Improving the viability and sustainability of perennial grasses for bioenergy

By

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Dedication

To Kristin, Baby J, Bob and Julie

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Abstract

Improving the viability and sustainability of perennial grasses for bioenergy

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The adoption of perennial warm-season grass crops for bioenergy production faces significant social, economic, and agronomic challenges. To overcome these hurdles, three separate studies were completed that evaluated alternative plant breeding and agronomic approaches. The first study evaluated the tradeoffs required for breeding programs to incorporate selection for reduced biomass nitrogen concentration. Biomass Yield and nitrogen concentration had a negative genetic correlation (-0.43), but a 50% increase in biomass yield will result in a 37% increase in nitrogen removal with direct selection for biomass yield. Incorporating the goal of reducing nitrogen concentration into a breeding program would result in improving nitrogen reduction by 225% per cycle as compared to direct yield selection, with only a 26% reduction in biomass yield gains. Reducing nitrogen concentration in biomass will improve quality for use of biomass in a combustion system and have minimal effects on quality in an ethanol conversion system.

An alternative approach to reduce inputs is to incorporate legumes into warm-season grass crops. Once established, red clover addition increased biomass yields in unfertilized swards to levels equivalent to fertilization of 112 kg nitrogen ha^{-1} and reduced weed cover by 7%. The yield gains with clover addition were consistent regardless of the warm-season grass accession tested and were due to

the production of biomass by the clover. The incorporation of legumes in mixtures with perennial warm-season grasses can and should play a part in improving the viability of these cropping systems.

The purpose of the third study was to determine whether increasing genetic diversity can increase biomass yields and reduce weed pressure at the population-level using switchgrass monocultures, big bluestem monocultures, and mixtures of the two species. Genetic diversity was the best predictor of productivity and weed cover, with increased diversity resulting in an increase in productivity of up to 6% and a reduction in weed cover of up to 18.4% in switchgrass and big bluestem monocultures. Only switchgrass genetic diversity was predictive of productivity in species mixtures, but total genetic diversity in species mixtures reduced weed cover by 8.7%.

Chapter 1

Can biomass yield of switchgrass be increased without increasing nitrogen requirements?

1.1 Abstract

Switchgrass has been identified as a cellulosic bioenergy feedstock crop due to its potential to produce high biomass yields on marginal lands. Several breeding programs are focused on increasing biomass yields to improve the economic viability of the crop. However, increasing biomass yields without selecting for reduced biomass nitrogen concentration will result in substantial increases in nitrogen removal at harvest. The objectives of this study were to (i) determine the current trajectory of biomass yield and nitrogen removal from breeding programs, (ii) estimate the reduction in the rate of genetic gain for biomass yield when nitrogen concentration was incorporated into a breeding program, and (iii) estimate the effects reduced nitrogen concentration will have on biofuel quality. Biomass yield and nitrogen concentration have a negative genetic correlation (-0.43), but a 50% increase in biomass yield will result in a 37% increase in nitrogen removal with direct selection for biomass yield. Incorporating the goal of reducing nitrogen concentration into a breeding program would result in a 225% greater reduction in nitrogen concentration while achieving 74% of the biomass yield gains of direct selection for biomass yield. Reducing nitrogen concentration in biomass will improve quality for use of biomass in a combustion system and have minimal effects on quality in an ethanol conversion system. Simultaneous selection for increased biomass yield and reduced nitrogen concentration is both a worthy and feasible objective for switchgrass breeding programs that will assure that the benefits of higher yields achieved by breeders are not undermined by increased fertilization requirements.

1.2 Introduction

Switchgrass (*Panicum virgatum* L.) has been identified as a promising bioenergy feedstock crop and has become the focus of several major research efforts. The species is a C4 perennial grass that is widely adapted in North America and capable of achieving high yields on marginal lands (Vogel et al. 2002). Biomass yields range widely from 5 Mg ha^{-1} up to 25 Mg ha^{-1} depending on the location and germplasm used (Wullschleger et al. 2010). This variability and the current average biomass yields are a major limitation to the economic viability of switchgrass as a bioenergy crop (Perrin et al. 2008). As a result, the major breeding efforts of switchgrass have focused almost exclusively on improving biomass yield (Casler 2012). Biomass yield gains have been approximately 1-8% $year^{-1}$ using traditional breeding methods (McLaughlin and Kszos 2005, Casler & Vogel *In Press*). As more diverse germplasm is incorporated into breeding programs and biotechnology tools become more available for switchgrass, annual yield gains are likely to increase (Casler 2012). However, increased biomass yields will increase nitrogen removal at harvest proportionally in the absence of selection for reduced biomass nitrogen concentration. To maintain economic viability and reduce fertilizer requirements, nitrogen concentration must be reduced in proportion to yield gains. The goal of this study is to determine the feasibility of simultaneous selection for increased biomass yields and reduced nitrogen concentration.

The development of inexpensive nitrogen for fertilizing croplands has been one of the major drivers of yield improvements across many agricultural crops (Evans 1998). Improvements in yield during the Green Revolution were reliant upon increasing a crop's response to nitrogen addition and ability to tolerate rapid growth (Khush 2001). However, the conversion of atmospheric nitrogen to the usable forms of nitrate or ammonia is an energy-intense process subject to volatile price fluctuations

(Economic Research Service 2012). For high-value crops where the return on the investment of applying inorganic nitrogen is sufficient, farmers will continue to be able to apply nitrogen, although their profit margins will be reduced. For low-value bioenergy crops, where profit margins are likely to be low and/or variable, reducing the need for inorganic nitrogen fertilization, while maintaining high yields is critical (Jain et al. 2010).

Nitrogen use efficiency is broadly controlled by two components: nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUtE) (Xu et al. 2012). Nitrogen uptake efficiency is defined as the capacity of plant roots to acquire nitrogen from the soil. Nitrogen utilization efficiency is defined as the fraction of plant-acquired nitrogen to be converted to total plant biomass. Throughout this manuscript, we will focus exclusively on NUtE in switchgrass. NUtE is defined here as the amount of aboveground biomass produced per unit of nitrogen in aboveground biomass at harvest specifically relating biomass yields and biomass nitrogen concentrations (Good et al. 2004) and treating NUtE as a ratio criterion (Rowe 1996). NUtE is controlled by complex interactions between genetic and environmental factors. A rich body of research is focused on unraveling the physiological and genetic mechanisms controlling NUtE in many crops (Hirel et al. 2007). Experiments in maize (*Zea mays* L.) suggest that it is possible to increase NUtE until a critical nitrogen concentration is reached, defined as the minimum concentration of nitrogen in shoots required to maximize biomass production (Plénet and Lemaire 2000).

Perennial crops such as switchgrass have more complex interactions with nitrogen due to their perenniality and ability to retranslocate nitrogen from aboveground tissue to belowground tissue during senescence (Clark 1977). An estimate of retranslocation efficiency in switchgrass suggests that up to

50% of nitrogen in aboveground tissue can be retranslocated to belowground tissue at the end of the season (Garten Jr. et al. 2010), although estimates have been as low as 10 to 30% (Clark 1977). The heritability of retranslocation efficiency in switchgrass has not been estimated, but the heritability of nitrogen concentration during the growing season has been estimated to be 0.74 (Talbert et al. 1983). In maize, the heritability of whole-plant nitrogen concentration has been estimated to be 0.39 (Coque and Gallais 2007).

Previous efforts to evaluate the heritability and identify genomic regions associated with improved nitrogen use efficiency in other crops have shown promising results, although parsing out the roles of improved uptake efficiency and improved utilization efficiency remains challenging (Hirel et al. 2007). Studies have identified quantitative trait loci associated with improved plant performance due to improved nitrogen use efficiency in maize, bread wheat (*Triticum aestivum* L.), and rice (*Oryza sativa* L.) (Agrama et al. 1999; Coque et al. 2008; Ladha et al. 1998). Significant variation for nitrogen use efficiency among cultivars has also been identified in perennial ryegrass (*Lolium perenne* L., Wilkins et al. 2000), Kentucky bluegrass (*Poa pratensis* L.*,* Jiang and Sullivan 2000), and timothy (*Phleum pratense* L., (Michaud et al. 1998). Forage breeders have successfully altered nitrogen concentration while altering forage quality in several species of cool-season grasses and legume crops (Casler 2001; Casler and Vogel 1999; Phillips et al. 1982) . Selection for improved forage quality in switchgrass was successful, but led to significant impacts on 28 biomass quality traits and winter survival (Vogel et al. 2013).

There were three objectives of this research. First, we estimated current rates of nitrogen removal and the potential impact of simultaneous selection for increased biomass yield and decreased nitrogen removal by estimating genetic parameters from half-sib family field evaluations. Second, we

estimated the reduction in the rate of genetic gain for biomass yield when nitrogen concentration was incorporated into a breeding program. Third, we estimated the effects of reducing nitrogen concentration in biomass on biofuel quality traits for combustion and ethanol conversion.

1.3 Methods

Data Description

Data used in this study was derived from cycle zero of a seven-year study with three cycles of selection for increased yield originally described by Casler (2010). Briefly, families were derived from seed from 150 half-sib families from the WS4U germplasm produced at Arlington, WI in 2000 by bulking from two vegetative replicates (Casler et al. 2006). This germplasm is exclusively upland and tetraploid (2n = 4x = 36) from a wide geographic range. Only 144 half-sib families were used in this study due to missing data.

Plot-level yield trials were established at two locations using a seeding rate of 400 pure live seeds m^{-2} in April 2002. Seeds were planted in 0.9 x 1.4 m plots consisting of five drilled rows, spaced 15 cm apart with seed placed at a depth of 0.5 to 1.0 cm at two locations. Locations were Arlington, WI [Plano silt loam (fine-silty, mixed, mesic Typic Argiudoll); 43.302°N, 89.353°W; USDA Hardiness Zone 5] and Marshfield, WI [Withee silt loam (fine-loamy, mixed, superactive, frigid Aquic Glossudalf); 44.643°N, 90.138°W; USDA Hardiness Zone 4]. The experimental design was a randomized complete block with one replication at Arlington and two replications at Marshfield. Plots were allowed to establish during the growing season following planting. Plots were fertilized with 112 kg N ha^{-1} prior to the initiation of growth. Plots were harvested with a flail-type harvester in late August, approximately 2 weeks post-anthesis, at a cutting height of approximately 8 cm in 2003 and 2004. A biomass sample of 400 to 500 g

was taken from each plot for dry matter determination and near-infrared reflectance spectroscopy (NIRS) analysis after drying at 60°C in a forced-air dryer.

Samples for NIRS analysis were prepared according to standard guidelines (Murray and Cowe 2004). Biomass samples were ground through a 1-mm screen and scanned using the methods described by Shenk and Westerhaus (1994). Biofuel quality traits and nitrogen concentration values (mg N g^{-1} biomass) on each plot were estimated from NIRS spectra using previously developed NIRS predictions (Núñez-Regueira et al. 2001; Vogel et al. 2010). Ash content (mg g^{-1} biomass) and energy content (kj g^{-1} biomass) were used to estimate biomass quality in a combustion bioenergy system. Potential ethanol (mg g^{-1} biomass), Klason lignin (mg g^{-1} biomass), and total sugars (mg g^{-1} biomass) were measured to estimate biomass quality in an ethanol conversion system.

Statistical Analysis

All analyses were performed using R 2.15.2 (R Development Core Team 2012). Narrow-sense heritabilities were calculated using variance components for all measured traits using mixed models in the *lme4* package treating family, location, block within location, and stand age as random effects (Bates et al. 2012). Heritability confidence intervals were calculated by combining variance component estimates from the mixed models with the coefficients from the expected mean squares calculated in ANOVA and a Satterthwaite approximation of degrees of freedom (Holland et al. 2003). Simple, phenotypic, and genetic correlations were calculated between nitrogen concentration and biomass yield, ash, energy content, Klason lignin, total sugars, and potential ethanol. Simple correlations were calculated as Pearson correlation coefficients using the raw values from each plot. Phenotypic and genetic correlations, response to selection, and correlated response to selection were estimated with

standard equations using the variance components from the mixed models described above (Bernardo 2010). All response to selection estimates assumed a selection intensity of 10%. Bootstrap confidence intervals for genetic correlation were estimated from 1000 bootstrap replicates (Hui Liu et al. 1997). Best linear unbiased predictions (BLUPs) for each half-sib family were estimated for all traits from the mixed models.

Selection response for biomass yield and nitrogen concentration were compared between direct selection for yield and two multiple trait selection approaches. The direct response to selection was calculated as

$$R_x = k_p h_x \sigma_{A(X)}$$

where R_x is the response to selection for trait *X*, k_p is the standardized selection differential, h_x is the heritability of trait *X*, and $\sigma_{A(X)}$ is the additive genetic variance of trait *X*. The correlated response to selection was calculated as

$$R_Y^C = k_p h_x r_A \sigma_{A(Y)}$$

where R_Y^C is the response of trait *Y* to selection on trait *X*, k_p is the standardized selection differential, h_x is the heritability of trait *X*, r_A is the genetic correlation between the two traits, and $\sigma_{A(Y)}$ is the additive genetic variance of trait *Y*. A base index value was calculated giving equal weighting to increasing biomass yield and reducing nitrogen concentration (Williams 1962). The base index was compared to ratio criterion selection, defined as *Biomass Yield (Mg ha^{-1}) /N Concentration (% N)*. Ratio criterion selection has been shown to maximize selection response when the breeding goal is to increase the ratio value and there is a strong negative genetic correlation between the traits (Rowe 1996; Rowe

1995). An added benefit of ratio criterion selection is that the selection focus will shift to value yield gains exclusively once a critical nitrogen concentration is reached.

1.4 Results

Yield and nitrogen removal varied widely among families and locations (Figure 1). The average biomass yield at Arlington and Marshfield was 12.0 and 9.9 Mg ha^{-1} ($p < 0.001$). The average nitrogen concentration at Arlington and Marshfield was 7.3 and 7.9 mg g-1 ($p < 0.001$). The average nitrogen removal at Arlington and Marshfield was 87.0 and 73.5 kg ha^{-1}. The heritability of biomass yield and nitrogen concentration, based on half-sib family means, was 0.34 and 0.25 respectively (Table 1). The heritability of biofuel quality traits ranged from 0.10 for ash content to 0.35 for potential ethanol.

The genetic correlation between nitrogen concentration and biomass yield was -0.42, suggesting it is possible to simultaneously increase yields and reduce nitrogen concentrations (Figure 2). The range of the BLUPs across the 144 families was relatively small for biomass yield and nitrogen concentration, ranging from 9.28 to 12.40 Mg ha^{-1} and 6.8 to 8.6 mg N g^{-1} biomass, respectively. Nitrogen concentration had relatively strong genetic correlations with many of the biofuel quality traits, but the favorability of these correlations was mixed (Table 2). Both energy content (0.66) and ash content (0.89) had positive genetic correlations with nitrogen concentration (Figure 3). Similar results were found for the correlations of nitrogen concentration with biofuel quality for ethanol conversion (Figure 4). Potential ethanol had a weak positive correlation with nitrogen concentration (0.11), but Klason lignin (-0.02) and total sugars (-0.83) had negative genetic correlations with nitrogen concentration.

The three selection programs resulted in substantial differences in the selection response of biomass yield and nitrogen concentration (Figure 5). Conventional breeding for improved biomass yield

with a 10% selection intensity resulted in estimated gains of 0.29 Mg ha^{-1} $cycle^{-1}$ (2.6 % $cycle^{-1}$ or 1 % $year^{-1}$). This selection program would also result in an indirect reduction of nitrogen concentration of -0.04 mg N g^{-1} biomass $cycle^{-1}$ due to the negative correlation between the traits. Therefore, a yield gain of 2 Mg ha^{-1} would result in a reduction in nitrogen concentration of only 0.24 mg g^{-1} and would require approximately seven cycles of selection. However, the reduction in nitrogen concentration due to indirect selection is not great enough to offset the yield gains. An increase in biomass yield of 2 Mg ha^{-1} in a breeding program selecting only for yield would result in an average increase in nitrogen removal of 12 kg ha^{-1}. Over the same seven cycles of selection, base index selection would result in a biomass yield increase of 1.12 Mg ha^{-1}, a reduction in nitrogen concentration of 0.42 mg N g^{-1} biomass, and an increase in nitrogen removal of 3.8 kg ha^{-1}. Ratio criterion selection over seven cycles of selection would increase biomass yield 1.47 Mg ha^{-1}, reduce nitrogen concentration 0.63 mg N g^{-1} biomass, and a reduction in nitrogen removal of 1.5 kg ha^{-1}. Ratio criterion selection resulted in greater improvement of both biomass yield and nitrogen concentration than base index selection.

1.5 Discussion

Breeding for increased biomass yields in switchgrass without also selecting for reduced nitrogen concentrations at harvest will result in a substantial increase in the amount of nitrogen removed during biomass harvest. Present day yields were approximately 10 Mg ha^{-1} with nitrogen removal of 75 kg ha^{-1}. The favorable negative genetic correlation between nitrogen concentration and biomass yield will enable efficient selection for both traits simultaneously. Unfortunately, direct selection on both traits will be necessary because relying on indirect selection of nitrogen concentration in breeding programs selecting only on biomass yield is not sufficient to maintain current levels of nitrogen removal. For

example, increasing biomass yield by 50%, while ignoring N concentration in the selection phase, is expected to increase N removal by 27.5 kg ha^{-1} (37 %). Either a reduction in nitrogen concentration of biomass at harvest must become a priority in breeding programs or alternative agronomic strategies need to be developed to achieve economically viable and high yielding switchgrass crops.

