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Abstract

Development of perennial biomass cropping systems is focused on maximizing biomass yield with minimum inputs, particularly nitrogen (N) fertilizer. Historical breeding efforts have focused on increasing biomass yield but have ignored N-use efficiency. The purpose of this study was to quantify the increased N demand associated with realized gains in biomass yield from big bluestem (*Andropogon gerardii* Vitman) and switchgrass (*Panicum virgatum* L.) breeding programs. Nitrogen demand was highly variable across locations and years, ranging from -1.7 to $+6.8$ kg N Mg^{-1} DM, with an average of 2.2 kg N Mg^{-1} DM. Increases in N demand were closely associated with realized gains in biomass yield and were observed for all types of switchgrass (upland, lowland, and hybrid) as well as for big bluestem. Attenuation of these responses will require alternative breeding schemes that are focused on evaluation of switchgrass genotypes and progeny under low-N conditions and include a high-throughput tissue N analysis as a component of future selection criteria, designed to develop new cultivars with high biomass yield and low tissue N.

Keywords *Panicum virgatum* L. · *Andropogon gerardii* Vitman · Nitrogen-use efficiency · Breeding · Genetics

Introduction

Development of sustainable perennial grass production systems is a key element of many proposed cellulosic biomass systems [1]. Switchgrass has been the model herbaceous species for these systems since 1992 [2]. Although it receives considerably less attention and funding, big bluestem is also a candidate for biomass production, owing to high yield in

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monocultures and candidacy as a component of high-diversity biomass polycultures [3, 4]. The general strategy for producing high-diversity polycultures is similar to that used to develop high-yield forage mixtures: select the highest-yielding cultivars of each species, each developed by breeding for high biomass yield and adaptation to the desired target population of environments [5, 6]. The best mixtures or polycrosses are then likely to be derived from the best components available.

Breeders have been extremely successful increasing biomass yield of switchgrass and, to a lesser extent, big bluestem. Genetic gains in biomass yield have been documented from several breeding programs [7, 8]. These and earlier studies have documented the strong genotype × environment (GE) interactions that exist for switchgrass, primarily associated with temperature and photoperiod, secondarily associated with moisture availability across a longitudinal gradient, and occasionally associated with soil characteristics [9]. These interactions appear to exist for big bluestem as well but are not as well characterized as for switchgrass [9, 10]. These studies have been critical in determining adaptation zones for individual cultivars and for helping to define the most efficient breeding methods to develop more broadly adapted cultivars.

Despite these gains and advancements, the role of N fertilizer in both commercial production systems and in breeding nurseries remains hotly debated and largely unresolved [1, 11]. Removal of N from the system is one of the major disadvantages of N fertilization. Increased rates of N fertilizer lead to increased nitrous oxide emissions, increased nitrate leaching, increased N removal in harvested biomass, and decreased N-use efficiency [1, 12, 13]. Furthermore, switchgrass biomass yield responds to increased N fertilizer only in about 50% of published N-rate experiments [14].

The purpose of this study was to determine the extent to which increases in biomass yield associated with breeding gains during the past 30 years have led to increased N demand for biomass crops of switchgrass and big bluestem. The experiments were conducted under "optimal" nitrogen conditions, using a fertilization rate of 112 kg ha^{-1} as recommended by Vogel et al. [15]. As such, the results and conclusions are intended to be focused on soil for which nitrogen is non-limiting for growth and biomass accumulation.

Materials and Methods

Field Sites and Plant Materials

Switchgrass and big bluestem field experiments were planted at 13 locations in April or May 2012 and 2014 [8]. Experiments planted in 2012 included 22 switchgrass populations and 12 big bluestem populations. Eight of the switchgrass populations planted in 2012 were proprietary and bred outside of the North Central region, so they were not used in

computing any of the statistical comparisons described later. Data from these eight populations were used in the analyses of variance for the sake of completeness and to improve the power of hypothesis tests. Experiments planted in 2014 included 20 switchgrass populations and 11 big bluestem populations [8], all of which were used for data analysis and included in the statistical data analyses reported herein.

Each experiment was designed as a randomized complete block with four replicates and plot sizes that varied with location, depending on local equipment and preferences, with details of the locations in Table 1 [16] and details of the populations in Tables 2 and 3 [8]. All plots were established with either 5-row or 7-row drill planters (0.9-m or 1.2-m width) at a seeding rate of 600 pure live seeds per meter squared. Germination percentages [17], and 1000-seed mass were used to adjust seeding rates to a pure-live-seed basis. Pre-emergence herbicide treatments were 0.56 kg ha^{-1} quinclorac [3,7-dichloroquinoline-8-carboxylic acid] plus 1.1 kg ha^{-1} atrazine [1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine] for switchgrass or 0.28 kg ha^{-1} imazamethapyr [3-pyridinecarboxylic acid, 2-(4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl)-5-methyl-] for big bluestem. All 2012 plantings were successfully established, except for the big bluestem trial at Brookings, SD. In 2014, trials at Brookings, SD, and all three Wisconsin locations, plus the big bluestem trial at Chatham, MI, failed to establish due to severe weed pressure. Of these, the only trial to recover was the 2014 switchgrass trial at Spooner, WI, which was delayed by 1 year due to slow establishment. The 2014 big bluestem trial at South Charleston, OH, was not harvested due to severe lodging which prevented accurate yield measurements.

