Title: Drought minimized nitrogen fertilization effects on bioenergy feedstock quality

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Abstract:

Switchgrass (*Panicum virgatum*) is one of the leading candidates for sustainable lignocellulosic biofuel production in North America. Most current management recommendations for switchgrass include applications of synthetic nitrogen (N) fertilizers to increase production, particularly when grown on marginal lands. However, this management can be costly to growers and have negative impacts on ecosystem functioning. Also, N-fertilization does not always result in higher yield in switchgrass and may have unintended effects on plant biomass quality, including cell wall composition, that can affect the efficiency of fermentation processes for biofuel production. Drought stress may reduce biomass responses to N-fertilization, further reducing the value of fertilizer application. To examine whether N-fertilization and reduced precipitation affected switchgrass productivity and cell wall composition, we conducted a two-year field experiment in mature stands of two switchgrass cultivars grown for bioenergy at the W.K. Kellogg Biological Station Long Term Ecological Research Site in Michigan, USA. Nitrogen was added at a rate of 56 kg N ha^{-1} (urea and ammonium nitrate), and precipitation was reduced using rainout shelters. Overall, we did not observe any effect of N-fertilization on biomass production. However, under ambient rainfall conditions, N-fertilization altered switchgrass biomass quality by reducing hemicellulose. Reduced precipitation minimized the effects of N-fertilization on switchgrass cell wall composition. Switchgrass is a relatively drought-tolerant species, and our results indicate that this crop will be a viable bioenergy feedstock even in a changing climate. However, in this study, N-fertilization had no effect on biomass quality or quantity under drought conditions.

Key Words: switchgrass, drought, lignin, nitrogen, bioenergy crops

1. Introduction

The growing interest in using perennial grasses such as switchgrass (*Panicum virgatum*) as bioenergy feedstocks [1] has motivated research into their sustainability. Perennial energy crops are predicted to be more efficient in terms of energy return, because their cultivation produces half of the greenhouse gas emissions compared to grain feedstock [2]. Also, many perennial species can be grown on marginal land conditions where food crops cannot be grown [3]. Switchgrass, a warm-season grass native to much of North America, is one of the leading candidates for sustainable lignocellulosic biofuel production due to its efficient nutrient and water use, and longevity of planted stands [10+ years of production; 4]. Switchgrass stands also can provide other ecosystem services that are increasingly seen as important for sustainability, including increased soil carbon sequestration, enhanced methane consumption, and providing habitat for populations of desirable organisms such as biocontrol agents and pollinators [5].

However, knowing how to manage these crops on marginal lands will be a challenge for producers, especially given the initial financial commitments that are needed to establish a productive stand. Growers need to consider which switchgrass cultivar to plant based on yield goals and environmental conditions [6], and whether or not to use synthetic fertilizers to increase yields [7]. While current management recommendations of switchgrass generally call for application of synthetic fertilizer, in particular nitrogen, these come with economic costs to growers (an estimated $37/ha) [8], and do not always deliver higher yields, especially on moderately-fertile lands or lands limited in other nutrients. For example, Kering et al. [9] found no effect of nitrogen addition (135 kg N ha^{-1}) on switchgrass yields in Oklahoma. Similarly, Garten et al. [10] found no effect of fertilizer (up to 202 kg N ha^{-1}) on switchgrass yields in Tennessee, and Roley et al. [11] found no effect of fertilizer (up to 196 kg N ha^{-1}) on switchgrass yields in Michigan. Also, even if fertilizer increases crop biomass, there may be negative impacts on other ecosystem services such as nitrate leaching and N_2O emissions [12, 13].

The use of N fertilizers to increase crop yield may also have unintended effects on plant biomass quality, including cell wall composition, that can affect the efficiency of fermentation processes for biofuel production [14]. A meta-analysis of 52 studies covering a wide range of plant species found that N-fertilization generally increased lignin in cell walls while decreasing hemicellulose due to tradeoffs in energetic costs of each component [15]. In general, increases in structural carbohydrates (cellulose and hemicellulose) in cell walls are favored for biochemical conversion [16], while lignin increases recalcitrance which can increase the cost of processing plant material [17]. These results suggest that N-fertilization could decrease the biomass quality of bioenergy crops.

Expected change in climate in the Midwestern USA, particularly precipitation patterns, may also alter recommendations for agricultural practices for bioenergy and other crops [18]. Regional climate models predict anywhere from 35% increased (PCM model) to 45% reduced (GFDL model) growing-season precipitation [19], along with increases in summer temperatures by 3.1-4.7°C over the next 100 years [20]. The changes in climate likely will results in crops experiencing increased water stress in the future [21]. Drought stress can reduce biomass responses to N-fertilization in switchgrass and other crops [22, 23], further reducing the value of fertilizer application.