Incorporating the goal of reducing nitrogen concentrations into breeding programs should result in only minor reductions in biomass yield gains. The favorable genetic correlation between the two traits enables efficient selection for both traits simultaneously. Ratio criterion selection was the most effective approach, improving nitrogen reduction by 225% per cycle as compared to direct yield selection with a 26% reduction in biomass yield gains. Base index selection was also effective, improving nitrogen reduction by 150% per cycle as compared to direct yield selection with a 43% reduction in biomass yield gains. Ratio criterion selection is not always more effective than index selection, but ratio criterion selection maximizes response when working to increase the ratio value and there is a strong negative genetic correlation between the traits (Rowe 1995). Ratio criterion selection has the added benefit of dynamically shifting breeding goals to emphasize yield gains once a critical minimum of nitrogen concentration is reached.

Our heritability of nitrogen concentration is relatively low at the plot level, suggesting that genotype x environment interactions and the environment heavily influence nitrogen concentration of the biomass (Bernardo 2010; Xu et al. 2012). However, the estimates of nitrogen concentration in this study are coarse and do not parse out the roles of retranslocation efficiency, nitrogen utilization efficiency during the growing season, or differences in senescence phenology between years and locations. We are unable to determine whether families with low nitrogen concentrations at harvest

time have low nitrogen concentrations throughout the season (high nitrogen utilization efficiency) or if some families retranslocate nitrogen more efficiently or rapidly during senescence (high retranslocation efficiency) with this dataset. Genetic variation in flowering time is minimal in this population (Casler 2010), eliminating flowering time as a potential factor causing families to vary in timing and efficiency of retranslocation.

The heritability of nitrogen concentration has been previously estimated to be 0.74 in individual space-planted switchgrass, although this value was overestimated because germplasm was evaluated at only one location and does not incorporate the spatial variability of plot-level experiments (Talbert et al. 1983). The relatively early harvest time in late August in our study (approximately two weeks following anthesis) allowed little time for plants to retranslocate nutrients, suggesting that families with a low nitrogen concentration at harvest may have had low nitrogen concentrations throughout the growing season. Retranslocation efficiency of nitrogen has been estimated to be approximately 50% in switchgrass, although we are unaware of any estimates of the heritability or range of variability for this trait in switchgrass (Garten Jr. et al. 2010). A comparison of retranslocation efficiency of stands of *Forestburg* (a composite of four native collections from Eastern South Dakota, Barker et al. 1988) under different fertilizer levels found no differences in final retranslocation efficiency (Smith 2012). However, major differences in the amount of time required to complete retranslocation were found, suggesting that genotype x management interactions can impact the rate of retranslocation. Heritable variation in the rate of retranslocation would be critical to selection for reduced nitrogen concentration at harvest because individuals capable of faster retranslocation would allow growers more flexibility in harvest timing. Greater nitrogen retranslocation may also improve retranslocation efficiency of many other

nutrients in the biomass, reducing fertilization requirements and improving biomass quality for use in a combustion system (Aerts 1996; Mclaughlin et al. 1999).

The concentration of nitrogen in biomass impacts the quality of the biomass for use as a bioenergy feedstock. Lower nitrogen concentration levels in biomass used for combustion will reduce fouling and nitrous oxide emissions (Lemus et al. 2008; Lewandowski and Kicherer 1997). The high ash content of grass biomass following combustion is a major inhibitor of the use of biomass in co-firing combustion systems. Selection for lower nitrogen concentration should reduce ash content directly due to the high correlation between these traits (Monti et al. 2008). These gains in quality may outweigh the cost of the reduced energy content associated with lower nitrogen concentration of biomass.

Reductions in nitrogen concentration of biomass will likely have minimal effects on the ethanol conversion potential of the biomass. Our results show a weak negative genetic correlation between nitrogen content and potential ethanol yield, suggesting that reducing nitrogen concentration will have little impact on the ethanol produced from the biomass. Although the effect of biomass nitrogen concentration varies depending on the ethanol conversion process (Lee 1997), an increase in carbon to nitrogen ratio is generally preferred in bioenergy crops (Lewandowski and Kicherer 1997). Nitrogen is a necessary component in the conversion process of biomass to ethanol, but the addition of nitrogen at the fermentation tank is a more efficient use of resources compared to relying on fertilizer application and uptake during plant growth (Lin and Tanaka 2006).

An additional benefit to reducing nitrogen concentration in biomass may be a reduction in disease and insect damage. Many studies have shown a strong correlation between the nitrogen content of a host plant and the level of herbivory (Mattson 1980; Moran and Hamilton 1980). Increases

in biomass quality (and nitrogen concentration) in forage crops have been associated with increased disease and insect damage (Casler 1997). Therefore, selection for lower nitrogen (reduced forage quality) may reduce the potential for disease and insect damage.

Conclusion

Simultaneous selection for increased biomass yield and reduced nitrogen concentration is both a worthy and achievable objective for switchgrass breeding programs. The economic benefit of yield gains achieved by breeding programs focused exclusively on improving biomass yield will be undermined by the accompanying increase in nitrogen removal at harvest. While incorporation of nitrogen concentration into a breeding program will slow yield gains, the favorable genetic correlation between biomass yield and nitrogen concentration will minimize the tradeoff. The development of switchgrass varieties that can produce high yields with minimal nutrient inputs is critical to achieve economic viability and environmental sustainability of switchgrass as a bioenergy feedstock.

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1.7 Tables

Table 1. Narrow-sense heritabilities of biomass yield and quality traits calculated using 144 half-sib families grown at two locations for two years in Wisconsin, USA. Confidence intervals (C.I.) are 95% confidence intervals.

Variable	h^2	C.I.
Biomass Yield (Mg ha^{-1})	0.34	0.12, 0.50
Nitrogen Concentration (mg g^{-1})	0.25	0.01, 0.43
Nitrogen Removal (kg $ha^{-1)}$	0.30	0.08, 0.47
Ash (mg g^{-1})	0.10	0.00, 0.32
Klason Lignin (mg g^{-1})	0.27	0.03, 0.45
Total Sugar (mg g^{-1})	0.26	0.02, 0.44
Ethanol (mg g^{-1})	0.35	0.14, 0.51
Energy Content (kj g^{-1})	0.25	0.01, 0.43

Table 2. Simple, phenotypic, and genetic correlations between nitrogen concentration and biomass yield and biofuel quality traits. Significance of p value indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 95% confidence intervals (C.I.) of genetic correlations are based on 1000 bootstrap replicates.

Variable	Simple	Phenotypic	Genetic	C.I.
Biomass Yield (Mg ha^{-1})	-0.58 ***	-0.54 ***	-0.43	-0.89, 0.24
Ash (mg g^{-1})	0.84 ***	0.79 ***	0.89	0.02, 0.99
Klason Lignin (mg g^{-1})	-0.70 ***	-0.47 ***	-0.02	-0.60, 0.83
Total Sugar (mg g^{-1})	-0.93 ***	-0.92 ***	-0.84	-0.93, -0.41
Ethanol (mg g^{-1})	0.51 ***	0.28 ***	0.11	-0.60, 0.58
Energy Content (kj g^{-1})	0.59 ***	0.19 *	0.66	0.07, 0.95
Nitrogen Removal (kg ha^{-1})	0.25 ***	0.07	0.19	-0.39, 0.62

1.8 Figures

Figure 1 Nitrogen removal (kg/ha) compared to biomass yields for 144 half-sib families grown at (A) Arlington, WI and (B) Marshfield, WI). Points plotted are the best linear unbiased predictors (BLUPs) for each family at each location. The dashed horizontal line is the annual fertilizer application level (112 kg/ha). The slope (+/- standard error), p value, and R^2 value are calculated for a linear regression between the BLUPs for each trait.

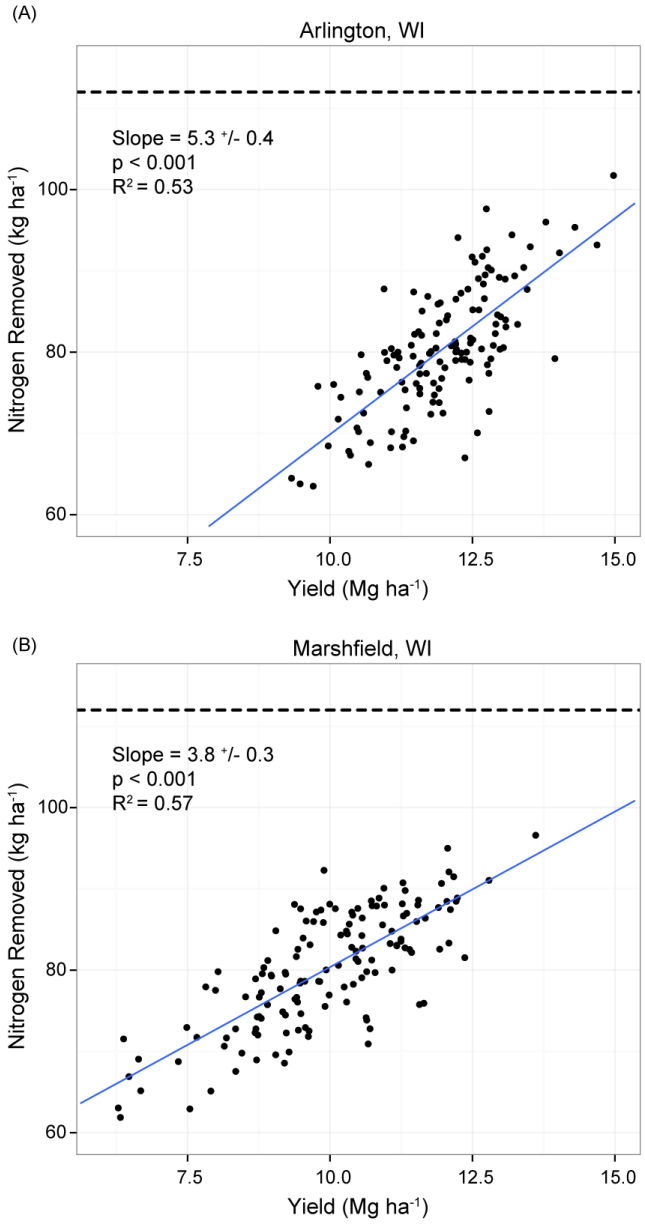


Figure 2. Biomass yield estimates as compared to nitrogen concentration in biomass using the best linear unbiased predictors (BLUP) estimates from 144 half-sib families. The p value and R^2 value are calculated for a linear regression between the BLUPs for each trait.

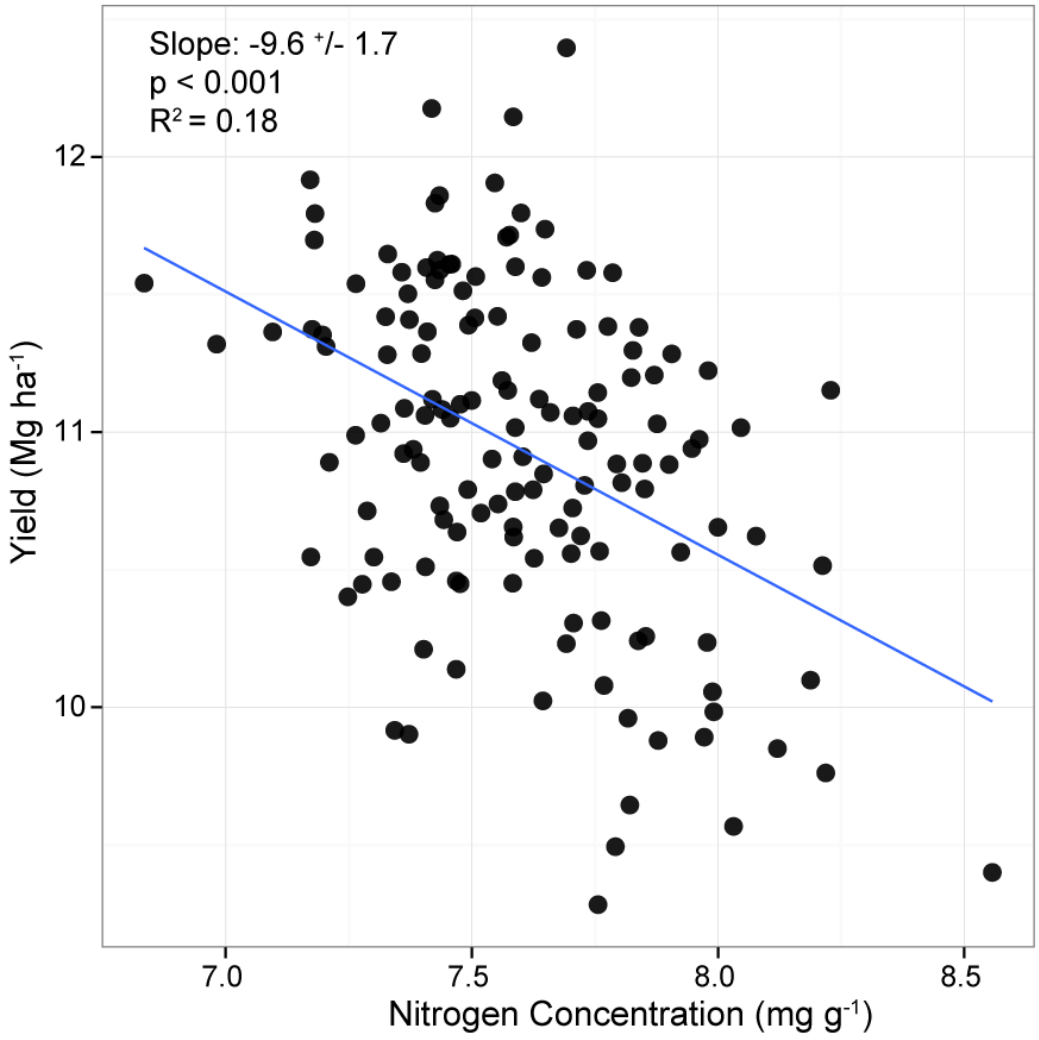


Figure 3. Combustion quality estimates compared to nitrogen concentration in biomass using best linear unbiased predictors (BLUPs) for 144 half-sib families. (A) Predicted energy content (kj g^{-1} biomass) plotted against nitrogen concentration (% N of biomass). (B) Predicted ash content (mg g^{-1}) plotted against nitrogen concentration (% N of biomass). The p value and R^2 value are calculated for a linear regression between the BLUPs for each trait.

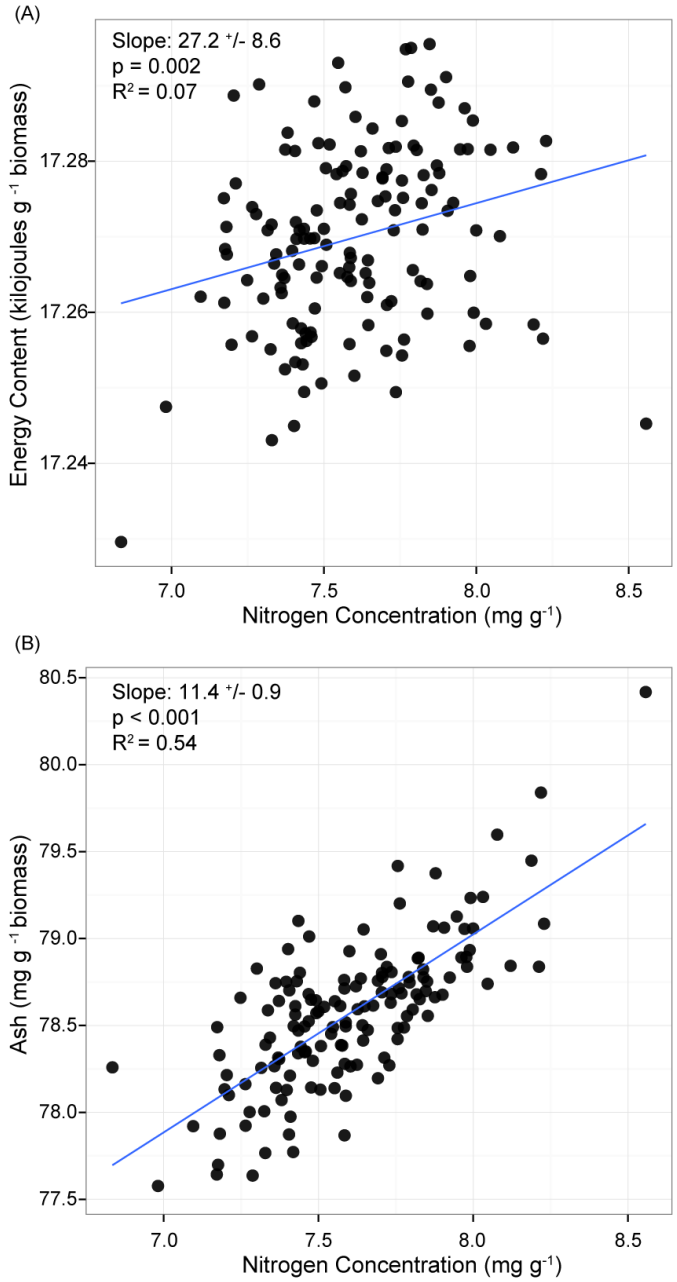


Figure 4. Ethanol conversion quality estimates compared to nitrogen concentration in biomass using best linear unbiased predictors (BLUPs) for 144 half-sib families. (A) Potential ethanol (mg g^{-1} biomass) plotted against nitrogen concentration. (B) Klason lignin (mg g^{-1}) plotted against nitrogen concentration. (C) Total Sugars (mg g^{-1}) plotted against nitrogen concentration. The p value and R^2 value are calculated for a linear regression between the BLUPs for each trait.

