.12	28.84 ± 1.26	10.67 ± 0.22
F statistic	0.42	0.30	0.20
P value	0.86	0.92	0.97

Table 3. The amounts of mineral N (N min), microbial biomass N (MBN), microbial biomass C (MBC) and potential denitrification (N_2O) among switchgrass monocultures. The values are shown as means ± SE ($p > 0.05$, n=4, Cave in Rock n=3).

Cultivar	N min	MBN	MBC	N_2O
	µg N /g soil	µg N /g soil	µg C /g soil	µg N /g soil
Blackwell	13.58 ± 4.22	33.05 ± 9.04	385.40 ± 34.58	4.52 ± 0.38
Cave in Rock	3.99 ± 7.60	38.22 ± 12.49	382.48 ± 54.36	5.32 ± 4.22
Dacotah	11.32 ± 1.47	25.88 ± 2.97	386.89 ± 83.73	12.51 ± 3.23
Forestburg	15.61 ± 4.00	17.34 ± 2.98	338.61 ± 39.74	5.99 ± 0.90
Kanlow	11.76 ± 3.52	38.30 ± 13.76	343.39 ± 13.30	5.40 ± 1.72
Southlow	13.01 ± 2.55	18.25 ± 4.16	363.98 ± 34.48	5.99 ± 2.14
Sunburst	10.00 ± 7.11	31.30 ± 10.37	309.32 ± 69.95	5.51 ± 2.33
F or Kruskal X^2	2.90	7.09	0.41	4.66
P value	0.82	0.31	0.86	0.59

Table 4. Relative amounts of ^{15}N in the mineral (^{15}N min) and bulk soil pools and biomass concentrations of ^{15}N of switchgrass cultivars. Values are shown as means ± SE (n=4, Cave in Rock n=3).

Cultivar	^{15}N min µg N /g soil	Bulk Soil [^{15}N] µg N /g soil	Shoot [^{15}N] µg C /g shoot	Root [^{15}N] µg N /g root
Blackwell	0.12 ± 0.12	0.41 ± 0.03	10.98 ± 1.84	9.32 ± 1.36
Cave in Rock	0.06 ± 0.07	0.41 ± 0.05	11.91 ± 1.19	8.59 ± 1.03
Dacotah	0.08 ± 0.03	0.41 ± 0.08	15.02 ± 3.20	18.61 ± 4.05
Forestburg	0.21 ± 0.21	0.44 ± 0.03	9.67 ± 0.85	10.38 ± 1.14
Kanlow	0.15 ± 0.16	0.39 ± 0.08	6.05 ± 1.46	14.36 ± 4.02
Southlow	0.23 ± 0.10	0.46 ± 0.06	8.96 ± 1.18	9.20 ± 1.88
Sunburst	0.09 ± 0.10	0.30 ± 0.03	11.02 ± 2.68	10.82 ± 1.87
F or Kruskal X^2	1.85	0.89	2.04	9.69
P value	0.93	0.52	0.11	0.14

Table 5. Regression results of mineral N and ^{15}N, microbial biomass C and N and potential denitrification (N_2O) with increasing intraspecific diversity. Data shows the regression analysis results (n= 63).

	Units	Slope	Standard Error	F	P
Soil ^{15}N	μg ^{15}N/ g soil	-0.01	0.04	0.93	0.34
Soil N	mg N/g soil	0.00	0.06	0.42	0.86
Soil C	mg N/g soil	0.04	0.84	0.30	0.93
Soil C/N	-	-0.00	0.10	0.00	0.95
Mineral ^{15}N	μg ^{15}N/ g soil	0.45	0.05	0.59	0.45
Mineral N	μg N/ g soil	1.03	2.66	2.42	0.13
Microbial Biomass N	μg N/ g soil	-1.04	3.30	1.60	0.21
Microbial Biomass C	μg N/ g soil	1.24	37.85	0.02	0.90
N_2O	μg N_2O/ g soil	0.79	23.01	0.02	0.89