Data Collection

Plots were fertilized at a rate of 112 kg N ha^{-1} in early spring, prior to initiation of growth, of each harvest year. Plots were mechanically harvested about 7 to 20 days after killing frost for up to 4 years (2012 trials) or 2 years (2014 trials). Harvesting was accomplished with either a sickle-bar mower or flail chopper, depending on location. Plot sizes and harvested areas varied across locations, but an unharvested border area was always preserved between plots [8]. A dry matter sample of 200 to 500 g was hand-clipped from random positions within each plot immediately before harvesting, and its mass was added to the recorded plot mass for completeness. Samples were dried for a minimum of 5 days at 55 to 65 °C and used to compute dry matter concentrations and adjusting plot mass to a dry matter basis. Samples were ground through a 1-mm screen on a Wiley-type mill, shipped to Madison, WI, and scanned on a near-infrared reflectance spectrophotometer (NIRS). Nitrogen concentration was predicted using calibrations from a broad-based population of switchgrass biomass samples [18]. Nitrogen removal (kg ha^{-1}) was computed as

Table 1 Site characteristics of 13 field locations used to evaluate selected switchgrass and big bluestem populations

Location	Latitude °N	Longitude °W	USDA plant HZ[a]	Soil type and taxonomy
Grand Rapids, MN	47.18	93.53	3b	Itasca-Goodland sandy loam (coarse-loamy, mixed, superactive, frigid Haplic Glossudalf)
Spooner, WI	45.80	72.87	4a	Murrill silt loam (fine-loamy, mixed, mesic Typic Hapludult)
Marshfield, WI	44.65	90.13	4b	Withee silt loam (fine-loamy, mixed, superactive frigid Aquic Glossudalf)
Brookings, SD	44.37	96.80	4b	McIntosh silt loam (fine-silty, mixed, superactive, frigid Aquic Calciudoll); Badger silty clay loam (fine, smectitic, frigid Vertic Argiaquoll)
Chatham, MI	46.32	86.92	4b	Eben very cobbly sandy loam (sandy-skeletal, mixed, frigid Pachic Hapludoll)
Arlington, WI	43.33	89.38	5a	Plano silt loam (fine-silty, mixed, mesic Typic Argiudoll)
Ames, IA	42.01	93.74	5a	Webster silty clay loam (fine-loamy, mixed, superactive, mesic Typic Endoaquoll)
Mead, NE	41.17	96.42	5b	2012: Tomek silt loam (fine, smectitic, mesic Pachic Argiudoll) 2014: Filbert silt loam (fine, smectitic, mesic Vertic Argialboll)
Urbana, IL	40.07	88.21	5b	Drummer silty clay loam (fine-silty, mixed, superactive, mesic Typic Endoaquoll)
West Lafayette, IN	40.30	86.89	5b	Toronto silt loam (fine-silty, mixed, superactive, mesic Udollic Epiaqualf); Millbrook silt loam (fine-silty, mixed, superactive, mesic Udollic Endoaqualf)
Columbia, MO	38.09	92.18	6a	Mexico silt loam (fine, smectitic, mesic Vertic Epiaqualf)
South Charleston, OH	39.86	83.67	6a	2012: Crosby silt loam (fine, mixed, active, mesic Aeric Epiaqualf) 2014: Kokomo silty clay loam (fine, mixed, superactive, mesic Typic Argiaquoll)
State College, PA	40.72	77.94	6b	Hagerstown silt loam (fine, mixed, semiactive, mesic Typic Hapludalf)

[a] USDA plant hardiness zone [16]. Each location is shown on a map in Fig. 1 of [8]

the product of biomass yield (Mg ha^{-1}) and nitrogen concentration (mg g^{-1} or kg Mg^{-1}).

Statistical Data Analysis

Data were analyzed separately by location and species. Locations were maintained as a "sort" variable rather than an ANOVA factor, because of the large range in USDA plant hardiness zones (PHZ; zones 3 to 6; Table 3) and the anticipated impact of this effect on the results of fixed-effect comparisons among populations. Mixed models analysis of variance was used with harvest year as a repeated measure and a compound symmetry covariance structure with either homogeneous or heterogeneous variances, whichever fit best according to Akaike's Information Criterion [19]. Block and planting year were considered random effects, while population and harvest year were fixed effects, the latter decision due to the large impact of stand age at some locations.

Statistical comparisons among populations were focused strictly on estimation and testing of gains from selection during the past 30 years, using five different measures of gain. Summer-Late-Mat-C2 was compared with summer switchgrass, both of which were present in both 2012 and 2014 plantings (Table 2). Four WS4U-derived populations were compared with WS4U using data from only the 2014 plantings (Table 2). Three groups of improved switchgrass populations were compared with each other, essentially excluding the natural populations and forage cultivars: improved lowland populations and improved populations derived from

Kanlow × Summer hybrids were both compared with improved upland populations (Table 2). Improved upland populations are not the ideal control for the other two groups, but they were used because the unimproved lowland populations, including Kanlow, are not sufficiently adapted to PHZ 3 through 5 to have reasonable survival levels. Furthermore, improved upland populations represent an opportunity cost of breeding locally adapted germplasm vs. adapting "exotic" germplasm that has traits not present in local ecotypes. It is still not completely clear if breeding efforts in these regions should be focused on upland, lowland, or hybrid sources of germplasm. Lastly, improved biomass-type big bluestem populations were compared with big bluestem cultivars (Table 3). Detailed results of these statistical comparisons for biomass yield were reported by [8], with partial results repeated here only to assist in explaining changes in nitrogen demand.