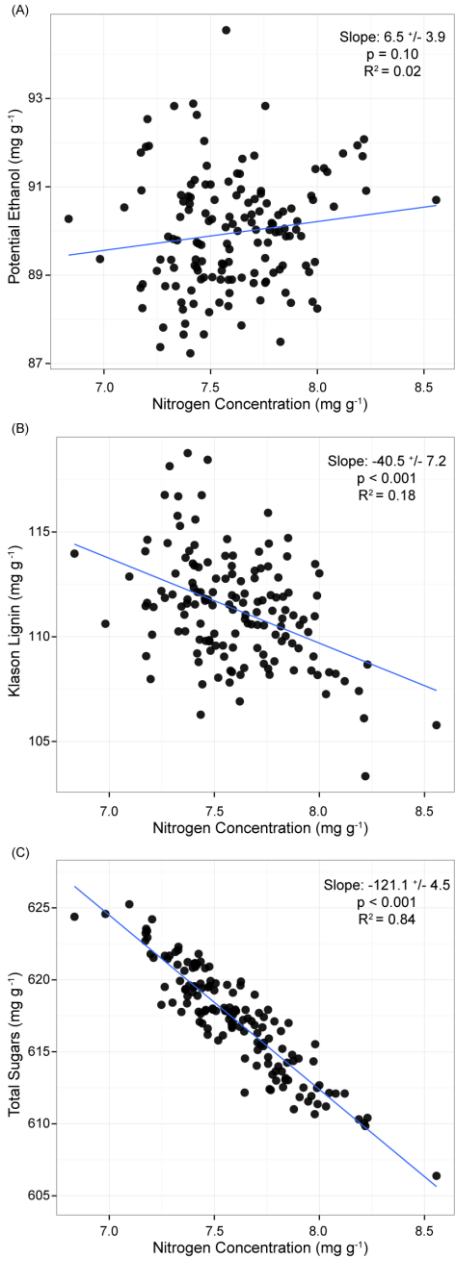
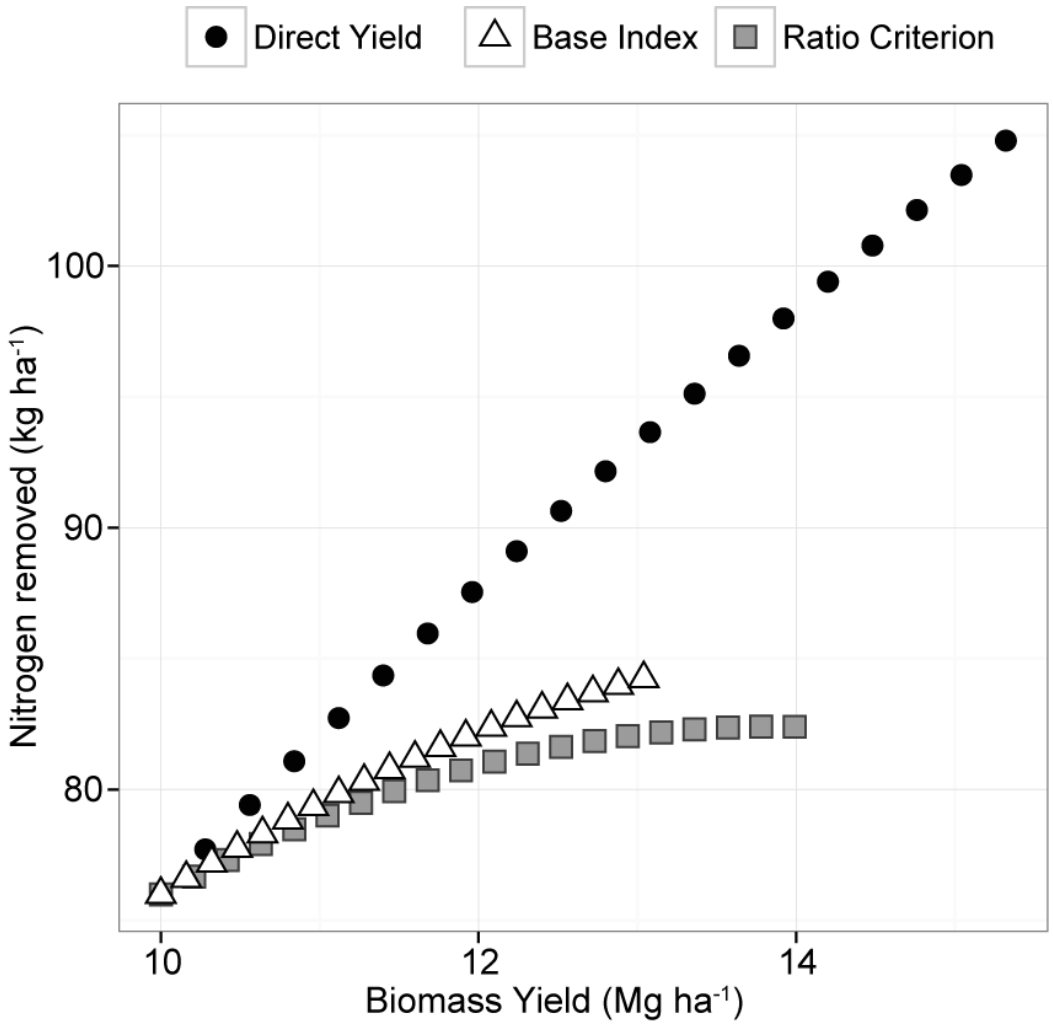


Figure 5. Selection response in biomass yield (Mg ha^{-1}) and nitrogen removal (kg ha^{-1}) over 20 cycles comparing direct selection for yield, base index selection with equal weighting on biomass yield and nitrogen concentration , and ratio criterion selection (Biomass yield / Nitrogen concentration (% N)).



Chapter 2

Legume addition to perennial warm-season grasses increases productivity and producer flexibility

2.1 Abstract

The adoption of perennial warm-season grass crops for bioenergy production faces significant social, economic, and agronomic challenges. The inclusion of legumes to warm-season grass crops may be able to increase productivity, reduce weed pressure and the need for fertilizer inputs, and allow farmers greater management flexibility. The objectives of this study were to (i) evaluate whether the overseeding of red clover in warm-season grass swards can improve biomass yields and reduce weed cover (ii) determine if biomass yield gains arose from increased nitrogen availability or the biomass production of the legume crop and (iii) determine whether switchgrass or big bluestem accessions respond differently to red clover overseeding. Once established, red clover addition increased biomass yields in unfertilized swards to levels equivalent to fertilization of 112 kg nitrogen ha^{-1} and reduced weed cover by 7%. The yield gains with clover addition were consistent regardless of the warm-season grass accession tested and were due to the production of biomass by the clover. The incorporation of legumes in mixtures with perennial warm-season grasses can and should play a part in improving the viability of these cropping systems. Future studies should consider alternative planting, harvesting, and weed control in a long-term experimental framework to refine management of legume and warm-season grass mixtures.

2.2 Introduction

The adoption of perennial warm-season grass crops for bioenergy production faces significant social, economic, and agronomic challenges. Perennial warm-season grasses, particularly in northern temperate regions, are slow to emerge in the spring and early to senesce in the fall. This phenology results in an incomplete use of the growing season that reduces yield potential and increases weed pressure. Biomass removal at harvest will remove essential nutrients from the cropping system, even with the ability of perennial crops to retranslocate nutrients to belowground tissues during senescence (Garten Jr. et al., 2010; Kering et al., 2011). To avoid waning yields over the long-term, nitrogen addition to these cropping systems is likely necessary. The use of inorganic fertilizer to offset nutrient removal will increase production costs and undermine global warming mitigation potential of these systems because of increased nitrous oxide (N_2O) emissions (Nikièma et al., 2011; Monti et al., 2012). Finally, the perennial nature of these crops reduces the flexibility of farmers to respond to changing crop markets because perennial warm-season grass crops will likely require a long-term commitment to become profitable (James et al., 2010; Egbendewe-Mondzozo et al., 2011). The introduction of legume crops to warm-season grass stands may be able to alleviate many of these concerns.

Legumes use C3 photosynthesis and experience peak productivity when warm-season grasses are dormant or less productive (Sleugh et al., 2000). Combinations of C3 and C4 species have been shown to increase overall productivity as compared to monoculture systems, with legume-C4 grass mixes having the greatest agronomic potential (Berdahl et al., 2001; Fornara and Tilman, 2008; Schipanski and Drinkwater, 2012). The growth of a legume prior to warm-season grass emergence and following fall harvest should reduce weed pressure by providing soil cover (Gettle et al., 1996; Hartwig and Ammon, 2002). Legume addition to cropping systems typically will result in atmospheric nitrogen fixation, which should reduce fertilizer requirements to maintain a level of productivity otherwise realized with inorganic fertilizer application. Reduced inorganic fertilizer should increase profitability

and reduce N_2O emissions (Rochette and Janzen, 2005). The production of a legume and warm-season grass mixture might also increase farmer flexibility because the legume crop can be harvested and used as a high quality animal feed (Bow et al., 2008).

The agronomic and environmental benefits of legume and cool-season grass mixtures in pasture systems have long been appreciated (Rochon and Doyle, 2004). Relatively few experiments have focused on field evaluations of legume warm-season grass mixtures, but previous experiments in animal forage production systems have shown promising results. Several legumes species have been found to be compatible in legume-switchgrass (*Panicum virgatum* L.) mixtures, although there is wide variation in biomass yields among the legume species (Blanchet et al., 1995). Of 11 legume-switchgrass mixtures evaluated in Ames, IA, those with red clover (*Trifolium pratense* L.) were among the most productive (George et al., 1995). Biomass yield gains of mixtures over unfertilized switchgrass monocultures were substantial and stemmed from legume biomass production rather than an increase in switchgrass production resulting from nitrogen fixation. However, red clover establishment success may differ depending on the warm-season grass cultivar used (Jung et al., 1985).

We had four specific objectives for this study. First, we evaluated whether the overseeding of red clover in warm-season grass swards improved biomass yield relative to that obtained from fertilizer addition in a single harvest production system. Second, we explored whether biomass yield gains arose from increased nitrogen availability or the biomass production of the legume crop. Third, we determined whether switchgrass or big bluestem (*Andropogon gerardii* Vitman.) accessions respond differently to red clover overseeding. Finally, we assessed weed pressure response to legume addition.

2.3 Methods

Six accessions of switchgrass and six accessions of big bluestem were established at Arlington, WI on 1 June 2010 (Table 1). The accessions comprised improved cultivars and seed increases of wild

populations. The soil type was a Plano silt loam (fine-silty, mixed, mesic Typic Argiudoll). The experimental design was a split-plot randomization restriction within a randomized complete block design with six replicates. Main plots consisted of a 2×2 factorial: nitrogen addition (112 kg N ha^{-1} and 0 kg N ha^{-1}, applied as ammonium nitrate) combined with red clover overseeding (7.2 kg pure live seed ha^{-1} and no clover addition). Grass seeding rates were adjusted for germination, using standard germination procedures (AOSA, 1998) and a seeding rate of 650 pure live seeds m^{-2}. Subplots were drill seeded in 10 rows with 15-cm spacing between rows and an overall size of 3.1 m^2. Plots were allowed to establish during 2010 with no main plot treatments or management other than clipping for weed control on 1 September 2010. The main-plot treatments were initiated 10 May 2011 following application of glyphosate at a rate of 1.2 kg ha^{-1} active ingredient for weed control. Ammonium nitrate was again applied on 1 May 2012. Red clover seed was broadcast seeded into main plots.

Biomass yields were measured on all plots in 2011 and 2012 using a flail-type harvester and a cutting height of approximately 8 cm. Harvest dates were 22 September 2011 and 29 August 2012. Dry matter concentration was determined on a sample of harvested biomass ranging from 100 to 300 g. Samples were dried at 60°C in a forced-air dryer. Sample dry matter concentration was used to adjust all biomass yields to a dry matter basis. Grass, legume and weed cover proportions were determined from 16-point estimates (15-cm spacing between points) in each subplot in early August of each year. Estimates of yield contribution for each of the above components was calculated using these cover estimates for each growing season. Soil cores to 15-cm depth were sampled in May 2012 to estimate nitrogen availability. Five subsamples from each main plot were consolidated. Inorganic N was extracted within 24 hours of sampling using 2 M KCL. Extracts were frozen before determination of NH_4^+ and NO_3^- on a Lachat flow injection analyzer (Robertson et al., 1999). Leaf area index (LAI) was measured for each plot in July 2011 using an Accupar LP-80 light ceptometer to determine the light competition faced by the establishing red clover (Decagon Devices, Inc., Pullman, WA). Likewise, LAI

was measured for each plot in late May 2012 to determine light availability for emerging warm-season grasses.

Statistical Analysis

Biomass yields were modeled using maximum likelihood mixed-effects models using the *lme4* package (Bates et al., 2012) in R 2.15.2 (R Development Core Team, 2012). The significance of fixed effects were tested using likelihood ratio tests (Crawley 2007). Biomass yields were square root transformed so that model residuals were normally distributed and homoscedastic. Years were analyzed separately in all analyses because year effects were significant for all analyses and clover cover increased substantially between years. The least-squares means were estimated for the effect of clover addition and for the magnitude of accession by fertilizer interactions using the *lsmeans* package in R (Lenth, 2012). Weed cover and red clover cover were treated as count data and were modeled using Poisson and negative binomial distributions (Hardin and Hilbe, 2007), with and without zero inflation, using the *glmmADMB* package in R (Skaug et al., 2011). The model type with the lowest Akaike Information Criterion (AIC) value was selected for each response variable. Red clover biomass yield was modeled using the same approach, although Poisson models were not tested. The significance of fixed effects were tested using likelihood ratio tests as described above.

2.4 Results

The red clover was slow to establish during the first season (2011) following spring planting. Red clover establishment was greater in unfertilized plots (8%) than in fertilized plots (0%, $p < 0.01$, Figure 1). By the second season (2012), average red clover cover was 62% in unfertilized plots and 30% in fertilized plots ($p < 0.01$, Figure 1). The difference in clover establishment between fertilizer treatments likely arose from the intensity of light competition during the establishment year. LAI and canopy height averaged 4.6 and 92.5 cm in fertilized plots, but was 2.6 and 62.9 cm in unfertilized plots

($p < 0.01$ for both comparisons). The addition of clover did not significantly alter LAI or canopy height during the establishment year ($p = 0.77$ and $p = 0.63$, respectively).

Biomass yields averaged 3.08 Mg ha^{-1} during the establishment year and did not vary with clover addition ($p = 0.65$, Figure 2). The addition of fertilizer doubled biomass yields (4.10 Mg ha^{-1} in fertilized plots, 2.07 Mg ha^{-1} in unfertilized plots, $p < 0.01$). By 2012 when the clover was established, unfertilized plots with clover (3.74 Mg ha^{-1}) produced more biomass than unfertilized plots without clover (1.46 Mg ha^{-1}, $p < 0.01$) by a factor of 2.6. Unfertilized plots with clover were as productive as fertilized plots without clover (3.77 Mg ha^{-1}) and fertilized plots with clover (4.12 Mg ha^{-1}, $p = 0.48$).

Each accession had a similar increase in biomass yield with clover addition, increasing biomass yield by 2.17 Mg ha^{-1} on average (accession × clover addition interaction, $p = 0.83$, Figure 3). The magnitude of biomass yield response to fertilizer addition varied widely among the accessions (accession × fertilizer interaction, $p < 0.01$). Chiwaukee switchgrass had the smallest response to fertilizer addition in 2012 (an increase of 0.53 Mg ha^{-1} in fertilized plots) while Central Iowa big bluestem (3.46 Mg ha^{-1} increase) and Blackwell switchgrass (3.41 Mg ha^{-1} increase) had the largest responses to fertilizer addition in 2012

The increase in biomass yield associated with addition of red clover to unfertilized plots in 2012 arose from the biomass production of clover, not an increase in warm-season grass production. The estimated biomass production of the warm-season grasses was not significantly affected by clover addition (Figure 4, $p = 0.14$). However, red clover produced on average 1.18 Mg ha^{-1} in fertilized plots and 2.40 Mg ha^{-1} in unfertilized plots ($p < 0.01$), leading to an increase in total biomass yield in unfertilized plots to levels equivalent to those of fertilized plots without clover. Soil nitrogen availability was not affected by clover addition (data not shown). There was no increase in available soil nitrogen in

spring 2012 from clover addition ($p = 0.40$). As expected, fertilizer addition increased available soil nitrogen ($p < 0.01$).

Clover addition did not reduce weed pressure during the 2011 establishment year ($p = 0.72$, Figure 5). Once established, clover addition reduced weed pressure in both fertilized plots (7% with clover versus 14% without clover) and unfertilized plots (2% weeds with clover versus 8% weeds without clover, $p < 0.01$). The effect of fertilizer addition switched between years. In 2011, weed pressure was lower in fertilized plots (5% weed cover) versus unfertilized plots (10% weed cover, $p < 0.01$). In 2012, fertilizer addition increased weed cover by approximately 6% ($p < 0.01$). In 2012, clover addition increased LAI from 1.8 to 4.2 prior to warm-season grass emergence ($p < 0.01$).