FIGURES

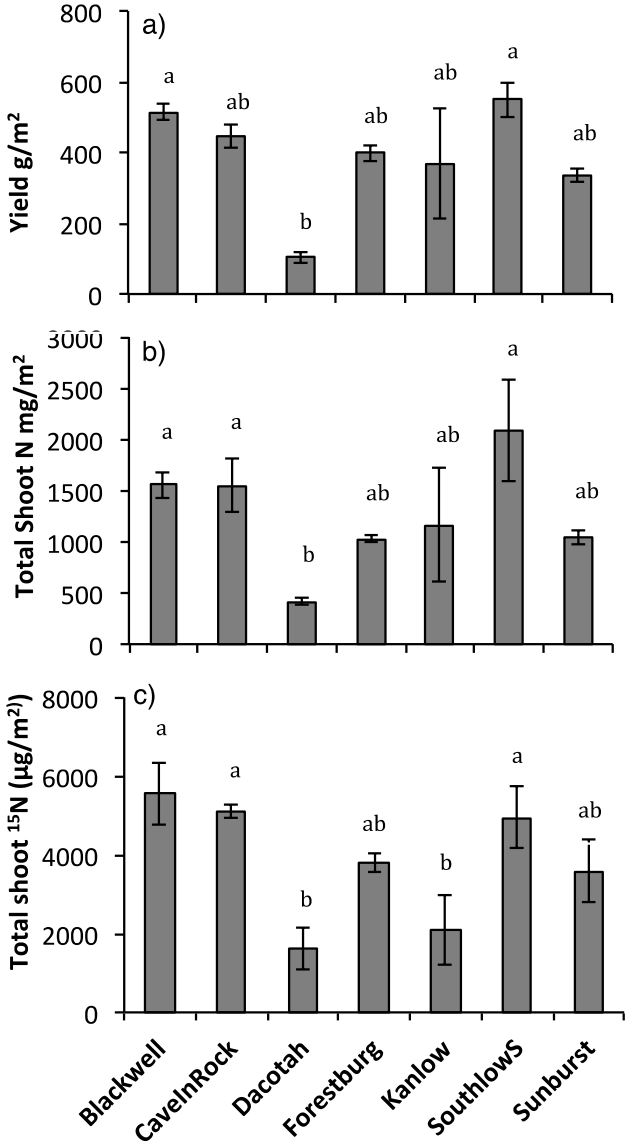

Figure 1. The relative a) yield b) total shoot N, and total shoot 15N for monocultures of switchgrass cultivars. Grey bars denote mean values ± SE and different letters represent significant differences between monocultures (Tukey's HSD, α = 0.05, n=4, Cave in Rock n=3).