The latter two groups of comparisons were not perfectly balanced between the 2012 and 2014 plantings due to changes in the specific populations included in the trials, but the results of these comparisons were sufficiently similar between the 2012 and 2014 plantings that they could be pooled across plantings without any significant loss of information. Most of the reason for the imbalance between the 2012 and 2014 plantings is the essential purpose of repeated plantings of regional trials, to allow the newest and most recent candidate cultivars to be tested in a timely manner, of which there were numerous candidates produced in 2012 and 2013.

All statistical comparisons were computed as contrasts within mixed models analyses combined over 2012 and

Table 2 Names, origins, and breeding histories of switchgrass populations planted in 2012 or 2014 experiments

Population	Year[a]	Origin[b]	Ecotype[c]	Type[d]	Breeding history
Cave-in-Rock (CIR)	2012, 2014	Illinois	Upland	Natural	None
Shawnee	2012, 2014	Nebraska	Upland	Bred cultivar	Selection from CIR for high digestibility (IVDMD) and yield in Nebraska
Summer	2012, 2014	South Dakota	Upland	Bred cultivar	Selection for early flowering and rust resistance in South Dakota
Sunburst	2012, 2014	South Dakota	Upland	Bred cultivar	Selection for large seed size in South Dakota
Kanlow	2012	Oklahoma	Lowland	Natural	None
Kanlow N2	2012, 2014	Nebraska	Lowland	Breeding	Selection from Kanlow for winter survival, biomass yield and quality
Summer-Late-Mat. C2	2012, 2014	Nebraska	Upland	Breeding	Two cycles of selection for late flowering from Summer
K×S HP1 NETO2 C2	2012, 2014	Nebraska	Hybrid	Bred cultivar	Selection for biomass yield from Kanlow x Summer (K×S) hybrids
NE 2010 8X HYLD-HDMD C1	2012	Nebraska	Upland	Breeding	Selection for biomass yield and IVDMD from three upland populations
CIR C4	2012, 2014	Nebraska	Upland	Breeding	Four cycles of selection for high biomass yield and high IVDMD from CIR
Liberty (K×S HP1 NETO2 C1)	2012, 2014	Nebraska	Hybrid	Breeding	Selection for high biomass yield and other traits from K×S hybrids
K×S HP1 High Yield C1	2012	Nebraska	Hybrid	Breeding	Selection for high biomass yield and other traits from K×S hybrids
Kanlow N1 Late Mat-High Yield	2012	Nebraska	Lowland	Breeding	Selection for high biomass yield and late flowering from Kanlow N1
Kanlow N1 Early Mat-High Yield	2012	Nebraska	Lowland	Breeding	Selection for high biomass yield and early flowering from Kanlow N1
WS-10L	2014	Wisconsin	Lowland	Breeding	Selection for winter survivorship from Kanlow
WS-11L Syn2	2014	Wisconsin	Lowland	Breeding	Selection for winter survivorship from various lowland accessions
WS-12L	2014	Wisconsin	Lowland	Breeding	Selection for winter survivorship from various lowland accessions
WS-12L Syn2	2014	Wisconsin	Lowland	Breeding	Selection for winter survivorship from various lowland accessions
WS-13LU44	2014	Wisconsin	Upland	Breeding	Selection for late flowering and tall plants from WS4U in Wisconsin
WS-13LU44B	2014	Wisconsin	Upland	Breeding	Selection for late flowering and tall plants from WS4U in Wisconsin
WS-13LUDLP	2014	Wisconsin	Upland	Breeding	Selection for late flowering and tall plants from WS4U in Wisconsin
WS-13LUL10	2014	Wisconsin	Upland	Breeding	Selection for late flowering and tall plants from WS4U in Wisconsin
WS4U	2014	NC USA	Upland	Breeding	Broad tetraploid germplasm pool (Casler et al., 2006)
WS8U	2014	NC USA	Upland	Breeding	Broad octoploid germplasm pool (Casler et al., 2006)
ILSW-KG2B	2014	Kansas	Lowland	Breeding	Selection for survivorship and high plant biomass from Kanlow

[a] Planted in 2012, 2014, or both

[b] Origin of natural population or location of the breeding program (NC, north central)

[c] Upland ecotype, lowland ecotype, or selected population from within Kanlow × Summer (K×S) hybrids

[d] Natural, natural population; bred cultivar, cultivar developed by breeding and selection, then released; breeding, unreleased breeding population from USDA Lincoln, NE; USDA Madison, WI; or University of Illinois (details in the last column)

2014 plantings. All contrasts were computed for each individual trial-year, after which they were combined over years and trials following verification that results were largely homogeneous across years and trials. Data presentations were focused largely on pooled results across all trial-years within each of the 13 locations.

The relationship between N removal and biomass yield was quantified by linear regression for each species-location-planting year combination (42 total field trials; 23 for switchgrass and 19 for big bluestem). Slopes of these regressions, representing N demand (kg N Mg^{-1} DM), were analyzed by linear mixed models with species, location, and year as fixed effects. Increased N demand, as represented by an increased slope of these linear regressions, is analogous to decreased internal N-use efficiency, iNUE [20], i.e., the change in nitrogen removed by harvest per unit increase in dry biomass yield.