2.5 Discussion

The overseeding of legumes into perennial warm-season grass stands can help to alleviate some of the concerns that inhibit the adoption of warm-season grasses as dedicated bioenergy crops. A mixed crop has the potential to increase the productivity, economic viability, and the environmental benefits of warm-season grass crops grown for bioenergy. Additional research is necessary to determine the management strategies that ensure long-term viability of mixed stands. However, our results suggest that the legumes can help to overcome the substantial hurdles inhibiting perennial bioenergy crop adoption.

Agronomic benefits

Once established, red clover increased yields in unfertilized plots to levels equivalent to those produced with 112 kg N ha^{-1} fertilization. An additional benefit of planting a legume crop rather than fertilizing is that clover addition resulted in stable yield gains of 2.17 Mg ha^{-1} over unfertilized plots, irrespective of the accession used. Conversely, the range of fertilizer response varied widely by

accession, ranging from 0.53 to 3.46 Mg ha^{-1} increase in biomass yield. Increased yield stability of grass-legume mixtures across years and location has been observed in pasture and grassland systems (Tilman, 1996; Tracy and Sanderson, 2004; Sanderson et al., 2005). Previous research found clover establishment to be dependent upon the accession used, but did not explicitly test whether biomass yield gains were accession dependent (Jung et al., 1985).

The biomass yield gains associated with clover addition were the result of clover biomass production rather than increased warm-season grass production in response to increased soil nitrogen availability. Clover production did not significantly reduce warm-season grass production on average, but may begin to outcompete the warm-season grasses over a longer period (Tow and Lazenby, 2001). Weed competition during emergence of switchgrass has been shown to reduce stand quality and annual yields (Buhler and Netzer, 1998; Miesel et al., 2012). Clover cover averaged 61% of the cover in unfertilized plots in 2012, with 26% of these plots having clover cover exceeding 80%. While clover cover was lower in fertilized plots, this was most likely the effect of slower establishment during the summer of 2011. Two alternative approaches may be necessary to maintain coexistence of the clover and warm-season grasses. A shift to a two-cut system with late spring harvest may be a preferred approach to maintaining coexistence of red clover and warm-season grasses (George et al., 1995). The competitive ability of red clover in northern temperate regions in the spring results in a significant canopy (average LAI of 4.2 in late May) prior to warm-season grass emergence. A harvest of the red clover at this time would provide the yield benefits from early season biomass production by the red clover and weed suppression, but reduce competition with the warm-season grasses. An alternative approach would be to use an annual-type red clover that is not winter hardy. Establishment would be required each season, but no additional harvest would be required. More study is necessary to determine the long-term viability of legume and warm-season grass mixtures.

While there was no evidence of increased soil nitrogen availability or increased warm-season grass production with clover addition in our study, biologically fixed nitrogen from the legume should result in an increase in available nitrogen over time (Herridge et al., 2008; Jensen et al., 2012). The length of our study may not have been sufficient for nitrogen fixation to affect nitrogen availability. The magnitude of nitrogen fixation varies widely depending on the environment and management practices, but legume-grass mixtures have been shown to fix between 125 and 300 kg N ha^{-1} in unfertilized systems and between 50 and 125 kg N ha^{-1} in fertilized systems (Boller and Nösberger, 1987, 1994; Nesheim and Øyen, 1994; Høgh-Jensen et al., 2004). Chopping the clover biomass as a green manure during a spring harvest, rather than removing it for animal feed, would maximize the amount of nitrogen made available to the warm-season grass crop (Cherr et al., 2006).

Legume addition cannot reduce weed pressure during the establishment year when the issue is of greatest concern (McLaughlin and Kszos, 2005). The simultaneous establishment of warm-season grasses and legumes has been shown to be ineffective because of the rapid emergence and high competitive ability of the legumes (Bow et al., 2008). In the cropping system evaluated in this study, clover addition did not reduce weed pressure during its establishment year (second year of growth for the warm-season grasses), but was effective in reducing weed cover once established. Early- and late-season weed pressure can be managed with broad spectrum herbicides prior to warm-season grass emergence (Mitchell et al., 2010; Sanderson et al., 2012; Miesel et al., 2012), but clover could reduce the need for regular weed management.

Environmental Benefits

The environmental benefits of perennial warm-season grasses replacing annual cropping systems and the use of biomass for production of energy have been well documented (Robertson et al., 2008; Gelfand et al., 2013). Incorporating legumes into these cropping systems may increase these

benefits further. Reduced fertilizer application and replacement with biological nitrogen fixation may result in reduced N_2O emissions from agricultural systems, increasing the favorable greenhouse gas balance of these crops (Crews and Peoples, 2004; Davis et al., 2012). Appropriately managed mixtures may also achieve higher levels of primary productivity and increase soil carbon sequestration potential (Ingram and Fernandes, 2001; Kong et al., 2005; Fornara and Tilman, 2008). Using the legume as a cover crop or green manure rather than for harvest may increase carbon sequestration further, helping to alleviate concerns regarding carbon storage potential from annually harvested perennial crops (Aulakh and Khera, 2001; Jarecki and Lal, 2003; Sartori et al., 2006).

While significant challenges must be overcome for perennial warm-season grasses to become a viable cropping system, the incorporation of legumes in mixtures with perennial warm-season grasses can and should play a part in improving the viability of these cropping systems. Future studies should consider alternative planting, harvesting, and weed control in a long-term experimental framework to refine management of these systems, but it is clear that legume mixtures can increase yields and reduce production costs of a warm-season grass crop. Legumes can also increase the environmental benefits of perennial warm-season grass crops. Perhaps most important, the increased flexibility gained by farmers growing a legume-grass mixture can help to overcome the social and economic hurdles inhibiting adoption of warm-season grasses.

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2.7 Tables

Table 1. The origin of the 12 accessions of warm-season grasses used in the study.

Acc. Number	Species	Name	Geographic Info	State	Lat	Long
1	*A. gerardii*	Chiwaukee	Chiwaukee prairie	WI	42.53	-87.82
2	*A. gerardii*	Central Iowa Ecotype	Central Iowa	IA	-	-
3	*A. gerardii*	Shooting Star	Houston County, MN	MN	43.59	-91.36
4	*A. gerardii*	'Southlow'	Southern Michigan	MI	-	-
5	*A. gerardii*	'Niagra'	New York	NY	42.70	-78.72
6	*A. gerardii*	'Kaw'	Flint Hills, KS	KS	38.37	-96.54
7	*P. virgatum*	Chiwaukee	Chiwaukee prairie	WI	42.53	-87.82
8	*P. virgatum*	Central Iowa Ecotype	Central Iowa	IA	-	-
9	*P. virgatum*	Shooting Star	LaCrosse County, WI	WI	43.79	-91.13
10	*P. virgatum*	'Southlow'	Southern Michigan	MI	-	-
11	*P. virgatum*	'Shelter'	St Marys, West Virginia	WV	39.40	-81.20
12	*P. virgatum*	'Blackwell'	Blackwell, OK	OK	36.81	-97.28
	T. pratense	'Cardinal' Red Clover				

2.8 Figures

Figure 1. Red Clover cover (%) by main-plot treatment in years 2011 and 2012. Error bars represent standard errors and p-values are shown in the legends (C = clover, F = fertilizer).

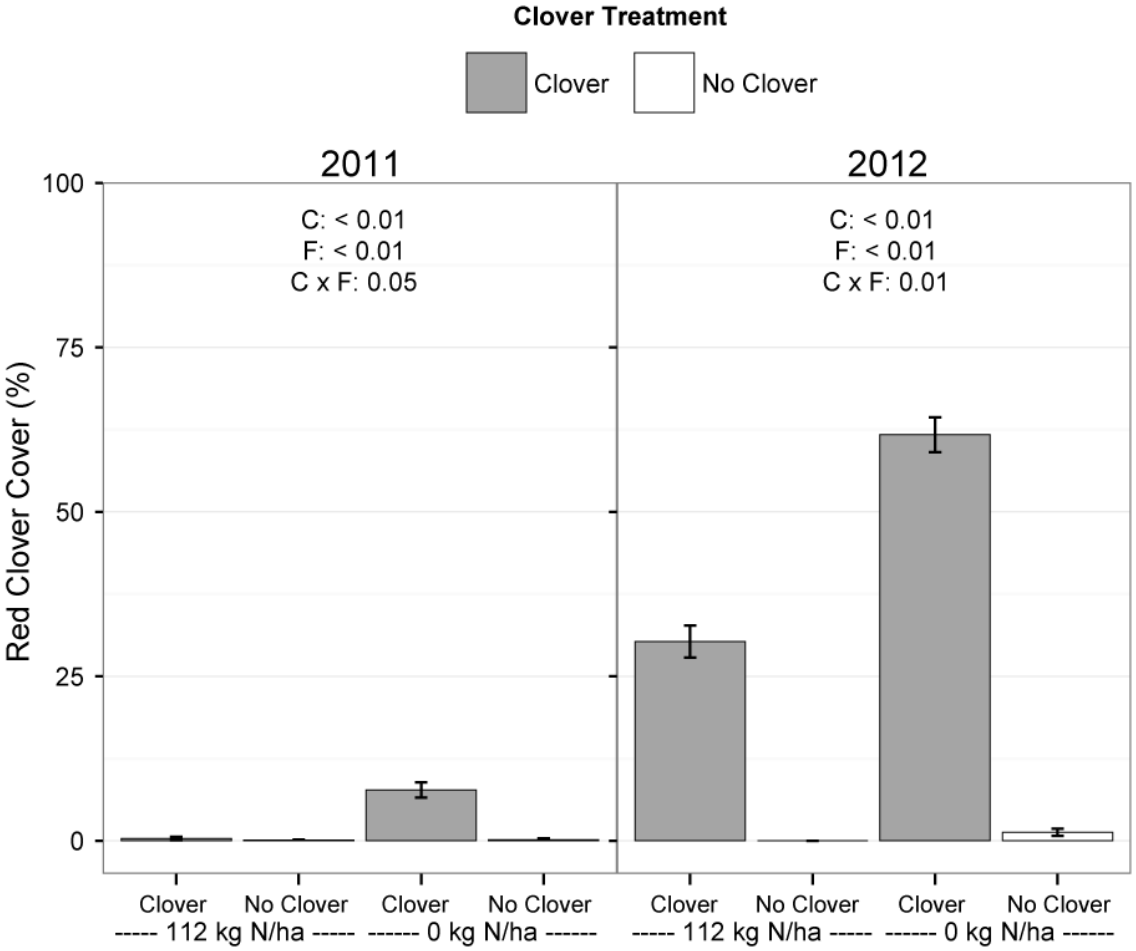


Figure 2. Biomass yields (Mg ha^{-1}) by main-plot treatments in years 2011 and 2012. Error bars represent standard errors and p-values are shown in the legends (F = fertilizer, and C = clover).

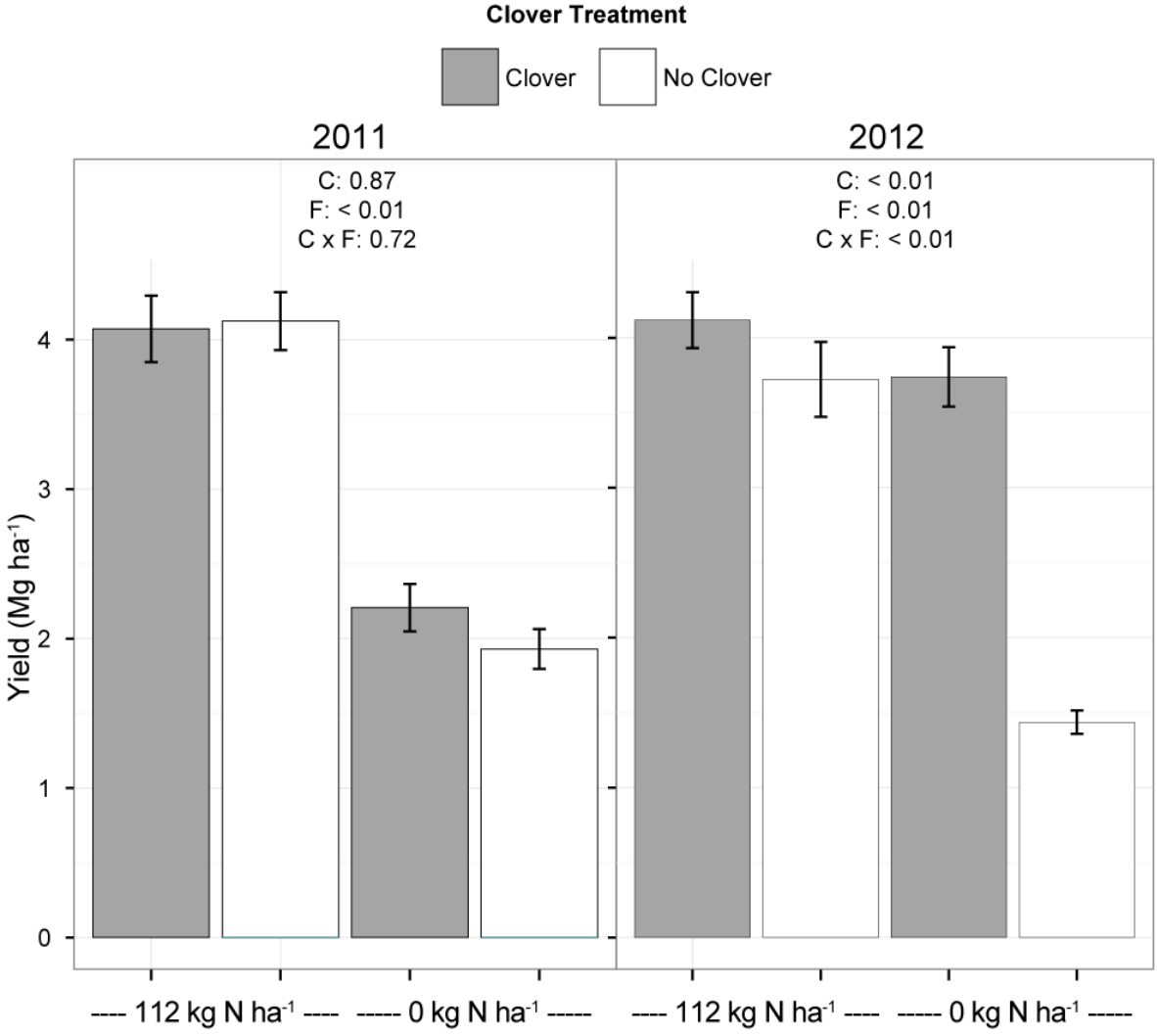


Figure 3: Biomass yield response of (A) fertilizer addition and (B) clover addition plotted as least-squares means in 2012. Numbers refer to the accession number in Table 1. p-values are shown in the legend (Acc = accession, F = fertilizer, and C = clover).

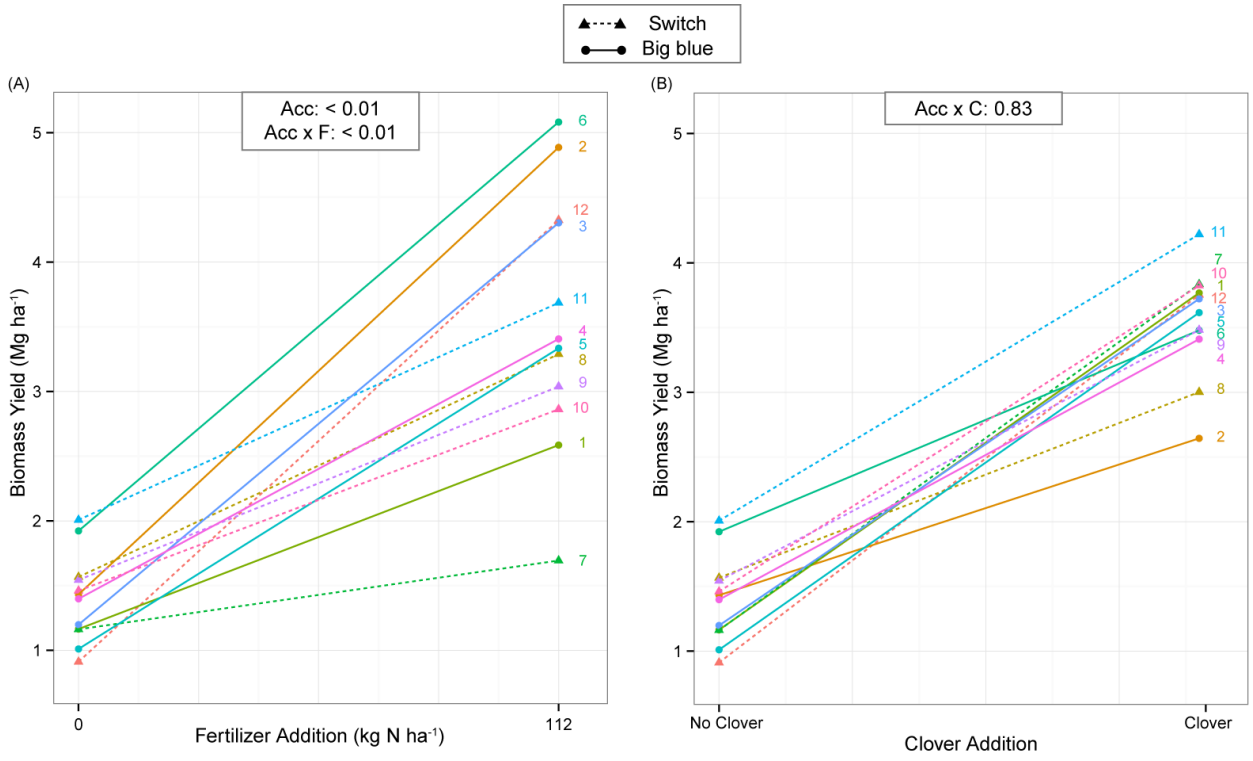


Figure 4. Biomass yield contributions (Mg ha^{-1}) of the warm-season grasses (WSG) and red clover (RC) in 2012. Error bars represent standard errors and p-values are shown in the legends (F = fertilizer, and C = clover).