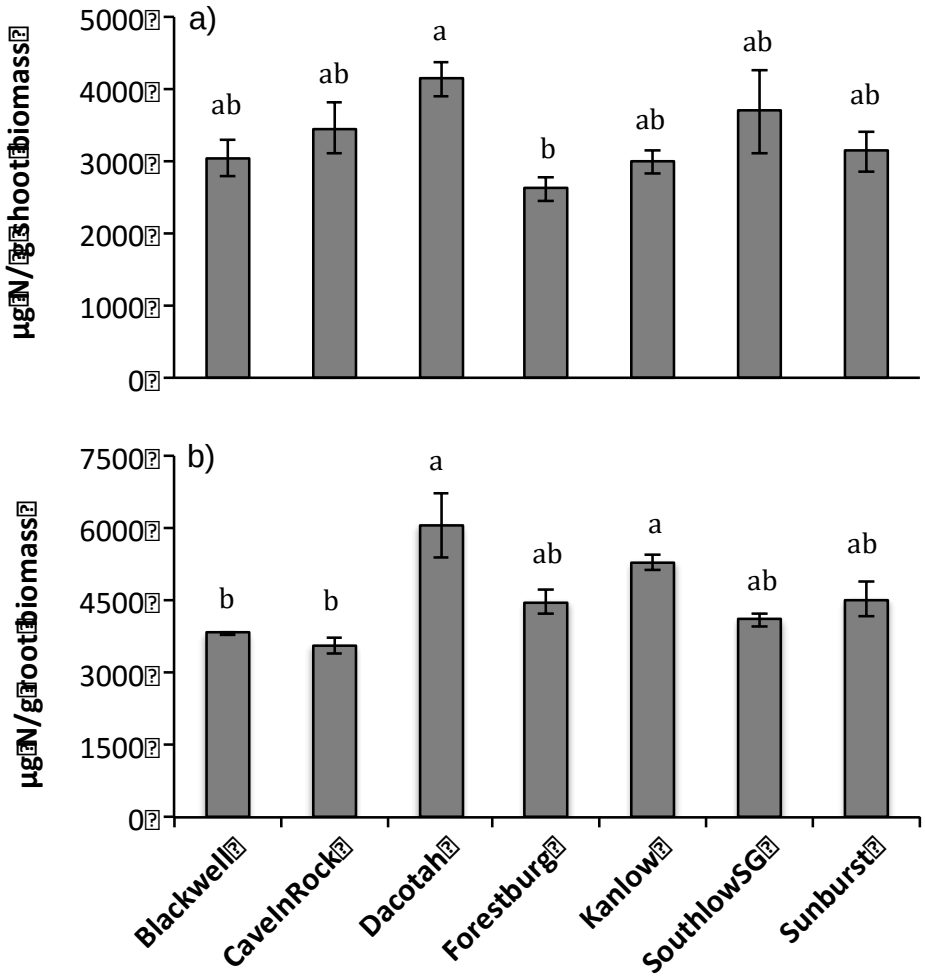

Figure 2. Concentration of µg N g^{-1} biomass for a) shoots and b) roots for switchgrass monocultures. Grey bars represent the mean ± SE values. Different letters denote significant differences (Tukey's HSD, α = 0.05, n=4, Cave in Rock n=3).

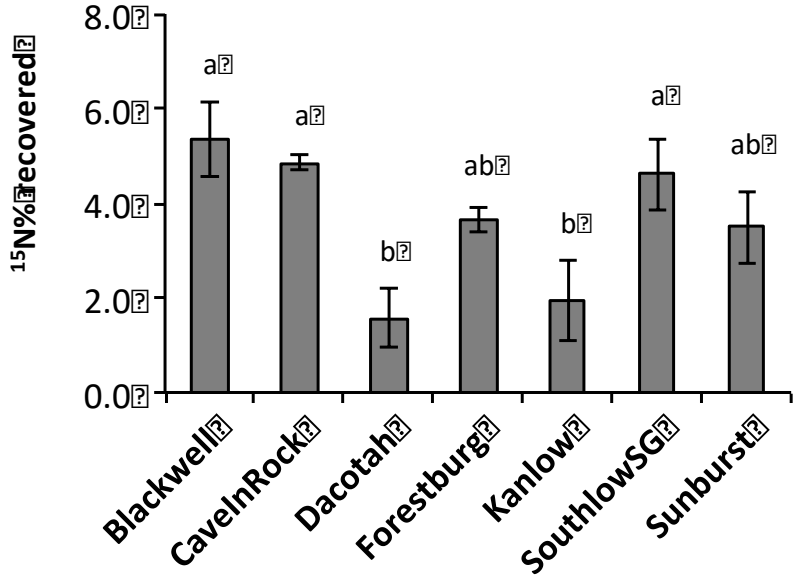

Figure 3. Nitrogen use efficiency compared between switchgrass cultivar monocultures represented by percent ^{15}N recovered in the aboveground biomass. Grey bars denote mean values ± SE and different letters represent significant differences between monocultures (Tukey's HSD, $\alpha = 0.05$, n=4, Cave in Rock n=3).