Finally, an attempt was made to relate mean N removal and N demand to soil characteristics of the 13 sites, which were highly variable and are shown in Supplemental Table 1 [21]. Soil characteristics were taken from the USDA-NRCS Web

Table 3 Names, origins, and breeding histories of big bluestem populations planted in 2012 or 2014 experiments

Population	Year[a]	Origin [b]	Type [c]	Breeding history
Rountree	2012, 2014	Iowa	Natural	None
Goldmine	2012, 2014	Nebraska	Bred cultivar	Selection for high yield and digestibility (IVDMD) from Kaw, three breeding cycles
Bonanza	2012, 2014	Nebraska	Bred cultivar	Selection for high yield and digestibility from Pawnee, three breeding cycles
Kaw	2012, 2014	Kansas	Bred cultivar	Selection for tall and leafy plants, medium to late flowering
Niagara	2012	New York	Natural	None
Sunnyview	2012	South Dakota	Bred cultivar	Selection for plant vigor, leaf rust, leafiness, and large seed size
Champ	2012, 2014	Nebraska	Bred cultivar	Selection for high crown diameter and large seed size
Kaw HYLD-HDMD C5	2012	Nebraska	Breeding	Selection for high yield and digestibility from Kaw, five breeding cycles
Pawnee HYLD-HDMD C5	2012	Nebraska	Breeding	Selection for high yield and digestibility from Pawnee, five breeding cycles
Bamboo C1	2012	Nebraska	Breeding	Plants from HZ5 collections with thick, coarse stems (bamboo appearance)
MW5A C1	2012, 2014	Nebraska	Breeding	Broad germplasm pool representing USDA hardiness zone 5a in the NC USA
MW5B C1	2012, 2014	Nebraska	Breeding	Broad germplasm pool representing USDA hardiness zone 5b in the NC USA
WBB-11L Syn2	2014	Wisconsin	Breeding	Selection for late flowering and high plant biomass from broad germplasm pool
WBB-12L Syn1	2014	Wisconsin	Breeding	Selection for late flowering and high plant biomass from broad germplasm pool
WBB-12L Syn2	2014	Wisconsin	Breeding	Selection for late flowering and high plant biomass from broad germplasm pool
WBB-12LLOK	2014	Wisconsin	Breeding	Selection for late flowering and high plant biomass from broad germplasm pool

[a] Planted in 2012, 2014, or both

[b] Origin of natural population or location of the breeding program

[c] Natural, natural population; bred cultivar, cultivar developed by breeding and selection, then released; breeding, unreleased breeding population from USDA Lincoln, NE, or USDA Madison, WI, as noted in the third column

Soil Survey [21]: cation exchange capacity, electrical conductivity, available water storage, and soil texture (percentages of sand, silt, and clay). Soil samples, taken to a depth of 0.7 m at the time of planting, were analyzed by the University of Wisconsin Soil and Forage Lab [22] for total nitrogen [23], Bray 1 P and K [24], pH [25], and OM [26]. Relationships of all soil variables to mean N removal values and to N demand were analyzed by simply correlation coefficients and by multiple regression analysis using forward stepwise regression.

Results

Variation among cultivars or populations was significant for nearly all locations and establishment years (Table 4). There was a slight tendency for lower significance levels (higher p values) for big bluestem compared with switchgrass at a few locations. This was probably a reflection on the smaller number of populations, with fewer degrees of freedom and lower power, combined with less overall effort on breeding big bluestem during the past 30 years, compared with switchgrass.

The path analysis of N removal was homogeneous across locations and establishment years, so Fig. 1 shows the results pooled across all locations and years. Biomass yield and N concentration were negatively correlated with each other, as expected for at least a partial dilution effect. Both N concentration and biomass yield had strong positive effects on N removal with biomass yield having a 25 to 45% higher impact than N concentration.

Most of the GE interactions associated with biomass yield were associated with differences in hardiness zone, specifically hardiness zones 3/4 vs. 5/6 [8]. There were few GE interactions associated with locations within these two groups of hardiness zones and few GE interactions associated with repeated years of harvesting or years of establishment (2012 vs. 2014). Therefore, the contrast analyses for N concentration and removal, which are focused on genetic responses to selection and population improvement, were pooled across locations and establishment years within the two groups of hardiness zones (HZ 3/4 vs. HZ 5/6). As such, the biologically meaningful GE interactions can be seen as differential selection responses in Fig. 2.

Each of the ten contrast comparisons for biomass yield were significant ($p < 0.05$), with eight positive and two negative responses (Fig. 2). Breeding for increased biomass yield of upland ecotypes of switchgrass significantly improved biomass yield within all plant hardiness zones. Because the

Table 4 *p* values for biomass yield, nitrogen concentration, and nitrogen removal measured on cultivars and experimental populations of two species established at up to 22 locations in either 2012 or 2014 seeding years