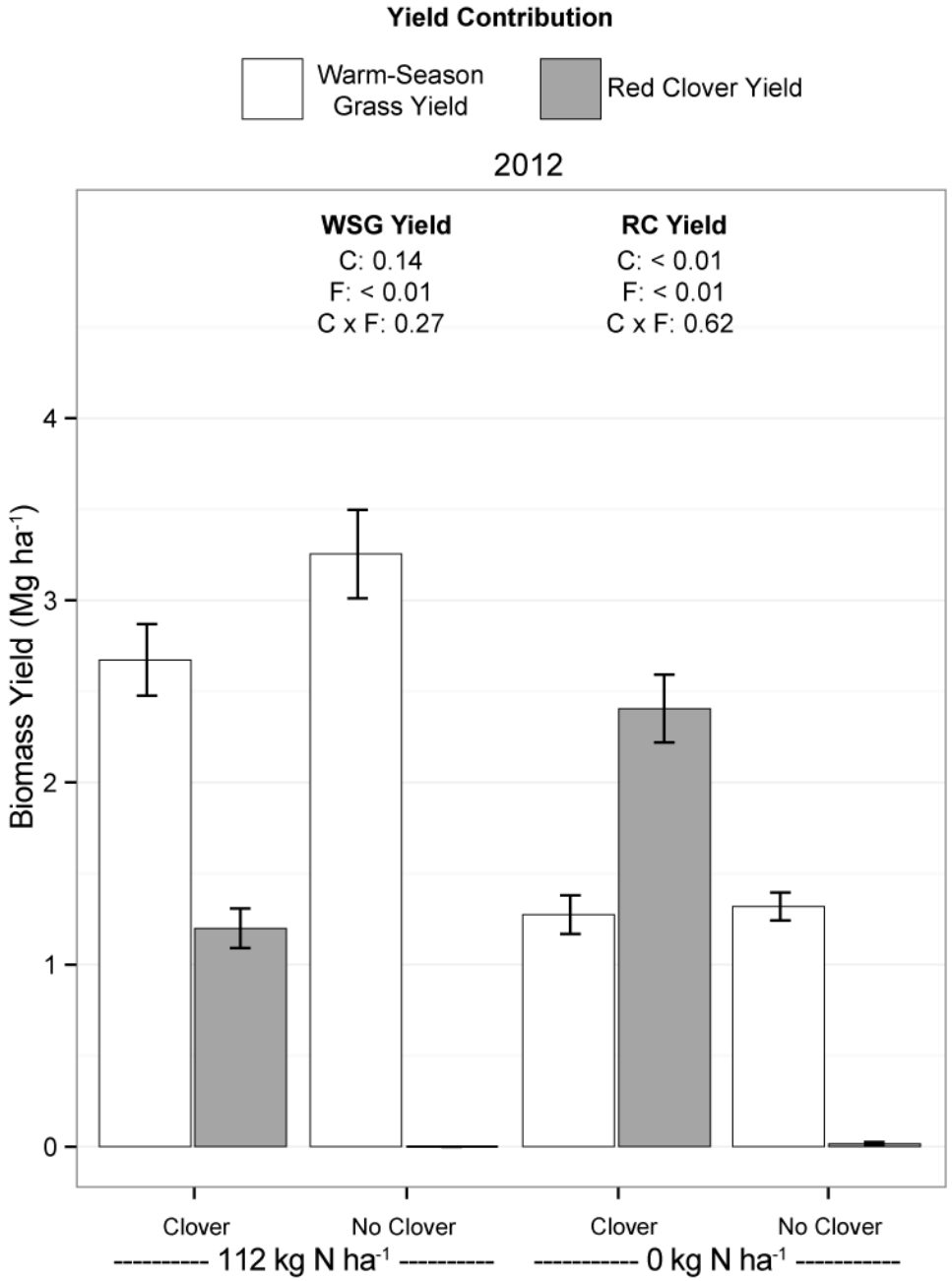
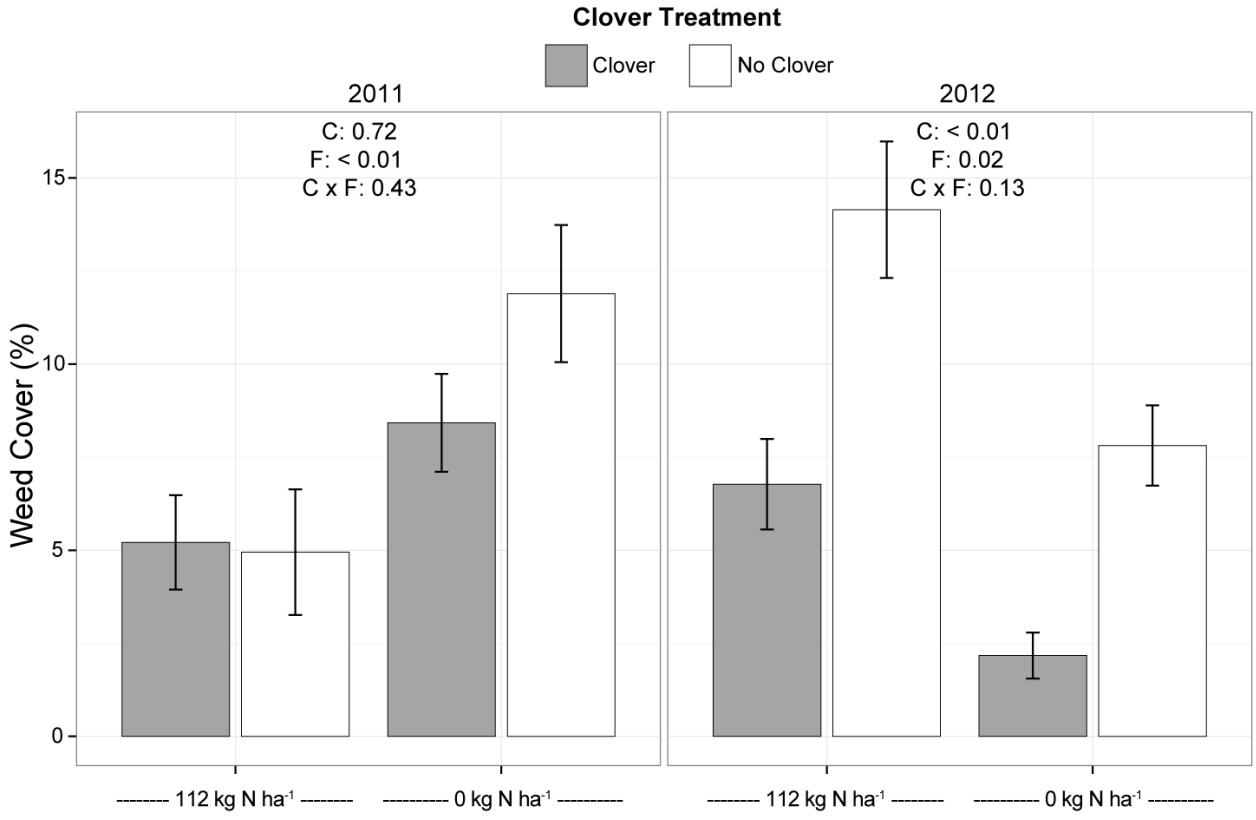


Figure 5.The impacts of clover addition and fertilizer on weed cover (%) during the 2011 establishment season and 2012. Error bars represent standard errors and p-values are shown in the legends (F = fertilizer, and C = clover).



Chapter 3

Increased genetic diversity improves productivity and invasion resistance in perennial warm-season grasses

3.1 Abstract

Exploring the impacts of genetic diversity in ecological systems is an exciting frontier of ecological study. Previous studies have identified benefits of genetic diversity, but done so using intensively managed mixtures of genotypes. The purpose of this experiment was to detect diversity effects when genetic diversity is manipulated at the population-level using switchgrass monocultures, big bluestem monocultures, and mixtures of the two species. The objectives of this study were to (i) determine whether genetic, morphological, and geographic diversity are predictive of productivity and weed cover and (ii) explore the mechanisms underlying the diversity effects. Genetic diversity was the best predictor of productivity and weed cover, with increased diversity resulting in an increase in productivity of up to 6% and a reduction in weed cover of up to 18.4% in switchgrass and big bluestem monocultures. Only switchgrass genetic diversity was predictive of productivity in species mixtures, but total genetic diversity in species mixtures reduced weed cover by 8.7%. Both the sampling effect and complementarity were found to be contributing to the genetic diversity effect. The benefits of increased genetic diversity were stable across fertility levels, although the occurrence of transgressive overyielding was more common in unfertilized crops. Increasing genetic diversity levels in agricultural systems may be a more palatable than increasing species richness to take advantage of the benefits of diversity to ecosystem functioning due to the simpler management strategies required.

3.2 Introduction

The relationships between biodiversity and ecosystem function, and the study of the mechanisms underlying these relationships, have been a focus of study in the ecological community for decades. Significant advances have occurred at the species-level where a range of ecosystem responses have been evaluated in field experiments with controlled diversity levels (Balvanera et al. 2006; Naeem 1998; Tilman 1999; Tilman 1996). This work has identified general positive correlations between species richness and measures of productivity, stability, and resilience (Cardinale et al. 2006; Naeem and Wright 2003), although many studies have identified no or a negative effect of species richness (Cardinale et al. 2007). More recently, functional and phylogenetic diversity have been found to be more informative metrics than species richness for predicting diversity effects (Cadotte et al. 2009; Petchey and Gaston 2006; Srivastava et al. 2012). However, stability of community- and ecosystem-level properties may come at the expense of individual species persistence (Hector et al. 2010; Loreau and de Mazancourt 2013)

Researchers have also designed experiments to link genetic diversity with ecosystem function (Hughes et al. 2008b). Results have shown intraspecific diversity to be positively correlated with ecosystem recovery (Hughes and Stachowicz 2004; Reusch et al. 2005), productivity (Crawford and Whitney 2010), maintenance of species-level diversity (Booth and Grime 2003), resistance to invasion (Crutsinger et al. 2008), and altered arthropod and plant community structure (Crutsinger et al. 2006). However, most studies have tested genetic or species diversity separately. The importance of genetic richness in ecosystem functioning relative to species richness is debated and remains a fertile research area (Bolnick et al. 2011).

The mechanisms underlying diversity effects have been linked to three general ecological phenomena: the sampling effect, niche complementarity, and facilitation (Hooper et al. 2005). The sampling effect, also known as the selection probability effect, is expected to increase productivity based on probability. As more species or genotypes are included in a community or population, the higher the likelihood of including the most productive species or genotypes in the community (Loreau and De Mazancourt 2013; Münzbergová et al. 2009). Complementarity occurs when species or genotypes partition resources so that the total productivity is greater than would be expected by combining the traits of the two species or genotypes individually (Ellers et al. 2011; Hughes and Stachowicz 2010; Loreau and Hector 2001). The more complete resource use in mixtures results in increased productivity and resistance to invasion and is identified by productivity in mixtures exceeding the average of the species or genotypes when grown in monoculture (overyielding Ewel 1986; Loreau 1998; Trenbath 1974). Complementarity of diverse mixtures is thought to be a critical way in which species utilize their genetic diversity to establish in new environments (Hughes et al. 2008b). Facilitation occurs when establishment of one species or genotype enables the establishment or success of other species or genotypes (Chapin et al. 1994; Fowler 1986; Mulder et al. 2001).

While the benefits of increased species richness on ecosystem function are widely appreciated in the ecological community, the agricultural community has been slow to embrace these concepts. The reluctance of farmers to use mixtures stems from three primary issues (Picasso et al. 2008; Vandermeer et al. 2004). First, intensive agricultural management (fertilization and defoliation) reduces plant species richness, making long-term management of diverse mixtures difficult (Gough et al. 2000; Kirchner 1977). Second, modern agricultural equipment is designed for use in monocultures (Tooker and Frank 2012), which creates significant social and economic hurdles that inhibit adoption of diverse cropping systems.

Third, yields of mixtures are generally not greater than yields of the best monoculture, i.e. transgressive overyielding (Trenbath 1974) is not a common phenomenon. A meta-analysis of 44 diversity experiments found that the most diverse mixture produced on average 12% less biomass than the best monoculture as compared to 170% more biomass than the average of all species when grown in monoculture (Cardinale et al. 2007). As a result, many intense agricultural systems continue to be monocultures. Pasture and perennial bioenergy cropping systems have been identified as the most feasible for taking advantage of the benefits of diversity (Bonin and Tracy 2012; Davis et al. 2012; Picasso et al. 2008; Sanderson et al. 2004; Tilman et al. 2006; Webster et al. 2010)

The agricultural community may be more amenable to increasing genetic diversity within monocultures (Hajjar et al. 2008). The increased stability of cultivar mixtures (population buffering) has a long history in agriculture, particularly when cultivar by environment interactions are substantial (Allard and Bradshaw 1964; Eberhart and Russell 1966). More recently, population buffering has been explored in mixed forage systems and has been found to stabilize production across varying environmental conditions (Smithson and Lennè 1996; Tooker and Frank 2012; Williams et al. 2003).

Although previous research has identified benefits associated with genetic diversity, the majority of this research has intensively controlled the establishment of diversity using cloned genotypes or tested effects at small spatial scales. This work has been critical in identifying the benefits of increased genetic diversity, but has not demonstrated the benefits of genetic diversity under management-relevant conditions.

We sought to determine whether genetic diversity benefits could be measured in agronomic trials of potential bioenergy crops. First, we evaluated whether genetic morphological diversity were

predictive of productivity and weed cover in switchgrass (*Panicum virgatum* L.) monocultures, big bluestem (*Andropogon gerardii* Vitman.) monocultures, and mixtures of these two species. Second, we determined whether the number of accessions present or the geographic diversity in a mixture could be used as a proxy for genetic and morphological diversity. Third, we explored the underlying mechanisms of the expressed diversity effects.

3.3 Methods

Field Trials

Monocultures and mixtures made up of six accessions of switchgrass and six accessions of big bluestem were established at Arlington, WI on 1 June 2010 (Table 1). The accessions consisted of improved cultivars and seed increases of wild populations. The soil type was a Plano silt loam (fine-silty, mixed, mesic Typic Argiudoll). The experimental design was a split-plot randomization restriction within a randomized incomplete block design with six replicates. Main plots consisted of a 2×2 factorial: nitrogen addition (112 kg N ha^{-1} and 0 kg N ha^{-1}, applied as ammonium nitrate) to test the stability of diversity effects across fertility levels combined with red clover overseeding as a weedy invader (7.2 kg pure live seed ha^{-1} and no clover addition). Subplots consisted of 25 combinations of monocultures and mixtures of switchgrass and big bluestem accessions (Supplemental Table 1) Twenty-one treatments were fully replicated, while the remaining four treatments were made up of pairwise mixtures of big bluestem, switchgrass, and two types of mixtures of the species. Grass seeding rates were adjusted for germination, using standard germination procedures (AOSA 1998) and a seeding rate of 650 pure live seeds m^{-2}. In mixtures, all accessions present were planted using equal numbers of pure live seeds. Subplots were drill seeded in 10 rows with 15-cm spacing between rows and an overall size of 3.1 m^2.

Plots were allowed to establish during 2010 with no main plot treatments or management other than clipping for weed control on 1 September 2010. The main-plot treatments were initiated 10 May 2011 following application of glyphosate at a rate of 1.2 kg active ingredient ha^{-1} for weed control. Ammonium nitrate was again applied on 1 May 2012.

Harvestable biomass was measured on all plots in 2011 and 2012 using a flail-type harvester with a cutting height of approximately 8 cm and was used as a proxy for aboveground net primary productivity. Harvest dates were 22 September 2011 and 29 August 2012. Dry matter concentration was determined on a sample of harvested biomass ranging from 100 to 300 g. Samples were dried at 60°C in a forced-air dryer. All biomass yields were adjusted to a dry matter basis using the dry matter concentration. Weed cover proportions were determined from 16-point estimates (15-cm spacing between points) in each subplot in early August of each year.