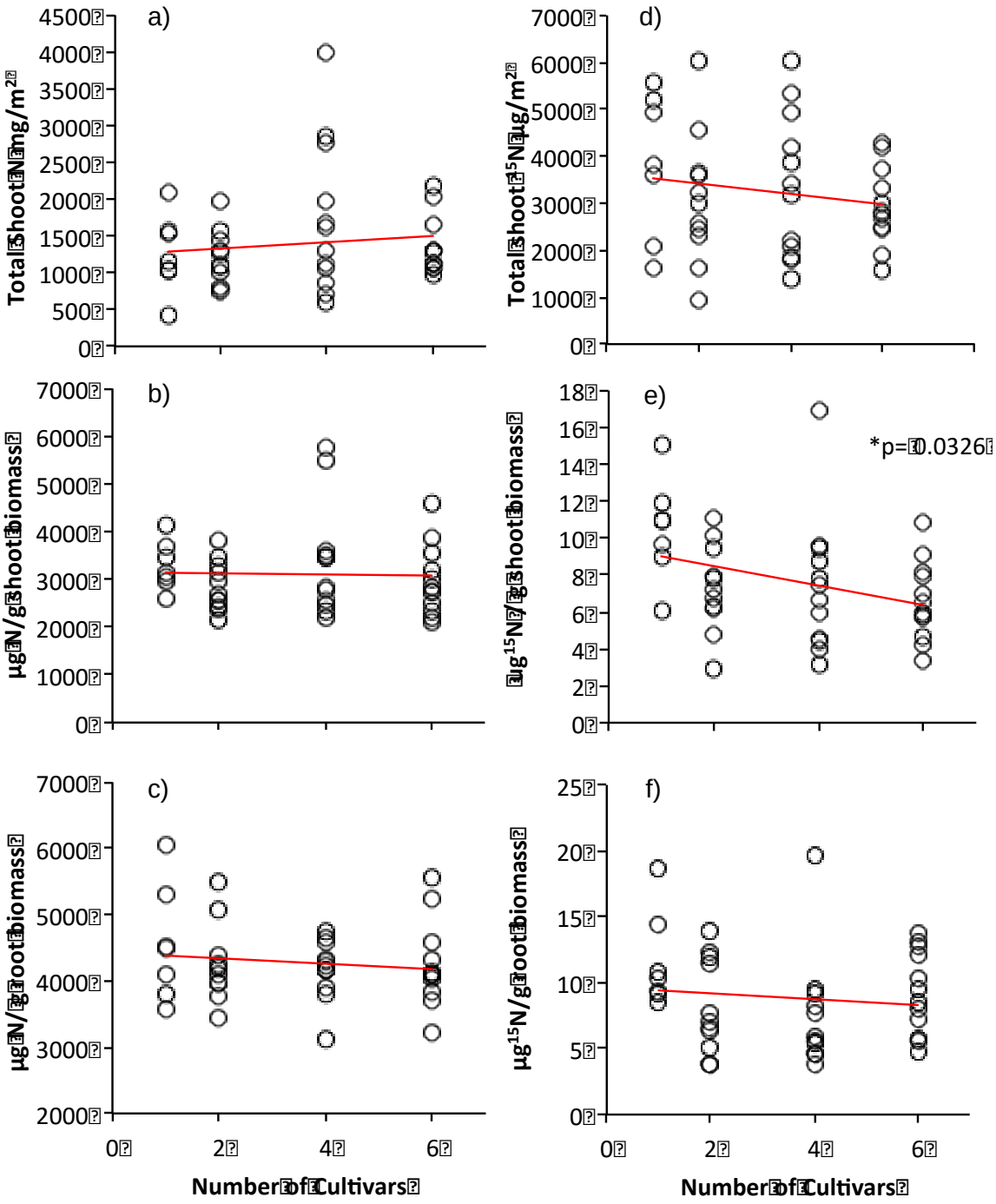

Figure 4. Regressions relating shoot N (a, b and c) and ^{15}N (d,e and f) content and concentration to intraspecific diversity of switchgrass cultivars (α = 0.05, n=40).