We conducted a two-year field experiment to determine how N-fertilization affected crop productivity and biomass quality (i.e., cell wall composition) in mature stands of two switchgrass cultivars grown for bioenergy in Michigan, USA. We also evaluated whether drought diminished these N-fertilization effects. Although yield responses to N-fertilization in switchgrass can vary, we predicted that N-fertilization would increase switchgrass yield, as shown in a wide variety of studies [24] and would alter switchgrass cell wall composition, generally making it more unfavorable for biofuel production by increasing lignin and decreasing hemicellulose. We also predicted that drier soil conditions would minimize effects of N-fertilization on switchgrass biomass quantity and quality, as increased abiotic stress can interfere with N-uptake, as shown in other crops [25-27].

2. Methods

2.1 LTER Cellulosic Biofuel Diversity Experiment Design

This research was conducted at the W.K. Kellogg Biological Station Long Term Ecological Research (KBS LTER) site in southwest Michigan USA (42°23047″N, 85°22026″W). This site averages 810 mm yr^{-1} of precipitation and soils are Kalamazoo series fine loamy, mixed, mesic Typic Hapludalfs [28]. The Cellulosic Biofuels Diversity Experiment was established as part of the KBS LTER in 2008 and designed to compare production of 12 different biofuel cropping systems varying in species composition and nitrogen input. The experiment included two cultivars of switchgrass (“Cave-in-Rock” and “Southlow”; each planted at a rate of 3.9 kg ha^{-1}) grown at two levels of fertilization (56 kg N ha^{-1} and unfertilized) which we used for this study. The four switchgrass treatment combinations were planted in 9 m x 27 m plots, replicated four times in a randomized block design interspersed with the other 8 treatments. Fertilizer (28% N; Urea + Ammonium nitrate) was applied to relevant treatment plots every year in the spring. All switchgrass plots were treated with Drive (quinclorac; BASF Corp, NC) at 0.56 kg ha^{-1} for broadleaf weed control during the first two years following planting (2009-10). Switchgrass plots were harvested annually at the end of each growing season (Oct/Nov) using a John Deere 7330 tractor with 7.5 ft wide Kemper head. Additional experiment details on the management of this experiment can be found at: https://lter.kbs.msu.edu/datasets/109.

2.2. Precipitation Manipulation

In May 2015, we established a precipitation reduction ("drought") treatment using rainout shelters placed in either the northwest or southwest corner of each of the switchgrass treatment plots (n=16). The rainout shelters were based upon the design of Yahdjian and Sala [29] and were 3 m × 2.5 m × 1.8 m, constructed using 3.8 cm PVC. Roofing material was made of 3 mm thick clear polycarbonate (no UV filter) that was cut into strips 246 cm x 12.7 cm and bent lengthwise down the center to a final angle of 105˚. Twenty-four bent pieces of polycarbonate were evenly spaced across each roof for a target rainfall reduction of approximately 80%. Gutters with downspouts were attached to one edge of each shelter to remove intercepted rainfall from subplots. In the alternate west corner of each switchgrass plot, a 3 m × 2.5 m "ambient" subplot was established to compare plant growth in non-shelter conditions. Shelters were installed May-October 2015 and 2016 and removed at the end of each growing season.

To monitor the effects of the rainout shelters on soil moisture, we buried soil moisture sensors (Watermark Model 200SS; Irrometer Company, Inc., Riverside, California) to a depth of 15 cm in the center of each drought and ambient subplot in May 2015. Soil moisture readings were taken every 3-5 days during the growing seasons (May-October) 2015 and 2016 (80 readings total). We also recorded light levels (AccuPAR LP-80 light wand; Decagon Devices; Pullman, WA, USA) above the vegetation under the shelters and in ambient subplots 8 times over the two growing seasons to evaluate any effects of the shelter structures on light availability. Soil water tension was, on average, 40% lower (more negative) under shelters compared to ambient plots (-39.5 vs. -28.2 centibars; paired t-test t_{1023}=11.68, $p<0.001$). Shelters also reduced light availability by 19% on average (1005.5 vs. 1239.5 μmol $m^{-2}s^{-1}$; $t_{143} = 12.12$, $p<0.001$).

To verify that our rainout shelters reduced water availability to plants, we measured pre-dawn and midday xylem pressure potential (Ψ_P) using a pressure chamber (Model 600; PMS Instrument Company, Albany, OR, USA). These measurements were taken on two tillers per subplot in July 2015, August 2015, Sept. 2015, June 2016, and August 2016. All subplots were measured on a single night (2:00-4:00) and the following day (13:00-15:00) per sampling period. Overall, pre-dawn Ψ_P was 29% lower (more negative) under the rainout shelters compared to ambient subplots (-2.48 vs. -1.93 MPa; $t_{79} = 2.20$, $p = 0.03$), while midday Ψ_P was 15% lower under the rainout shelters compared to ambient subplots (-11.88 vs. -0.29 MPa; $t_{79} = 3.19$, $p = 0.002$).