Diversity metrics

Genetic diversity of each species was evaluated using 14 simple sequence repeat (SSR) markers in switchgrass and 16 SSR markers in big bluestem on 16 individual plants for each accession (Supplemental Table 2). The individual plants were sampled randomly from seeds grown from each accession. The forward primer for each SSR was synthesized with the universal M13 tail (CACGACGTTGTAAAACGAC) at the 5' end to facilitate fluorescent labeling (Schuelke 2000). The M13 tail was labeled either with carboxyfluorescein (FAM) or hexachlorofluorescein (HEX) fluorescent tags. In addition, to reduce polyadenylation and improve genotyping, the PIG sequence (GTTTCTT) was appended to the 5' end of the reverse primer (Brownstein et al. 1996). Polymerase chain reactions (PCR) were performed in 8 ul total volume using 3.5 ul 1X JumpStart REDTaq ReadyMix (Sigma, St. Louis,

MO, USA), 10 ng genomic DNA, 1.25 ul of H2O, 0.5 ul 5 M M13-FAM/HEX primer, 0.5 ul 5 M reverse/0.5 M forward primer, 0.125 ul 5 M betaine (Sigma, St. Louis, MO, USA), and 0.125 ul 50 mg/ml BSA (CHIMERx, Milwaukee, WI, USA). Thermocycling conditions consisted of an initial melting step (94°C for 3 min), followed by 35 cycles of 94°C for 15 s, 54°C for 90 s, and 72°C for 2 min, and an elongation step (72°C for 20 min), followed by an indefinite soak at 4°C. PCR products (2 ul) using different fluorescent labels (i.e., FAM and HEX) were pooled and combined with 15 ul Hi-Di formamide (Applied Biosystems, Foster City, CA, USA) and 0.15 ul of carboxy-X-rhodamine (ROX) custom size standard (Custom MapMarker, BioVentures, Murfreesboro, TN, USA). SSR allele genotyping was performed using an ABI 3730 fluorescent sequencer (POP-6 and a 50-cm array; Applied Biosystems, Foster City, CA, USA). Alleles were scored in a binary format as presence (1) or absence (0) of bands due to potential ploidy differences among accessions and the inability to determine allele dosage using GeneMarker Software version 1.7 (SoftGenetics, State College, PA, USA).

The genetic diversity for each accession was calculated as the total number of unique alleles (allelic richness) identified within the 16 samples of each accession divided by the total number of unique alleles identified across all accessions within a species (Allendorf 1986; Frankel et al. 1995; Hughes et al. 2008a). In intraspecific mixtures, genetic diversity was calculated as the total number of unique alleles present across the accessions present in the mixture divided by the total number of unique alleles identified in the species. Genetic diversity in species mixtures was calculated similarly, where the number of unique alleles in a mixture was summed for all included accessions and the denominator was the sum of the total number of unique alleles identified in both species. Principal component analyses (PCA) were plotted to identify patterns of genetic diversity within each species using the base package in R 2.15.2 (R Development Core Team 2012).

Morphological diversity within and among accessions was calculated by measuring the following variables on six unfertilized plots of each accession grown in monoculture: inflorescence number, average inflorescence height, average sward height, average panicle or spikelet length and width, seed weight and area, biomass yield, biomass moisture content at harvest, and flowering time across both season (obtained from 8 weekly measurements in both seasons from July to August). A PCA was used to reduce the dimensions of this dataset across species, keeping 10 principal components, using the base package in R. Within-accession morphological diversity was calculated as the average distance among the six measured plots using 10 principal components. In mixtures, the morphological diversity was calculated as the average distance among all individuals within each accession present in the mixture using 10 principal components.

To examine whether the geographic distance of the accessions in a mixture could be used as a proxy for genetic or morphological diversity, a geographic diversity metric was calculated for each treatment. The geographic distance in mixtures with two accessions present was the distance between the origins of the accessions. For mixtures with more than two accessions, the length of the shortest one-way route between all accession origins was used. The length of the shortest one-way route was determined using the *traveling salesman* algorithm as implemented in the R package *TSP* (Hahsler and Hornik 2011; Hahsler and Hornik 2007). The correlation among genetic diversity, morphological diversity, geographic distance, and the number of accessions in a plot was calculated using Pearson correlation coefficients.

Statistical Analysis

Maximum likelihood mixed-effects models were developed using the *lme4* package (Bates et al. 2012) in R 2.15.2 for predicting harvestable biomass. Harvestable biomass values were square root transformed so that model residuals were normally distributed and homoscedastic. For all analyses, big bluestem, switchgrass, and species mixtures were treated as separate datasets. The different diversity metrics were tested individually by adding each term to a model with main-plot and the appropriate blocking terms incorporated. Each diversity effect was log transformed because we expected an asymptotic response at high diversity levels (Hooper et al. 2005). Significance of the diversity effect was determined by comparing a fully specified model with the diversity effect term to the model without the diversity effect term using a likelihood ratio test (Crawley 2007). The fertilizer by diversity metric interaction was also tested to determine whether diversity effects were stable across fertility levels.

Weed cover was treated as count data and modeled by comparing models with Poisson and two types of negative binomial distributions (Hardin and Hilbe 2007), with and without zero inflation, using the *glmmADMB* package in R (Skaug et al. 2011). The model type with the lowest Akaike Information Criterion (AIC) value was selected for modeling the distribution for each dataset. Diversity effects on weed cover were tested using the same approach used to evaluate their effect on harvestable biomass. Parameter estimates for significant diversity metrics were normalized to represent the magnitude of the effect of the diversity metric moving from the least diverse treatment to the most diverse treatment in the respective dataset. For geographic distances, the parameter estimate was normalized to the effect per 1000 km of distance.

The presence of an individual accession in a mixture was tested for predictive ability for harvestable biomass and weed cover to test for the presence of a sampling effect. A two-factor term

defining the presence or absence of an accession in a mixture was added to a fully defined model and tested for significance using a likelihood ratio test.

The magnitude of overyielding in mixtures was compared to the average of all monocultures and the best monoculture across years (transgressive overyielding) separately for each dataset and fertility level. The least-squares means were estimated for each monoculture and mixture from a full-mixed effect model using the *lsmeans* package in R (Lenth 2012). In species mixtures, the average and best monoculture value was determined using all 12 accessions.

3.4 Results

Diversity metrics

The genetic markers identified similar diversity patterns in both species among the accessions tested (Table 2). For switchgrass, the PCA clustered the four accessions collected in the Upper Midwest closely, while the two remaining accessions ('Blackwell' and 'Shelter') were more divergent (Figure 1a). The number of alleles identified in switchgrass accessions varied from 51 (Chiwaukee) to 102 ('Blackwell') alleles present (of 113 unique alleles), averaging 70 alleles per accession. A similar pattern of genetic diversity was present in big bluestem. The PCA clustered the four Upper Midwest collections, while 'Kaw' and 'Niagra' were divergent (Figure 1b). The number of alleles identified in big bluestem accessions varied from 63 ('Niagra') to 93 ('Kaw') alleles present (of 116 unique alleles), averaging 78 alleles per accession.

The morphological diversity metric separated the two species and grouped samples from each accession closely (Figure 2, Table 2). The ten principal components used to calculate distances

accounted for 78% of the variation in the morphological measurements. The average distance within each switchgrass and big bluestem accession was 0.40 and 0.43, respectively. 'Shelter' switchgrass and 'Niagra' big bluestem had the largest within-accession diversity at 0.48 and 0.73, respectively. The average morphological distance among mixtures of switchgrass, big bluestem, and species mixtures was 0.48, 0.52, and 0.78, respectively. The average geographic distance of mixtures was 556 km, 554 km, and 709 km for switchgrass, big bluestem, and species mixtures, respectively.

The genetic, morphological, and geographic distance metrics were correlated with one another and with the number of accessions in a mixture. Genetic diversity and morphological diversity were highly correlated with a Pearson correlation coefficient of 0.75. The correlation between genetic and geographic distance was weaker with a value of 0.48. The correlation coefficient between geographic and morphological distance was 0.49. The correlation between the number of accessions in a mixture and genetic, morphological, and geographic diversity was 0.63, 0.47, and 0.73, respectively.

Diversity impacts on aboveground biomass production and weed cover

The benefits of increased diversity on harvestable biomass varied by metric and species (Table 2). For big bluestem, the genetic diversity metric was significant with a normalized estimate of 0.18 Mg ha^{-1} ($p < 0.01$, Figure 3a). The genetic diversity effect did not vary by fertilizer levels ($p = 0.98$). Geographic diversity was significant, but had a minor effect, increasing harvestable biomass by 0.001 Mg ha^{-1} per 1000 km of distance ($p = 0.03$). In switchgrass, the genetic diversity metric was significant, increasing biomass production by a normalized estimate of 0.08 Mg ha^{-1} ($p < 0.01$, Figure 3b). Increasing morphological diversity had a similar effect as genetic diversity in switchgrass, increasing production by 0.06 Mg ha^{-1} ($p < 0.01$). Geographic diversity was near significant, but again had a minor effect of 0.005

Mg ha^{-1} per 1000 km of distance ($p = 0.05$). In species mixtures, total genetic diversity did not significantly increase harvestable biomass ($p = 0.10$, Figure 3c). However, the amount of switchgrass genetic diversity within the species mixture did have a minor effect, increasing production by a normalized estimate of 0.03 Mg ha^{-1} (Table 3). Geographic diversity was a significant predictor of harvestable biomass, but had a small effect (0.003 Mg ha^{-1} per 1000 km, $p = 0.02$). The number of accessions present in a mixture was not a significant predictor of production in any of the datasets ($p = 0.07$ in big bluestem, $p = 0.08$ in switchgrass, and $p = 0.57$ in species mixtures).

The benefits of increasing diversity for reducing weed cover were similar to the effects on harvestable biomass (Table 3). In big bluestem, genetic diversity was a significant predictor of weed cover, reducing weed cover by a normalized estimate of 9.4% ($p = 0.03$, Figure 4a). No other diversity metrics were significant predictors of weed cover in big bluestem. In switchgrass, the genetic diversity metric was significant, but was modified depending on fertility level. In unfertilized switchgrass, increasing genetic diversity reduced weed cover by 11.2% ($p < 0.01$, Figure 4b). In fertilized switchgrass, increasing genetic diversity reduced weed cover by 18.4% ($p = 0.01$). Increasing morphological diversity also reduced weed cover in switchgrass by 6.4% ($p = 0.04$). In species mixtures, increasing total genetic diversity reduced weed cover by 8.7% ($p = 0.03$, Figure 4c). Increasing individual species genetic diversity in species mixtures significantly reduced weed cover as well (12.7% reduction, $p = 0.03$ for big bluestem; 6.0% reduction, $p = 0.05$ for switchgrass).

Mechanisms underlying diversity effects

In switchgrass, the observation of overyielding depended on fertility level. In unfertilized plots, six of nine mixtures yielded greater than the average of all monocultures, with five of those six yielding

greater than the best monoculture (Figure 5a). However, in fertilized plots, only two of nine mixtures yielded greater than the average monoculture, with neither of these mixtures yielding greater than the best monoculture (Figure 5b). The presence of five of the switchgrass accessions in a mixture had significant effects on harvestable biomass (Table 4). When Chiwaukee switchgrass was included in a mixture, it reduced biomass yields by 0.45 Mg ha^{-1} on average ($p < 0.01$). When 'Blackwell' was included, yields increased by 0.37 Mg ha^{-1} on average ($p < 0.01$). Four of the switchgrass accessions had significant effects on weed cover in mixtures as well (Table 3). When Chiwaukee switchgrass was present, weed cover increased by an average of 4.1% ($p < 0.01$). When 'Blackwell' was present, weed cover was reduced by 4.5% ($p < 0.01$).

Similar patterns of overyielding were found in big bluestem. In unfertilized plots, six of nine mixtures yielded greater than the average of monocultures, with two of those six yielding greater than the best monoculture (Figure 5c). In fertilized plots, six of nine mixtures yielded greater than the average of the monocultures, but no mixtures yielded greater than the best monoculture (Figure 5d). Inclusion of four of the big bluestem accessions had significant effects on harvestable biomass (Table 4). As in switchgrass, inclusion of Chiwaukee big bluestem in a mixture reduced the yield by 0.53 Mg ha^{-1} ($p < 0.01$). Inclusion of 'Kaw' increased yields by 0.44 Mg ha^{-1} ($p < 0.01$). Only Chiwaukee big bluestem had a significant effect on weed cover among big bluestem accessions, increasing weed cover by 2.6% when included in a mixture ($p = 0.04$).

In species mixtures, differences among fertility levels were less pronounced. In unfertilized plots, seven of fifteen mixtures yielded greater than the average of all monocultures, while two of these mixtures yielded greater than the best monoculture (Figure 5e). In fertilized plots, nine of 15 mixtures

yielded greater than the average of the monocultures, while one mixture yielded greater than the best monoculture (Figure 5f).

3.5 Discussion

Genetic diversity predicts harvestable biomass and weed resistance

Independent tests in two species found genetic diversity to be a significant predictor of harvestable biomass and weed cover. In both switchgrass and big bluestem, metrics of genetic diversity using neutral markers had a positive relationship with harvestable biomass and a negative relationship with weed cover. In both species, the directions of these relationships were stable across fertility levels. Within the diversity range of our experiment, increasing diversity from the lowest level to the highest level increased harvestable biomass by as much as 0.18 Mg ha^{-1} and reduced weed cover by as much as 18.4%. The productivity benefit of increasing genetic diversity was approximately 6% of the average harvestable biomass. This productivity increase is substantially smaller than the 70% increase found in a meta-analysis of species mixtures (Cardinale et al. 2007). The difference in size of diversity effects may be due to the difference in the magnitude of the range of functional traits within a species and among species. On average, there is likely a greater functional range among species compared to the functional range within a species (Hughes et al. 2008a). A greater functional range should lead to more pronounced sampling effects and a higher likelihood of complementarity between species (Díaz and Cabido 2001).

The number of accessions present in a mixture was not a significant predictor of harvestable biomass or weed cover in either species. The genetic and morphological diversity metric likely provided more information regarding the true diversity of a mixture than the number of accessions. If accessions

have similar genetic backgrounds and similar morphologies, a count of the number of accessions overestimates the functional diversity present within a mixture (Petchey and Gaston 2006; Srivastava et al. 2012). More precise metrics of the relationships among species or populations are better predictors of diversity effects than the number of species or populations in a mixture (phylogenetic diversity vs. number of species, Cadotte et al. 2009). The genetic diversity metric also broadens the range of diversity tested by enabling comparisons of diversity among single accession plots.

Contrary to our expectations, morphological diversity was not a better predictor than genetic diversity (Jousset et al. 2011). While genetic and morphological diversity were highly correlated, morphological diversity was only a significant predictor for weed cover in switchgrass. The reason morphological diversity was not as good of a predictor as genetic diversity is unclear. Competition among individuals may have occurred belowground, but none of the traits that made up the morphological metric incorporated belowground metrics (Casper and Jackson 1997). Alternatively, the plasticity of a population may have been better captured by the genetic diversity metric than the morphological diversity metric (Hughes et al. 2008b).

The number of accessions or the geographic diversity in a mixture were not good proxies for genetic diversity. Geographic diversity had minor effects relative to genetic diversity on harvestable biomass and was not significant for predicting weed cover. Neither geographic diversity or the number of accessions were highly correlated with genetic diversity. Several concepts help to explain this finding. Both metrics may overestimate diversity levels, particularly in the two species evaluated in this study. In previous research investigating the population genetics and structure of switchgrass and big bluestem, interbreeding populations occurred on a relatively large scale (greater than 500 km) with high levels of

diversity within a site (Price et al. 2012; Zalapa et al. 2011; Zhang et al. 2011). Four of the six accessions in each species that were collected in the Upper Midwest came from closely related populations. A mix of these four accessions in either species may not increase genetic diversity much over a monoculture of one of these accessions. However, this finding may be species specific and should be able to be inferred by the reproductive strategy of a species (Hamrick and Godt 1996; Leimu and Fischer 2008). Many population genetic studies in species with alternative reproductive strategies have found genetic and geographic distance to be highly correlated, although a measure of ecological distance may prove more useful (McKay et al. 2005).

While increasing genetic diversity within a species monoculture has significant effects on harvestable biomass and weed cover, the benefits in species mixtures were less pronounced. Total genetic diversity calculated across species was not a significant predictor of harvestable biomass and was a weak predictor of weed cover in species mixtures. However, the amount of switchgrass genetic diversity present in the species mixtures was a significant predictor of harvestable biomass and the genetic diversity within both species had significant effects on weed cover. These results suggest that diversity within a species (genetic diversity) and diversity among species (species richness) may not be of the same magnitude. The range of morphological and functional diversity among species should be much greater than the range within a species. Determining the relationships between these two levels of organization is an exciting frontier in ecology. Studies that experimentally control both genetic and species diversity on a large-scale will help untangle these relationships. A second approach would be to leverage existing species richness experiments by evaluating the genetic diversity within each species. To our knowledge, all tests of species diversity have been blind to the amount of genetic diversity present within the species used in experiments. There are likely to be unintentional differences among

the genetic diversity of species included in diversity experiments that can be leveraged to determine the importance of genetic diversity at higher levels of species richness.

Mechanisms underlying the diversity effects

Our experiment provides evidence that both sampling and complementarity are contributing to the genetic diversity effect. The inclusion of particular accessions in mixtures had a significant effect on both harvestable biomass and weed pressure, supporting the sampling effect. Across both species, the inclusion of nine of the 12 accessions had significant effects on the harvestable biomass, with five accessions reducing biomass and four increasing biomass. Effects of individual accessions on weed cover were less evident with five of 12 accessions having significant effects. The inclusion of three accessions increased weed cover and two accessions reduced weed cover.

'Kaw' big bluestem and 'Blackwell' switchgrass had the largest effects for increasing harvestable biomass and reducing weed cover. Both of these accessions come from south of the experimental site, but are winter hardy. These late-maturing accessions consistently produced higher biomass yields when grown in monocultures in northern latitudes because they more fully utilize the growing season than accessions collected in the Upper Midwest (Casler et al. 2007; Madakadze et al. 1998).