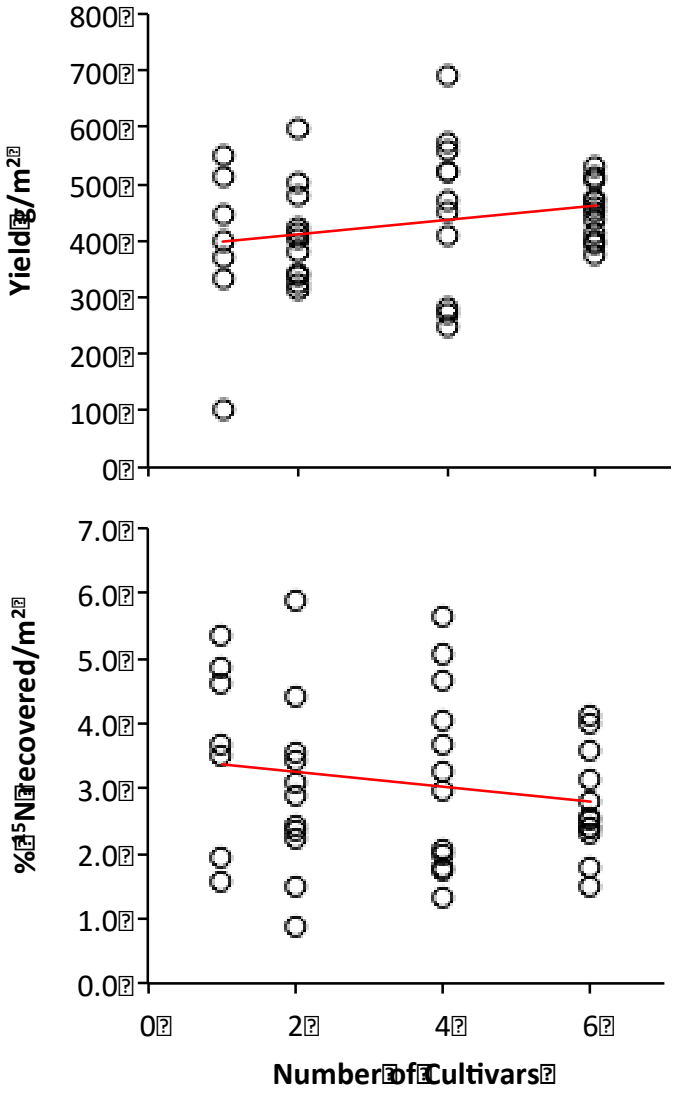

Figure 5. The a) yield and b) %^{15}N recovered (NUE) in the aboveground biomass with increasing levels of diversity (α = 0.05, n=40).

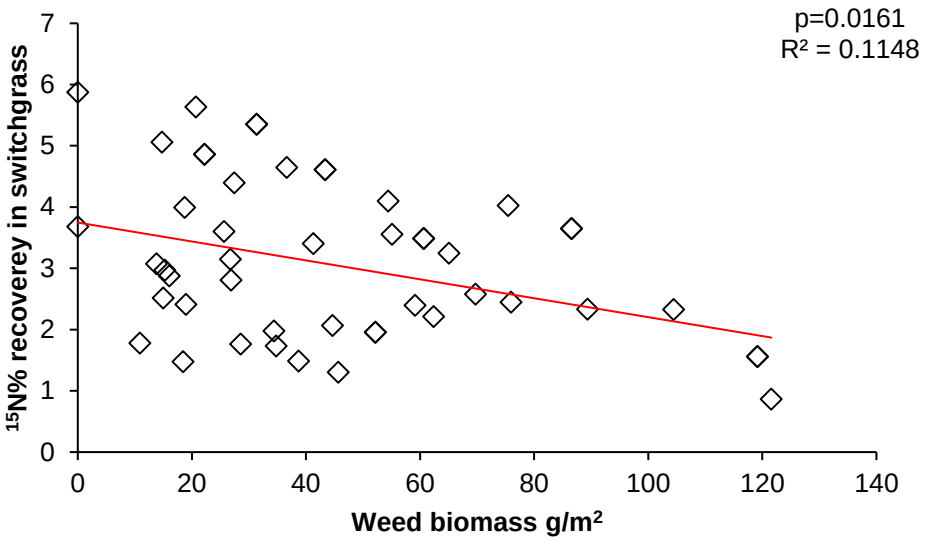

Figure 6. Correlation between percent ^{15}N recovered and the biomass of weeds. (p= 0.0161, df=40, adjusted R^2= 0.1148, Pearson's correlation coefficient= -0.369).