Year	Location[a]	Big bluestem			Switchgrass		
		Biomass yield	Nitrogen conc.	Nitrogen removal	Biomass yield	Nitrogen conc.	Nitrogen removal
2012	Grand Rapids, MN	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01
2014	Grand Rapids, MN	< 0.01	< 0.01	0.04	< 0.01	< 0.01	< 0.01
2012	Spooner, WI	< 0.01	0.31	< 0.01	0.01	< 0.01	0.01
2014	Spooner, WI	ND [b]	ND	ND	0.03	0.66	0.03
2012	Marshfield, WI	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
2012	Brookings, SD	ND	ND	ND	< 0.01	< 0.01	< 0.01
2012	Chatham, MI	< 0.01	< 0.01	0.01	< 0.01	< 0.01	< 0.01
2014	Chatham, MI	ND	ND	ND	< 0.01	0.16	0.06
2012	Arlington, WI	0.04	0.18	< 0.01	< 0.01	< 0.01	< 0.01
2012	Ames, IA	0.05	0.33	0.07	< 0.01	< 0.01	< 0.01
2014	Ames, IA	0.21	0.09	0.71	< 0.01	0.01	< 0.01
2012	Mead, NE	0.12	0.04	0.23	< 0.01	< 0.01	< 0.01
2014	Mead, NE	0.31	0.79	0.78	< 0.01	< 0.01	0.01
2012	Urbana, IL	0.04	< 0.01	0.27	< 0.01	< 0.01	< 0.01
2014	Urbana, IL	0.03	< 0.01	0.18	< 0.01	< 0.01	0.06
2012	West Lafayette, IN	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
2014	West Lafayette, IN	< 0.01	0.19	0.16	< 0.01	< 0.01	< 0.01
2012	Columbia, MO	< 0.01	0.30	0.64	< 0.01	0.19	< 0.01
2014	Columbia, MO	< 0.01	0.08	0.08	< 0.01	< 0.01	< 0.01
2012	South Charleston, OH	< 0.01	< 0.01	0.02	0.22	< 0.01	0.12
2014	South Charleston, OH	ND	ND	ND	< 0.01	0.03	< 0.01
2012	State College, PA	< 0.01	< 0.01	0.33	< 0.01	< 0.01	< 0.01
2014	State College, PA	0.45	0.92	0.97	< 0.01	< 0.01	< 0.01

[a] Locations are sorted by plant hardiness zone: PHZ 3b on top to PHZ 6b on bottom

[b] *ND* no data due to failed establishment or to severe lodging that prevented a yield measurement

lowland and K×S strains of switchgrass were still green and actively growing at the time of killing frost, they had not yet completed their annual life cycle for the locations within plant hardiness zones 3 and 4, reducing their potential for biomass accumulation. As such, selection responses for biomass yield within these two groups were negative, while gains in biomass yield were significant within plant hardiness zones 5 and 6, where these populations are normally adapted and they were able to complete their annual life cycle before killing frost. Results for big bluestem were intermediate between the upland switchgrass populations and the lowland or K×S populations: significant gains for both hardiness regions, but reduced for the northern regions where big bluestem had less time to complete its annual life cycle.

As expected, based on the negative correlations between biomass yield and N concentration, responses for N concentration were generally in the opposite direction as observed for biomass yield, with seven significant responses (five negative and two positive; Fig. 2). The significant increases in N concentration of the lowland and K×S in plant hardiness zones 3

and 4 was due to their late flowering nature and the inability to complete their annual life cycle, which includes recycling tissue N to belowground storage tissues. Contrast comparisons for N removal closely paralleled those for biomass yield in seven of ten cases (five positive and two negative), with partial attenuation of responses due to the negative correlation between biomass yield and N concentration. This partial attenuation can also be observed in the average absolute responses to selection as a percentage of the unselected controls, which were 38% for biomass yield and 25% for N removal.

Thirty-two of the 42 linear regressions of N removal on biomass yield were significant ($p < 0.05$), with nine nonsignificant values and one negative value (Table 5; Fig. 3). Nitrogen demand was highly variable, ranging from − 1.7 to 6.8 kg N for each megagram increase in biomass yield. There was no significant difference between big bluestem and switchgrass. The two establishment years were significantly different for N demand ($p < 0.01$), with means of 2.3 and 3.4 kg N Mg^{-1} DM for 2012 and 2014, respectively. Locations were highly variable for N demand, with mean
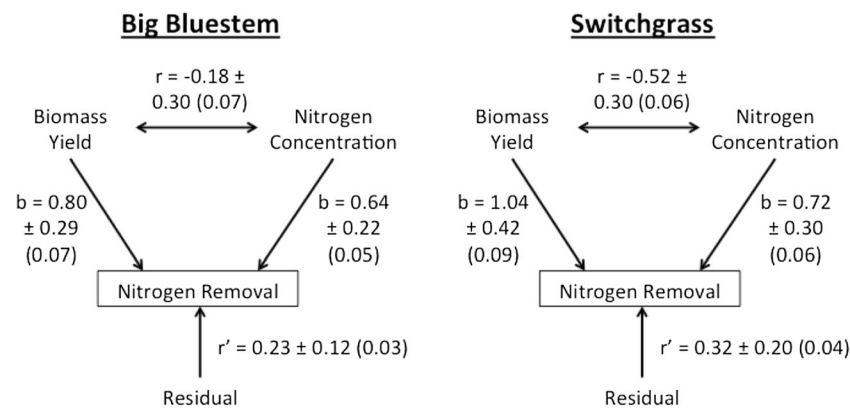
Fig. 1 Path analysis diagrams for switchgrass and big bluestem: standardized partial regression coefficients (direct effects) of biomass yield and nitrogen concentration on nitrogen removal, correlation coefficients between biomass yield and nitrogen concentration, and residuals. All values represent the mean over all trial-years for each species

values ranging from 0.4 to 4.8 kg N Mg^{-1} DM, but this variability was not associated with plant hardiness zone (Fig. 4). These differences were significant ($p < 0.01$) and Fig. 4 shows that there was some level of consistency or predictability for N demand between the 2012 and 2014 establishment years. However, none of the soil chemical or physical characteristics explained a significant portion of variability for either mean N removal or N demand across locations,