The presence of transgressive overyielding of mixtures also provides strong support for a complementarity effect (Finn et al. 2013). In unfertilized plots, transgressive overyielding was much more common, occurring in nine of 33 mixtures. The mixture exhibiting the greatest gain over the highest-yielding monoculture occurred in switchgrass with a yield gain of 32%. However, in fertilized plots, only one of 33 mixtures yielded greater than the best monoculture. This mixture had an estimated yield gain over the best monoculture of 5%. Previous studies have found overyielding is more

likely to occur in fertilized conditions because of resource partitioning (Fridley 2003; Gross et al. 2007). The reduced occurrence of transgressive overyielding in fertilized plots may stem from the dramatic differences in fertilizer responses of the accessions included in the study (Jakubowski et al., unpublished data). In fertilized mixtures, the accessions that had the greatest response to fertilizer may have displaced other accessions, reducing the relative diversity and minimizing overyielding potential. A measure of the relative abundance of each accession throughout the study would be necessary for confirmation.

Implications for management

Increasing genetic diversity within a species monoculture can provide ecosystem and agricultural benefits, although these benefits may not be as substantial in species-rich plant communities (Whitham et al. 2006). Harvestable biomass was increased and weed cover was reduced with increased genetic diversity. Increasing genetic diversity should improve pest and disease resistance as well, providing greater stability for crop production in the face of biotic pressure (Tooker and Frank 2012). The planting of genetically diverse mixtures in a species monoculture has a long history in agricultural systems, particularly in cases where the success of accessions is strongly influenced by the environment (Smithson and Lennè, 1996; Williams et al., 2003). If the farmer is unlikely to select the best monoculture accession with any certainty, mixtures are a viable option (Finn et al. 2013). In pasture and bioenergy cropping systems where crops are likely to be planted on marginal lands with high variability across sites, mixtures may produce more stable yields in environments with high biotic and abiotic stress.

Conclusion

The benefits of increasing genetic diversity within a species in agriculturally-relevant mixtures is measurable in both switchgrass and big bluestem monocultures. In single species genetic mixtures, the benefits of increased genetic diversity were stable across fertility levels, although the occurrence of transgressive overyielding was more common in unfertilized crops. Both sampling and complementarity effects appear to be contributing to the observed diversity effect. The benefits of genetic diversity in species-level mixtures are less clear and may not scale directly between levels of organization, but are likely of a smaller magnitude than species richness effects. Increasing genetic diversity levels in agricultural systems may be a more palatable approach to taking advantage of the benefits of diversity to ecosystem functioning because of the simpler management strategies required.

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3.7 Tables

Table 1. Origin and background information of the 12 populations included in the study.

Species	Name	Improved cultivar?	Info	State	Lat	Long
A. gerardii	Chiwaukee	No	Chiwaukee prairie	WI	42.53	-87.82
A. gerardii	'Central Iowa' Ecotype	No	Central Iowa	IA	-	-
A. gerardii	Shooting Star	No	Houston County, MN	MN	43.59	-91.36
A. gerardii	'SouthLow' Ecotype	No	Southern Lower Peninsula, MI	MI	-	-
A. gerardii	'Niagra'	Yes	New York	NY	42.70	-78.72
A. gerardii	'Kaw'	Yes	Flint Hills, KS	KS	38.37	-96.54
P. virgatum	Chiwaukee	No	Chiwaukee prairie	WI	42.53	-87.82
P. virgatum	'Central Iowa' Ecotype	No	Central Iowa	IA	-	-
P. virgatum	Shooting Star	No	LaCrosse County, WI	WI	43.79	-91.13
P. virgatum	'SouthLow' Ecotype	No	Southern Lower Peninsula, MI	MI	-	-
P. virgatum	'Shelter'	Yes	St Marys, West Virginia	WV	39.40	-81.20
P. virgatum	'Blackwell'	Yes	Blackwell, OK	OK	36.81	-97.28

Table 2. The average (minimum and maximum) of the diversity metrics across the 15 treatments of switchgrass monocultures, big bluestem monocultures, and mixtures of the two species.

Diversity Metric	Switchgrass	Big Bluestem	Species Mixtures
Genetic	0.75 (0.45, 1)	0.76 (0.54, 1)	0.69 (0.54, 1)
Morphological	0.48 (0.30, 0.63)	0.52 (0.27, 0.74)	0.78 (0.63, 0.95)
Geographic (km)	556 (0, 1981)	554 (0, 1812)	709 (0, 2418)

Table 3. The predictive ability of the diversity metrics for harvestable biomass and weed cover within each dataset. Estimates are normalized to represent the effect of moving from the least diverse to most diverse treatment within each dataset. For geographic distances, the estimate is calculated for the effect of 1000 km of distance. Estimates are included for any diversity metrics with p values less than 0.10.

		Harvestable Biomass (Mg ha^{-1})		Weed Cover (%)	
Species	Diversity Metric	p value	Estimate	p value	Estimate
Big Bluestem	Genetic	< 0.01	0.18	0.03	-9.4
	Genetic*Fert	0.98	-	0.74	-
	Number Acc's	0.07	0.01	0.20	-
	#Acc's*Fert	0.78	-	0.26	-
	Morphological	0.52	-	0.65	-
	Morpho*Fert	0.70	-	0.11	-
	Geographic	0.03	0.01 / 1000 km	0.09	-0.9 / 100 km
	Geo*Fert	0.72	-	0.24	-
Switchgrass	Genetic	< 0.01	0.08	< 0.01	-11.2
	Genetic*Fert(100)	0.09	0.03	0.01	-7.2
	Number Acc's	0.08	0.01	0.17	-
	#Acc's*Fert	0.60	-	0.40	-
	Morphological	< 0.01	0.06	0.04	-6.4
	Morpho*Fert	0.84	-	0.33	-
	Geographic	0.05	0.05 / 1000 km	0.25	-
	Geo*Fert	0.64	-	0.55	-
Species Mixtures	Total Genetic	0.10	-	0.03	-8.7
	Total Genetic*Fert	0.90	-	0.23	-
	BB Genetic	0.80	-	0.03	-12.7
	BB Genetic*Fert	0.92	-	0.08	-13.2
	SW Genetic	0.02	0.03	0.05	-6.0
	SW Genetic*Fert	0.70	-	0.48	-
	Number Acc's	0.57	-	0.16	-
	#Acc's*Fert	0.85	-	0.22	-
	Morphological	0.12	-	0.66	-
	Morpho*Fert	0.14	-	0.96	-12.7
	Geographic	0.02	0.03 / 1000 km	0.83	-
	Geo*Fert	0.46	-	0.58	-

Table 4. Estimates of yield and weed cover effects of individual accessions when included in mixtures.

		Yield Effect (Mg ha^{-1})		Weed Cover Effect (% Weeds)	
Species	Accession	P value	Effect	P value	Effect
Big Bluestem	Chiwaukee	< 0.01	-0.53	0.04	2.58
	'Central Iowa'	0.14	-0.18	0.31	1.30
	Shooting Star	< 0.01	-0.37	0.08	2.18
	'SouthLow'	0.14	0.18	0.39	-1.08
	'Niagra'	< 0.01	0.36	0.39	-1.11
	'Kaw'	< 0.01	0.44	0.07	-2.31
Switchgrass	Chiwaukee	< 0.01	-0.45	< 0.01	4.14
	'Central Iowa'	0.03	-0.28	0.02	3.21
	Shooting Star	< 0.01	-0.36	0.15	1.94
	'SouthLow'	0.15	0.18	0.11	-2.15
	'Shelter'	0.02	0.30	0.01	-3.42
	'Blackwell'	< 0.01	0.37	< 0.01	-4.28

3.8 Figures

Figure 1. The first two principal components plotted from a PCA based on genetic analysis of the six populations of (A) switchgrass (SW) and (B) big bluestem (BB). Variance explained: BB PC1:6%, PC2: 5%, SW PC1:8%, PC2: 6%.

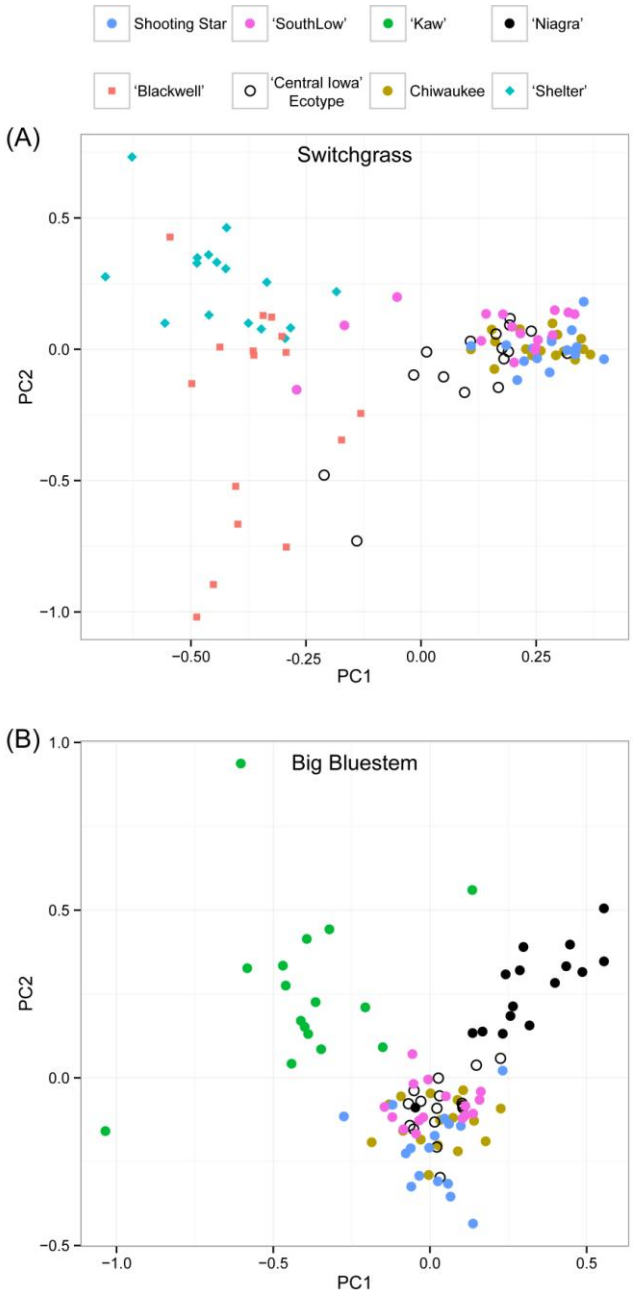


Figure 2. The first two principal components plotted from a PCA based on morphological analysis of the six accessions of switchgrass and six accessions big bluestem. Variance explained: PC1:37%, PC2: 9%.

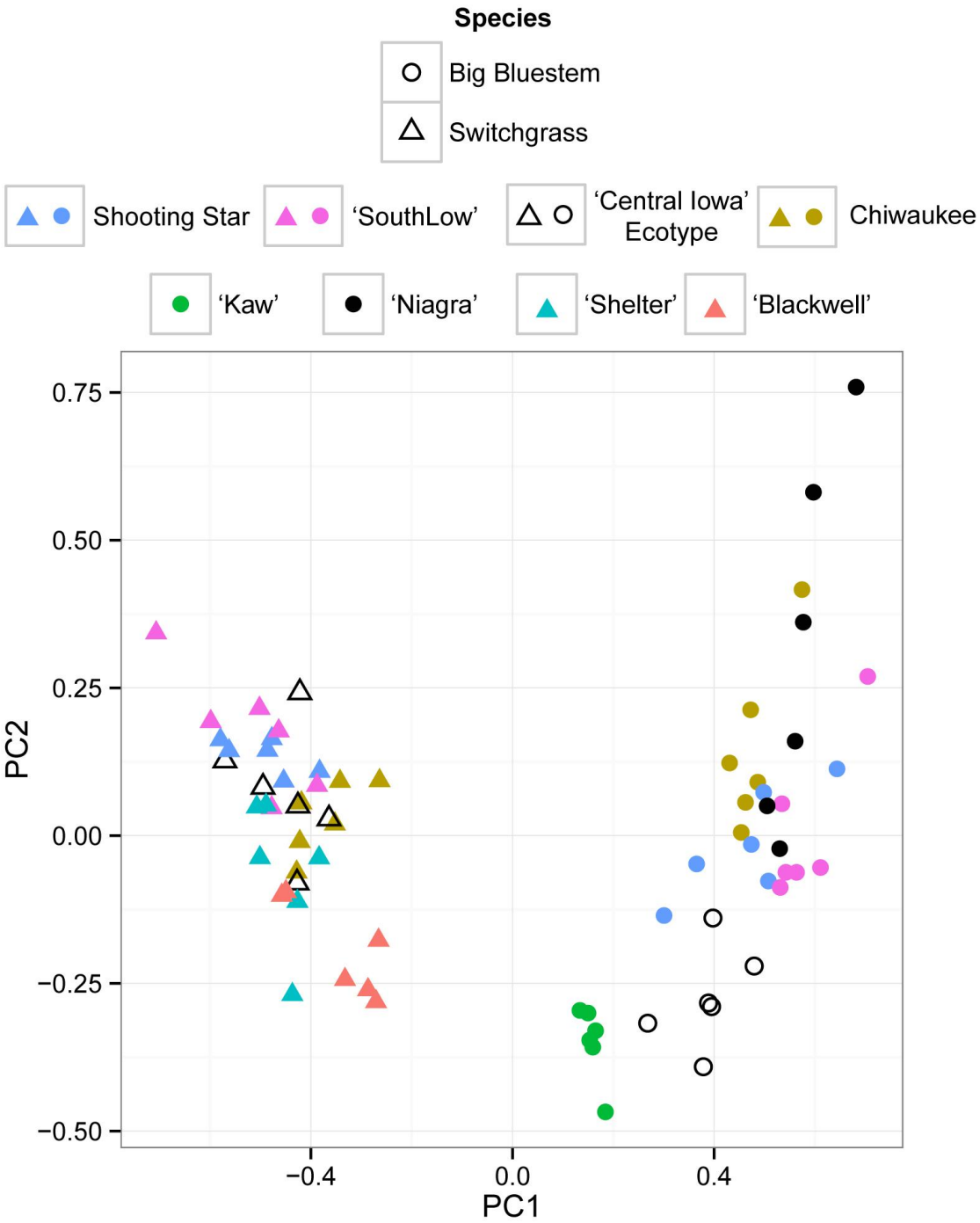


Figure 3. Harvestable biomass yields (Mg ha^{-1}) as a function of the amount of genetic diversity within a plot for (A) big bluestem ($p < 0.01$), (B) switchgrass ($p < 0.01$) , and (C) species mixture plots ($p = 0.10$). The genetic diversity metric was log transformed in each plot.

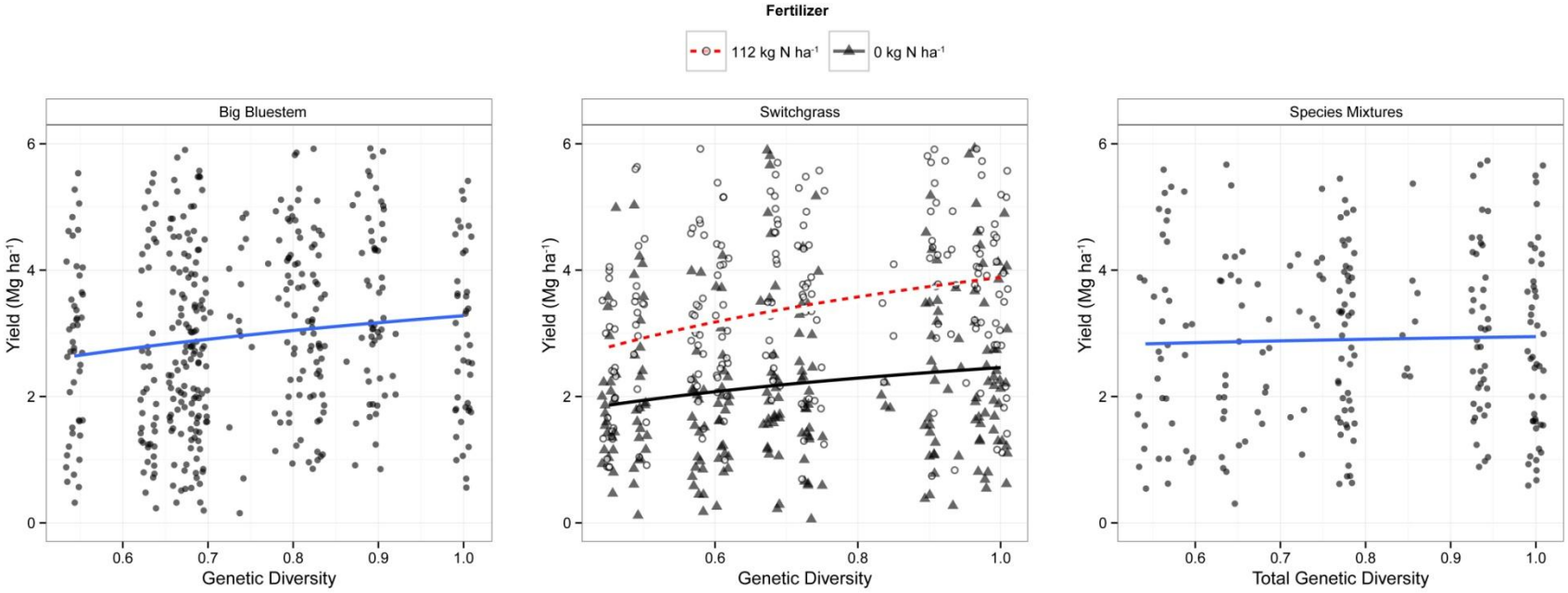


Figure 4. Weed proportion (% Weeds) as a function of the amount of genetic diversity within a plot for (A) big bluestem ($p = 0.03$), (B) switchgrass (genetic diversity: $p < 0.01$, genetic diversity x fertilizer: $p = 0.01$), and (C) species mixture plots ($p = 0.03$). The genetic diversity by fertilizer interaction was only significant for switchgrass. The genetic diversity metric was log transformed in each plot.