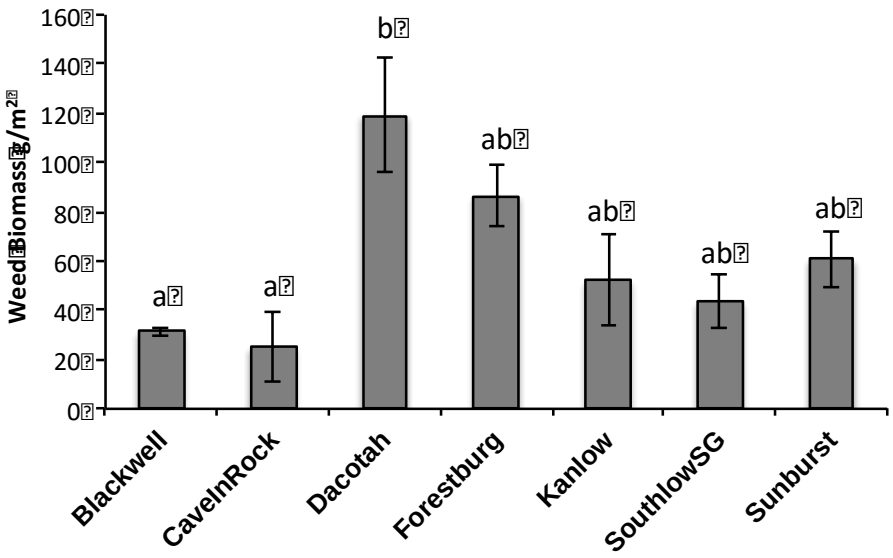

Figure 7. Weed biomass compared between switchgrass cultivar monocultures. Grey bars denote mean values ± SE and different letters represent significant differences between monocultures (Tukey's HSD, $\alpha = 0.05$, n=4, Cave in Rock n=3).

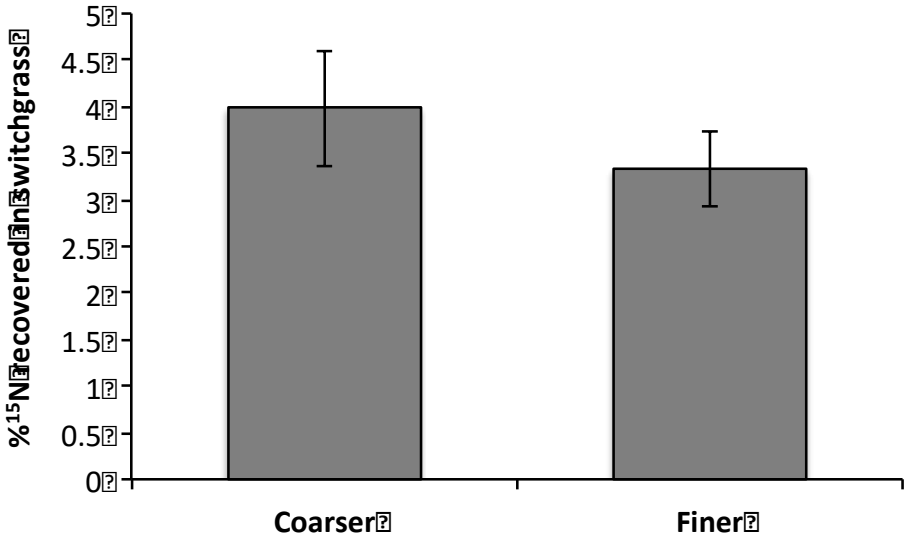

Figure 8. Percent ^{15}N recovered in switchgrass comparing coarser and finer root architectures. Grey bars denote mean values ± SE (Welch's t test, p= 0.442, α = 0.05, coarser n=11, finer n=16).