either individually or from the multiple regression analyses; the highest proportion of variability explained was < 0.25. Specifically, neither N removal nor N demand was related to total N concentration in the soil for either switchgrass or big bluestem ($r^2 < 0.02$). Despite the large range in mean soil N (663 to 1994 ppm), this was a strong indication that the use of nitrogen fertilizer in this experiment resulted in non-N-limiting conditions.
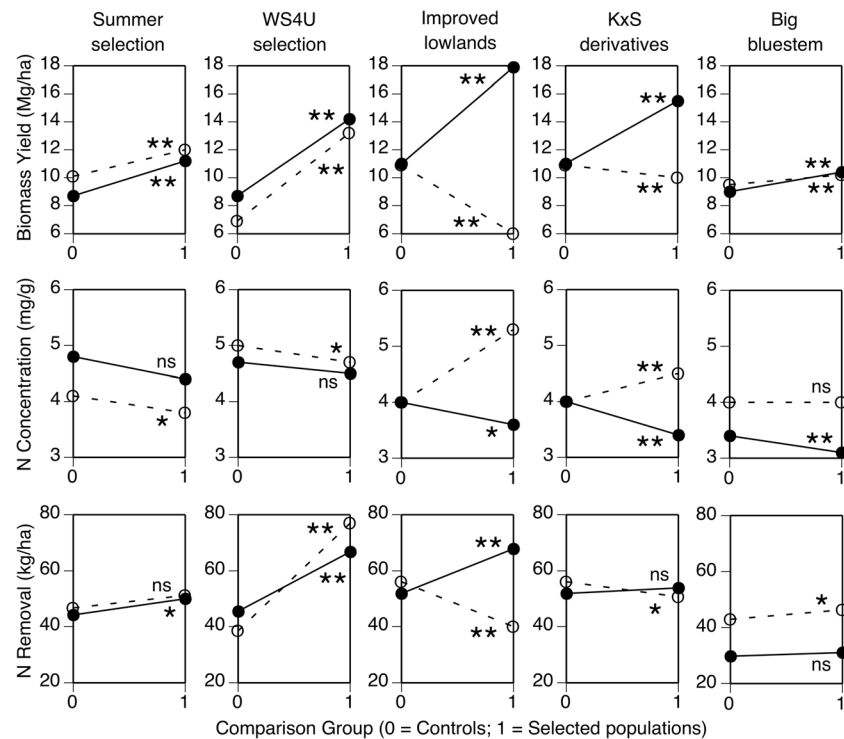

Fig. 2 Responses to selection for increased biomass yield of switchgrass or big bluestem germplasm (x-axis: 0 = unselected parent population or cultivars; 1 = progeny populations selected for increased biomass yield). The five columns, each representing a different set of control vs. selected populations, are described in detail in the text and in Tables 2 and 3. Open circles and dashed lines represent the mean of all trials located in USDA plant hardiness zones 3 and 4; closed circles and solid lines represent the mean of all trials located in USDA plant hardiness zones 5 and 6; *, **, and ns represent significance at 0.05 or 0.01, or non-significance, respectively, of the selection responses (0 vs. 1 on the x-axis)

Table 5 Statistics for the linear regressions of nitrogen removal (kg ha^{-1}) on biomass yield (Mg ha^{-1}) for 42 big bluestem or switchgrass trials, computed from population means over years

Year	Location[a]	Big bluestem				Switchgrass			
		r^2	Slope (b) kg N Mg^{-1} DM	SE$_b$	p value	r^2	Slope (b) kg N Mg^{-1} DM	SE$_b$	p value
2012	Grand Rapids, MN	0.80	2.78	0.43	< 0.01	0.90	5.66	0.41	< 0.01
2014	Grand Rapids, MN	0.58	4.11	1.18	0.01	0.94	6.81	0.40	< 0.01
2012	Spooner, WI	0.80	2.65	0.42	< 0.01	0.41	3.25	0.45	< 0.01
2014	Spooner, WI	ND ^{b}	ND	ND	ND	0.84	4.92	0.47	< 0.01
2012	Marshfield, WI	0.78	3.29	0.56	< 0.01	0.79	3.23	0.37	< 0.01
2012	Brookings, SD	ND	ND	ND	ND	0.44	1.65	0.41	< 0.01
2012	Chatham, MI	0.10	1.53	1.46	0.32	0.13	−1.72	1.06	0.12
2014	Chatham, MI	ND	ND	ND	ND	0.03	0.79	1.11	0.49
2012	Arlington, WI	0.73	4.81	0.93	< 0.01	0.34	3.72	1.17	< 0.01
2012	Ames, IA	0.82	3.62	0.53	< 0.01	0.26	3.16	1.25	0.02
2014	Ames, IA	0.44	2.63	1.00	0.03	0.89	3.70	0.31	< 0.01
2012	Mead, NE	0.73	2.94	0.56	< 0.01	0.10	1.13	0.78	0.16
2014	Mead, NE	0.58	5.71	1.62	0.01	0.53	2.38	0.52	< 0.01
2012	Urbana, IL	0.50	2.27	0.72	0.01	0.49	2.18	0.50	< 0.01
2014	Urbana, IL	0.28	3.45	1.83	0.09	0.81	3.39	0.39	< 0.01
2012	West Lafayette, IN	0.73	2.36	0.46	< 0.01	0.41	2.18	0.62	< 0.01
2014	West Lafayette, IN	0.56	1.79	0.53	0.01	0.43	1.51	0.41	< 0.01
2012	Columbia, MO	0.25	0.50	0.27	0.10	0.00	0.04	0.27	0.89
2014	Columbia, MO	0.29	0.81	0.42	0.09	0.75	1.36	0.18	< 0.01
2012	South Charleston, OH	0.26	1.78	0.96	0.09	0.39	2.92	1.09	0.02
2014	South Charleston, OH	ND	ND	ND	ND	0.80	4.69	0.55	< 0.01
2012	State College, PA	0.00	−0.05	0.34	0.87	0.18	1.35	0.63	0.05
2014	State College, PA	0.81	3.60	0.58	< 0.01	0.78	2.77	0.34	< 0.01
2012	Overall mean	0.61	1.57	0.40	< 0.01	0.27	2.08	0.76	0.01
2014	Overall mean	0.60	2.10	0.58	< 0.01	0.79	3.00	0.37	< 0.01