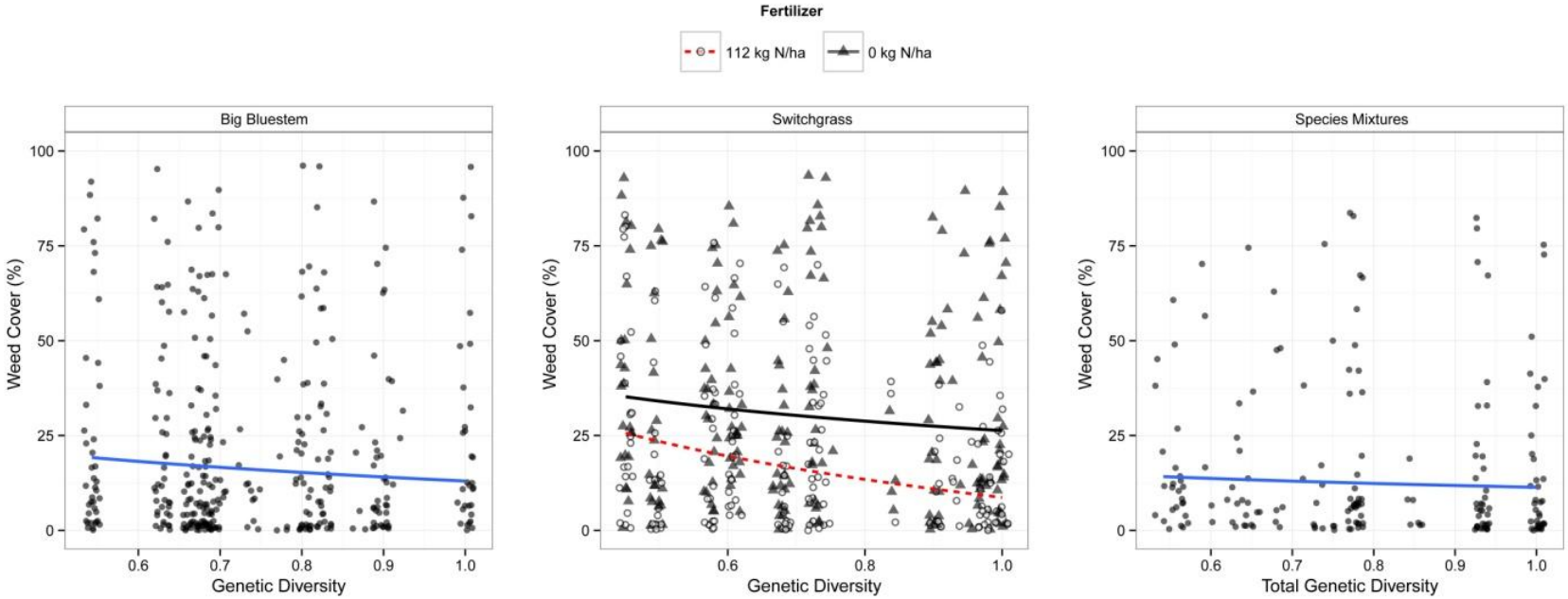
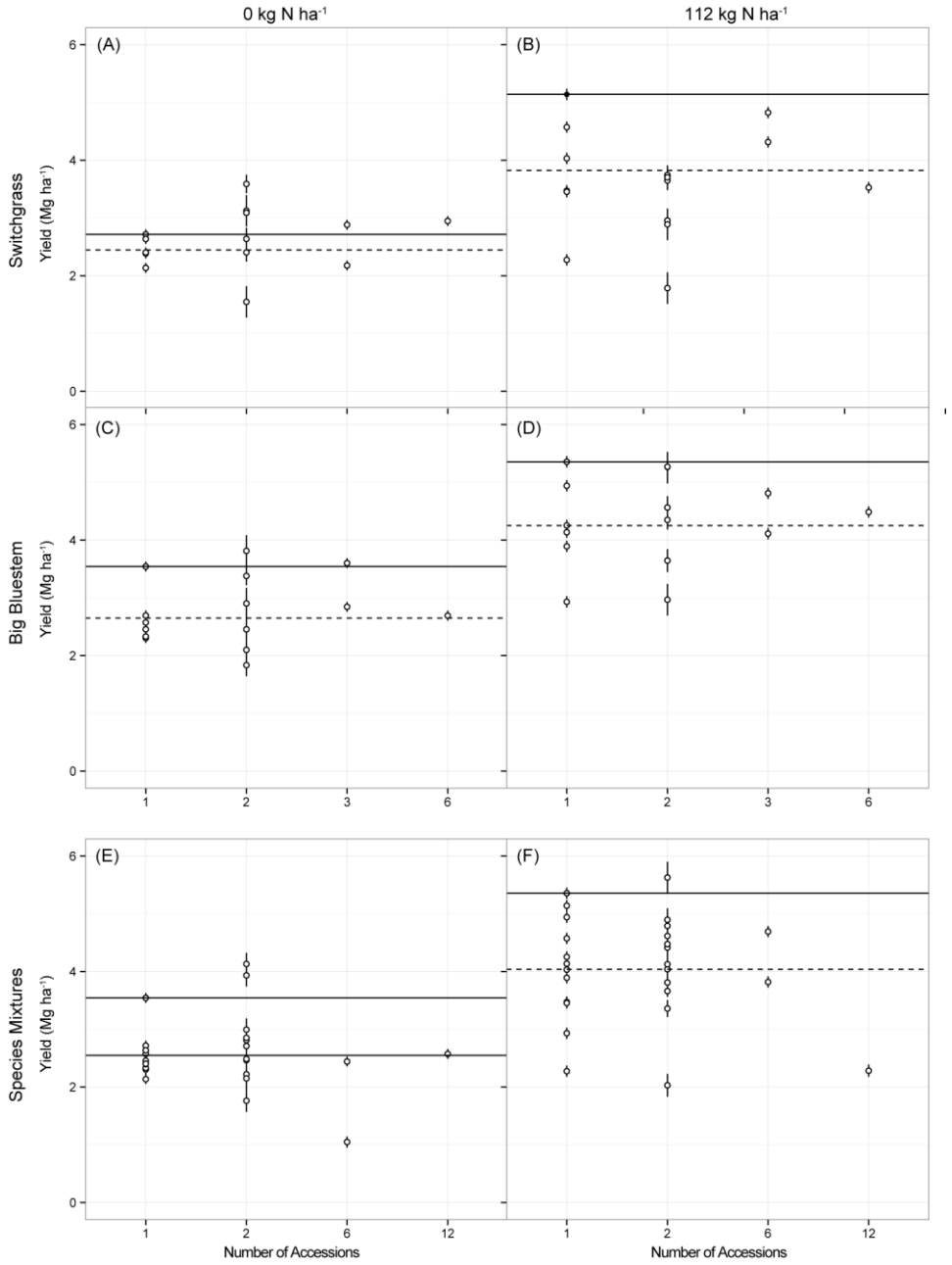


Figure 5. Comparisons of overyielding as compared to monoculture average (dotted line) and best monoculture (solid line) separated by fertility treatments for (A) unfertilized switchgrass, (B) fertilized switchgrass, (C) unfertilized big bluestem, (D) fertilized big bluestem, (E) unfertilized species mixtures, and (F) fertilized species mixtures. Least-squares means are plotted for each treatment. In species mixtures, both switchgrass and big bluestem monocultures are included.



3.9 Supplemental Tables

Supplemental Table 1. Treatment list

Treatment #	Dataset	# Acc	n	Accessions	Genetic Diversity	Geographic Distance (km)	Morphological Diversity
1	Big Blue	1	24	Chiwaukee BB	0.63	0	0.37
2	Big Blue	1	24	Central Iowa' BB	0.69	0	0.32
3	Big Blue	1	24	Shooting Star BB	0.66	0	0.41
4	Big Blue	1	24	Southlow' BB	0.68	0	0.45
5	Big Blue	1	24	Niagra'	0.54	0	0.74
6	Big Blue	1	24	Kaw'	0.80	0	0.27
7	Switch	1	24	Chiwaukee SW	0.45	0	0.40
8	Switch	1	24	Central Iowa' SW	0.61	0	0.42
9	Switch	1	24	Shooting Star SW	0.50	0	0.30
10	Switch	1	24	Southlow' SW	0.58	0	0.38
11	Switch	1	24	Shelter'	0.68	0	0.48
12	Switch	1	24	Blackwell'	0.90	0	0.44
13.1	Big Blue	2	4	Central Iowa BB, 'Kaw'	0.91	569	0.45
13.2	Big Blue	2	4	Shooting Star BB, 'Southlow' BB	0.78	572	0.47
13.3	Big Blue	2	4	Chiwaukee BB, 'Kaw'	0.87	870	0.58
13.4	Big Blue	2	4	Chiwaukee BB, 'Southlow' BB	0.70	275	0.48
13.5	Big Blue	2	4	Central Iowa BB, 'Niagra'	0.74	1072	0.72
13.6	Big Blue	2	4	Shooting Star BB, 'Niagra'	0.73	1030	0.66
14.1	Switch	2	4	Chiwaukee SW, 'Blackwell'	0.93	1029	0.56
14.2	Switch	2	4	Central Iowa SW, 'Southlow' SW	0.74	602	0.50
14.3	Switch	2	4	Chiwaukee SW, 'Shelter'	0.74	656	0.52
14.4	Switch	2	4	Central Iowa SW, 'Shelter'	0.84	930	0.56
14.5	Switch	2	4	Shooting Star SW, 'Southlow' SW	0.67	558	0.37
14.6	Switch	2	4	Shooting Star SW, 'Blackwell'	0.95	935	0.59
15	Big Blue	3	24	Chiwaukee BB, Central Iowa BB, Shooting Star BB	0.83	499	0.52
16	Big Blue	3	24	Southlow' BB, 'Niagra', 'Kaw'	0.90	1592	0.74
17	Switch	3	24	Chiwaukee SW, Central Iowa SW, Shooting Star SW	0.73	517	0.48
18	Switch	3	24	Southlow' SW, 'Shelter', 'Blackwell'	0.97	1716	0.63
19	Big Blue	6	24	Chiwaukee BB, Central Iowa BB, Shooting Star BB, 'Southlow' BB, 'Niagra', 'Kaw'	1.00	1812	0.66
20	Switch	6	24	Chiwaukee SW, Central Iowa SW, Shooting Star SW, 'Southlow' SW, 'Shelter', 'Blackwell'	1.00	1981	0.60

Treatment #	Dataset	# Acc	n	Accessions	Genetic Diversity	Geographic Distance (km)	Morphological Diversity
21.1	Species Mixtures	2	4	Central Iowa BB, Central Iowa SW	0.65	0	0.71
21.2	Species Mixtures	2	4	Shooting Star BB, Chiwaukee SW	0.56	311	0.69
21.3	Species Mixtures	2	4	Shooting Star BB, Central Iowa SW	0.64	188	0.74
21.4	Species Mixtures	2	4	Central Iowa BB, Shooting Star SW	0.59	213	0.69
21.5	Species Mixtures	2	4	Chiwaukee BB, Shooting Star SW	0.56	303	0.72
21.6	Species Mixtures	2	4	Chiwaukee BB, Chiwaukee SW	0.54	0	0.70
22.1	Species Mixtures	2	4	Kaw', 'Shelter'	0.74	1331	0.67
22.2	Species Mixtures	2	4	Southlow' BB, 'Shelter'	0.68	447	0.84
22.3	Species Mixtures	2	4	Kaw', 'Southlow' SW	0.85	186	0.63
22.4	Species Mixtures	2	4	Niagra', 'Southlow' SW	0.56	470	0.95
22.5	Species Mixtures	2	4	Southlow' BB, 'Southlow' SW	0.63	0	0.82
22.6	Species Mixtures	2	4	Niagra', 'Blackwell'	0.72	1712	0.94
23	Species Mixtures	6	24	Chiwaukee BB, Central Iowa BB, Shooting Star BB, Chiwaukee SW, Central Iowa SW, Shooting Star SW	0.78	520	0.77
24	Species Mixtures	6	24	Southlow' BB, 'Niagra', 'Kaw', 'Southlow' SW, 'Shelter', 'Blackwell'	0.93	2177	0.92
25	Species Mixtures	12	24	All	1.00	2418	0.87

Supplemental Table 2. List of primers used for genetic analysis

Species	Primer Name	# Alleles	Forward	Reverse	Source
Switchgrass	pvSSR132	8	TGCGGCTGCTTATCTAGCTT	CTTCTAGCTCGCCCCGTATCTAT	Zalapa et al 2011
Switchgrass	sw2455	5	CTCCGCAGTAGCAGCTTACAT	AGTTGTAGGAGGCGTTGGTCT	Zalapa et al 2011
Switchgrass	sw174	6	CCTTCCGTGTGCGACTCTAT	AAATAGCAACAGGCCGGATA	Zalapa et al 2011
Switchgrass	sw554	5	GATAGCCAGCACACCTGGTC	CCTAGCAGCCACTACCTTGC	Zalapa et al 2011
Switchgrass	sw438	11	AGAAGAAGCACAATCCGCAC	ATCCATCCAAATTCCAAACG	Zalapa et al 2011
Switchgrass	sw2376	8	CCCTCTCTTCCCTGTCAAATC	ACCATCAATCACCATGCAACT	Zalapa et al 2011
Switchgrass	sw583	9	TGCTAAATAACTGGAGCGCA	CTGATGAGCTGAAACGACCA	Zalapa et al 2011
Switchgrass	sw150	6	TGCAGCTGAATCCCTTCTTT	GAACTCCAGCCACCAGTTG	Zalapa et al 2011
Switchgrass	sw2312	10	TGGCATCTGGATGGATACAAG	GAACACGGTCCCATATGAAT	Zalapa et al 2011
Switchgrass	sw439	9	TTTCCCTTTCTCCTTCGGTT	TGCTTAAATTTACATGCCGC	Zalapa et al 2011
Switchgrass	sw2403	13	AAGGAAATCATCGTCTGATGC	AAGACAGCCCGTGGTTTATTC	Zalapa et al 2011
Switchgrass	sw2426	4	TGTCCCAAGTACCAATGTTCC	CAGGTGCTGACTGCTGTAGG	Zalapa et al 2011
Switchgrass	sw2422	6	GGTGAAGACTGAAGAGAGCA	ATCGCCTATGCACACAATTTC	Zalapa et al 2011
Switchgrass	sw2393	13	CTTCCTCATCGTCCACCAGT	CAGAGGGGGTACTCAAGCTC	Zalapa et al 2011
Big Bluestem	nc013	8	AATGGTTTTGAGGATGCAGCGTGG	CCCCGTGATTCCCTTCAACTTTC	Maize GDB
Big Bluestem	umc2323	5	TTCCCTATCAACTTCCATCCTGAA	ATCTGAAGCCAACTGTGTTCATTG	Maize GDB
Big Bluestem	phi071	14	GGAGTTCATCAGCTACCCCATCT	TTCTGCTTGTTGATCTGCACCCAC	Maize GDB
Big Bluestem	umc2272	15	GGAGGAAGCACAAGTACATGCAG	CCTGAACTTGGTCTGCTTCTCTG	Maize GDB
Big Bluestem	umc1035	5	CTGGCATGATCACGCTATGTATG	TAACATCAGCAGGTTTGCTCATTC	Maize GDB
Big Bluestem	umc2185	5	CTTCTTCTGCCACAGCACGAAC	CAAGGTTAAGAGCATGTACGCGAT	Maize GDB
Big Bluestem	umc1319	6	TGAGAGCCACCTTCTTGAGCTACT	TTCCTTGAAGGCGAAGGTAGGTAT	Maize GDB
Big Bluestem	umc2162	11	GATGCTGATGACGCTCTACAAGG	GACGATGACGAACACCGAGC	Maize GDB
Big Bluestem	umc1500	11	TCTCTGACTATTCCACGAGCTCAA	CTGGTGCGTGCTACAACTGTG	Maize GDB
Big Bluestem	umc1621	4	AGACTCTGAGGAGGTAGACGCTGA	GACTCGGAGGCCTTCTACATGAT	Maize GDB
Big Bluestem	umc1809	8	ACTCTTCTTCTTCCACCGGGAC	GTCGGACTCGAGAAACGACG	Maize GDB
Big Bluestem	umc2779	5	GACATCTTGAAGAAGAGCTCAGGC	GAGGATGGATTCTGCAACTTCG	Maize GDB
Big Bluestem	umc2567	6	CTCAATCTTTGCCCTCCGCT	TAGCTCGTCCAGGTCCAGGG	Maize GDB

Species	Primer Name	# Alleles	Forward	Reverse	Source
Big Bluestem	umc2266	4	GGACAGCTTGGCTTCGAGTG	ACGTTGGCCGTTAGTTCTTATCCT	Maize GDB
Big Bluestem	umc2788	4	TTCTCCTTTCTTGGTGGAGATGAC	TCTCTGAAACACAAAGACCCCAAC	Maize GDB
Big Bluestem	umc1479	5	CTGGCTCTTCAAGTGTAAAGGAGG	GGCCTTTTTCTTAGCTTCCTCATC	Maize GDB