2.3 Plant biomass responses

In October 2015 and 2016, we measured aboveground biomass production in each treatment by clipping at 5 cm above ground level all standing biomass from a 50 cm x 50 cm quadrat in the center of each subplot [30]. Switchgrass tillers were senesced and seeds were ripe at harvesting. Switchgrass stems were separated from weeds (that comprised, on average, 11% of total biomass in both drought and ambient rainfall treatments), and samples were dried (48 h at 60°C) and weighed. We determined cell wall composition of the switchgrass using dried subsamples collected from biomass harvests in both years. Samples were sent to the Michigan State University Cell Wall Facility (East Lansing, MI) and processed according to methods described in [31, 32]. For each sample, total lignin (consisting of individual lignin monomers syringyl (S), guaicyl (G) and p-hydroxyphenyl (H)), total hemicellulose (consisting of individual monomers arabinose, mannose, galactactose, xylose, and hemicellulosic glucose), and crystalline cellulose were quantified using GC-MS.

2.4. Data analyses

To determine N-fertilization effects on switchgrass productivity, cell wall composition, and physiology, we analyzed data from the ambient subplots first, using two-factor ANOVA (productivity) and MANOVAs (cell wall monomers and major cell wall components) with cultivar and fertilizer as main effects and experimental block and year as blocking terms. When MANOVAs showed significance based on Pillai's trace statistics, we followed-up with univariate analyses to determine which of the individual response variables was responsible for the differences [33]. We also examined differences in cell wall composition across cultivar and fertilizer treatments by conducting a linear discriminant analysis (LDA) [33], which determined how useful cell wall components were in separating treatments.

To evaluate whether drought minimized fertilizer effects on plant responses, we repeated the above analyses on the shelter subplot data. We did not have sufficient statistical power to examine fertilization × drought interactions explicitly due to low replication (8 main plots) in the long-term treatments. Sampling year had a significant effect on most measured responses, but with only two years in this experiment, we could not definitely attribute causes for this variation, and so do not discuss this effect further. All analyses were conducted in Systat v.12 [34].

3. Results

3.1. N-fertilization effects under ambient conditions

In the ambient precipitation subplots, fertilization did not have a significant effect on aboveground biomass (Fig. 1). However, cell wall monomer composition differed in response to fertilization (MANOVA $F_{11,14} = 4.21$, $p = 0.007$, Table A.1). Specifically, there was a 4% decrease in the major hemicellulose monomer xylose, while mannose, a minor hemicellulose monomer, increased by 42%. Syringyl (S), a lignin monomer, also increased by 12% (Table 1, A.1). Two linear discriminant functions explained 94% of the variation in cell wall monomer composition among cultivar and fertilization treatments, and placed plots in the correct treatment 81% of the time (Fig. 2a), further indicating that cell wall monomer composition differed among treatments. Discriminant function 1 was most strongly associated with galactose and mannose, and negatively associated with fucose, while discriminant function 2 was most strongly associated with xylose, fucose, and p-hydroxyphenyl, and negatively associated with arabinose (Table A.2).

The individual monomer responses to fertilization resulted in a 3% reduction in hemicellulose (MANOVA $F_{3,22} = 3.11$, $p = 0.047$, Table A.3). However, the minor changes in syringyl did not correspond to a change in total lignin (Fig. 3, Table A.3).

Independent of fertilization, Southlow had 25% more p-hydroxyphenyl, 15% more rhamnose, and 19% less mannose than Cave-in-Rock (Table 1, Table A.1), despite a lack of overall differences in major cell wall components.

3.2. N-fertilization effects under drought conditions

Above ground biomass also did not respond to fertilization in the drought subplots (Fig. 1). Fertilizer still impacted cell wall monomer composition in drought conditions (MANOVA $F_{11,14} = 4.02$, $p = 0.008$), but this was entirely due to a 6% reduction in glucose (Table 1, Table A.1). No other monomers varied in response to fertilizer in the drought subplots. Further, two linear discriminant functions explained 82% of the variation in cell wall monomer composition among cultivar and fertilizer treatments in drought conditions, respectively, but only placed plots in the correct treatment 31% of the time (Fig. 2b), indicating that treatments did not strongly differ in cell wall monomer composition. Discriminant function 1 was most strongly associated with mannose, and negatively associated with rhamnose and xylose, while discriminant function 2 was most strongly associated with galacose and xylose, and negatively associated with fucose (Table A.2).