^{a} Locations are sorted by plant hardiness zone: PHZ 3b on top to PHZ 6b on bottom

^{b} *ND* no data due to failed establishment

Fig. 3 Scatterplots and linear regressions of nitrogen removal as a function of biomass yield for big bluestem and switchgrass, illustrating the range of responses. Each panel shows the regressions with the minimum or maximum N demand within each establishment year (2012 and 2014), with regression statistics shown in Table 3
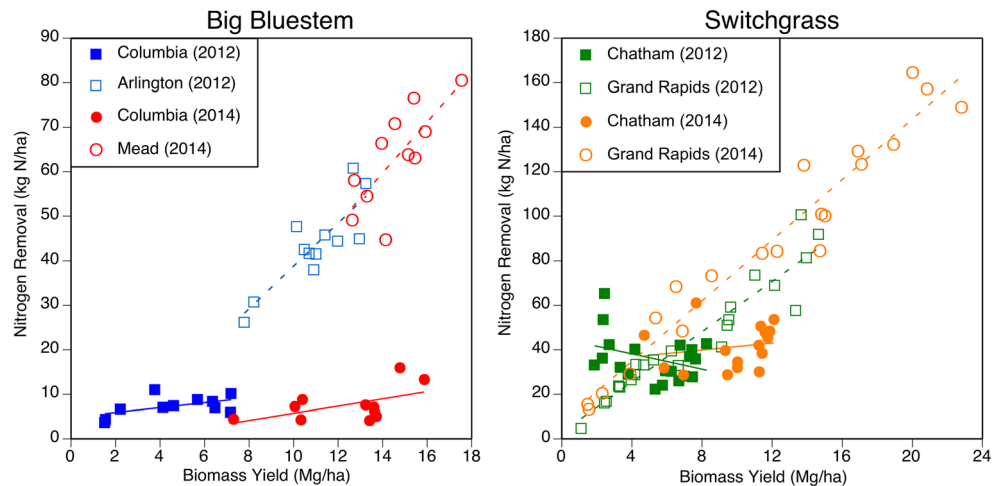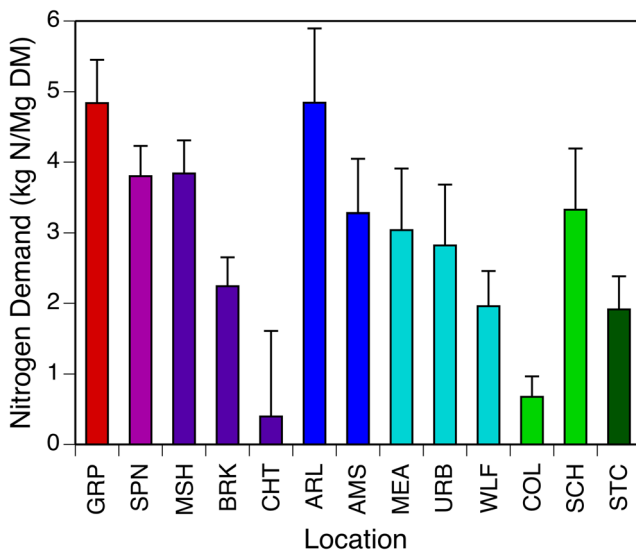
Fig. 4 Mean nitrogen demand for 13 field locations, as measured by the slope of linear regressions of nitrogen removal on biomass yield, averaged across two species and two establishment years. Locations are sorted as shown in Table 1, from hardiness zone 3b (left) to 6b (right) and each color represents a specific plant hardiness zone (GRP, Grand Rapids, MN; SPN, Spooner, WI; MSH, Marshfield, WI; BRK, Brookings, SD; CHT, Chatham, MI; ARL, Arlington, WI; AMS, Ames, IA; MEA, Mead, NE; URB, Urbana, IL; WLF, West Lafayette, IN; COL, Columbia, MO; SCH, South Charleston, OH; STC, State College, PA)

Discussion

Nitrogen is essential to life, being one of the most important constituents of amino acids, proteins, and enzymes. For this reason, breeding for increased N content has often been an objective of forage breeding programs aimed at developing forage crops with increased protein content [27–29]. Nitrogen concentration has a moderate to high heritability and it is relatively easy and rapid to achieve increases in protein concentration of both grasses and legumes [27–29].

Dedicated biomass crops represent a significant departure from this concept because N removed in the biomass is largely wasted for future use, except as a component of biochar, a byproduct of biomass pyrolysis, which can be used as a source of nutrients for future crops [30]. Essentially, N has little or no value for conversion of biomass to energy, except as a component of fertilizer co-products from the conversion process. Delayed harvesting is one of the principal short-term strategies to mitigate N loss from bioenergy cropping systems. Perennial energy grasses remobilize tissue N in response to decreased photoperiod and temperature during autumn [31]. Delaying harvest until after killing frost can result in significant reductions in tissue N, but additional significant reductions continue to occur during winter, even though active translocation has ceased [32, 33]. Some of these winter losses occur through leaf loss as litter, some of which are retained in the field [34, 35].

Historically, switchgrass and big bluestem have been bred under conditions where N has been non-limiting, e.g., conditions similar to the soils and production conditions utilized in this study. Under these conditions, increases in biomass yield have led to a clear increase in N demand, essentially a decrease in iNUE, as defined by [20]. While the increases in N demand were highly variable across locations and somewhat variable between different trials within each location, they were nearly always positive and significant, resulting in an overall mean of 2.2 kg N Mg^{-1} DM. While additional delays in harvesting, well after killing frost, will likely attenuate some of this increased N demand, it is important to realize that large-scale production of biomass from perennial energy grasses will require "just-in-time" harvesting approaches, where biomass is stored in the field and harvested over many months on an "as needed" basis [36]. Ideally, producers could rotate which fields are harvested early vs. late, thereby practicing a form of N conservation by rotating those fields that suffer significant nitrogen losses in any given year. Studies have shown that switchgrass biomass can be maintained at a high level on high fertility soils for up to 2 or 3 years after fertilization [37, 38], implying that an occasional or cyclic N loss from the system could be tolerated. However, this is a very idealistic scenario that increases the level of complication in a biomass production operation, increasing the likelihood that it will not become a standard operating procedure.

Alternatively, in the longer term, breeding programs for perennial energy grasses should begin to focus on improving N-use efficiency as a routine part of the breeding objective. For these two species, the negative genetic correlation between biomass yield and tissue N concentration is simply not large enough to rely on correlated responses to selection. While tissue N concentration tends to decline with selection for biomass yield, these decreases are insufficient to offset the relatively high rates of gain (and notable successes) from breeding for increased biomass yield. Rather, tissue N concentration should become part of the selection criterion, likely as part of a selection index or as a ratio criterion, e.g., biomass yield ÷ nitrogen concentration [39, 40].

Research on sugarcane (*Saccharum officinarum* L.) has demonstrated genetic variability for iNUE, which is expressed more under low-N compared with high-N conditions [20]. As observed in the present study of switchgrass and big bluestem for N demand, iNUE was strongly a function of biomass yield in sugarcane. Furthermore, iNUE was positively correlated between low-N and high-N conditions, a phenomenon commonly observed for biomass yield of switchgrass [8, 9], suggesting that iNUE is repeatable. While the results of this study suggest that iNUE of both switchgrass and big bluestem has been decreased by selection for increased biomass yield, the results for sugarcane suggest that perhaps this trend can be reversed by directed and focused selection aimed at

increasing biomass yield and decreasing tissue N concentration, as suggested above.

Collecting and processing biomass samples within an energy-grass breeding program require a massive time commitment, both for sampling tissue at harvest time and for processing samples during the offseason. The use of near-infrared reflectance spectroscopy (NIRS) is an essential component of this process, allowing breeding programs to process samples more rapidly than can be accomplished if each sample must be analyzed by wet chemistry [18]. Nevertheless, work-loads can be reduced and throughput can be increased if a hand-held NIRS can be adapted to predict tissue N based on real-time scans made on fresh tissue either at the harvest time or shortly beforehand. The latter would be advantageous because of the time and labor commitment required for harvesting biomass plots. Hand-held or portable NIRS units are capable of predicting chemical constituents of liquids, fruits, grains, and herbage [41–46]. Similar technologies have been used to scan forage-crop canopies to predict N concentration of standing alfalfa (*Medicago sativa* L.) or bermudagrass (*Cynodon dactylon* (L.) Pers.) forage crops, with $r^2 \approx 0.6$ [47, 48]. This technology would allow for selections to be made immediately after the biomass harvest, drastically reducing the need for time-sensitive offseason activities, which are often significant sources of stress for project personnel, as well as competition against glasshouse activities focused on establishment of new nurseries to advance the breeding program.

Most of the improved populations evaluated in this study were bred under non-N-limiting conditions, similar to those in the evaluation environments. This is similar to the philosophy of many plant breeding programs, which have often operated under the assumption that the most favorable environments favor expression of genetic variability, favoring the greatest rate of gain [49]. More recent results have demonstrated that the most effective selection for low-input conditions should be made under those same low-input conditions [50–55]. Quantitative genetic theory generally supports this philosophy, suggesting that the heritability and genetic correlation requirements to support indirect selection under high-input conditions are too great to provide a routine advantage compared with direct selection under low-input conditions [56–58].

These conclusions were supported by a switchgrass study in which direct selection under low-input conditions resulted in the greatest gain under those conditions [59]. From a broader perspective, the elimination of N fertilizer on switchgrass selection nurseries would increase the likelihood of identifying genotypes with enhanced breeding value for the ability to recycle N efficiently, to utilize N efficiently, and to scavenge N from low-N soils. This may even extend to the ability of breeders to identify switchgrass genotypes capable of forming beneficial associations with rhizobacteria or endophytic bacteria that may have growth promotion impacts, such

as biological N fixation, nutrient acquisition, and increased biomass production [60, 61]. Nutrient acquisition by host plants may also be enhanced by arbuscular mycorrhizal fungi [62]. Because host-microbe associations are under genetic control and heritable, it should be possible to identify genotypes with superior nutrient acquisition ability and nutrient-use efficiency [63–66]. Just as N fertilization may impair the ability of a soil microbiome to evolve into a diverse community capable of providing these functions [67], it may also limit our ability to break the link between increased biomass yield and increased N demand.

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