The reduction in glucose corresponded to a 2% overall reduction in hemicellulose in drought subplots (MANOVA $F_{3,22} = 7.54$, $p = 0.01$, Table A.3). There were no differences in lignin or cellulose, the other two major cell wall components.

Under drought conditions, Southlow had 12% more p-hydroxyphenyl than Cave-in-Rock, independent of fertilization effects (Table 1, Table A.1).

4. Discussion

4.1 Does N-fertilization affect switchgrass biomass quantity or quality?

Under ambient precipitation conditions at our site, fertilization did not increase biomass, but did alter cell wall composition of two switchgrass cultivars in ways that are likely to affect quality as a bioenergy crop. Changes in the proportion of the three major cell wall components: cellulose, hemicellulose, and lignin, as well as changes in the monomers contributing to hemicellulose and lignin, can influence biomass fermentation and ethanol yield. Generally, hemicellulose, especially the monomer xylose, is easily converted to ethanol [35], while lignin interferes with biochemical conversion processes by binding with cellulose and hemicellulose [16]. In this study, fertilization reduced total hemicellulose by decreasing xylose, the major component of hemicellulose, but also increasing mannose, a minor component of hemicellulose.

Hemicellulose monomers differ in their fermentation efficiency, with xylose more easily digested than mannose [36]. This indicates that while the effect size was not large, N-fertilization decreased switchgrass biomass quality in terms of hemicellulose. Other studies have also reported that fertilizer decreases hemicellulose in switchgrass [e.g., 37], though this is not always the case [35, 38]. A meta-analysis by Liu et al. [15] found that, on average, N-fertilization decreased hemicellulose by 4.39% in a wide variety of plant species, which is similar to the response (4% decrease) in our study.

For lignin, N-fertilization effects were slightly positive. While this study did not find any change in total lignin levels in response to fertilization, it did detect an increase in syringyl (S) compared to other lignin monomers. A high S:G ratio is desirable for bioenergy production as it is associated with decreased recalcitrance during pre-treatment [39, 40], so from this perspective our results indicate that fertilizer slightly improved biomass quality. There is considerable variability in prior research on the effects of fertilizer on switchgrass cell lignin. Studies have shown N-fertilization to have no effect on lignin [38, 41, 42], but also that N-fertilization can increase switchgrass lignin [37, 43, 44] due to stimulation of phenylalanine biosynthesis [45]. The meta-analysis by Liu et al. [15] found that N-fertilization increased lignin (~7%), but the magnitude of the response was contingent on study duration. Short-term fertilizer treatments (less than three years) showed stronger positive effects than long term (3-10+ years) application [15]. Our study was conducted in plots that had consistent fertilizer application for 9+ years, thus our results are consistent with the meta-analysis findings.

The lack of N-fertilization effects on switchgrass biomass we observed in this study is not uncommon, especially for switchgrass grown in more northern regions [46] and on more fertile soils [47]. The level of N we applied has been shown to consistently increase aboveground biomass production in successional fields at this site [48], suggesting that biomass production is N-limited on these soils. However, other experiments at this site have shown no effect of fertilizer (up to 196 kg N ha^{-1}) on switchgrass biomass production [11]. The soils at this experimental site are moderately fertile [total mineral N 3–30 μg/g; 49], and had previously been used for corn production. Our results indicate that switchgrass may not be very responsive to N-fertilization compared to other perennial herbaceous plants. Given that biomass yield is overall more important than plant cell wall composition in predicting ethanol production [43, 50], it is unlikely that the minor changes in plant cell wall composition due to fertilization will outweigh the financial costs of nitrogen fertilizer application in our study. However, fertilization may change other aspects of plant biomass quality such as soluble sugars, which we did not measure here [51].

While it was not a primary focus of our study to compare cultivar responses, we did find that the two switchgrass cultivars in our experiment differed slightly in their cell wall monomer composition. Switchgrass cultivars have been shown to vary in cell wall composition in other studies, though differences are usually stronger between ecotypes than between cultivars within a given ecotype [16, 52]. The two cultivars used in this study were both upland ecotypes, and this may be why differences in cell wall monomer composition did not translate to differences in the three major cell wall components.

4.2 Does drought affect switchgrass responses to fertilization?

Our drought treatment reduced soil moisture by 40% overall, which did not affect switchgrass biomass but did minimize the effects of N-fertilization on switchgrass cell wall composition. Fertilization caused changes in three cell wall monomers, including the important monomer xylose, under ambient conditions. However, glucose, a relatively minor hemicelluosic monomer, was the only component to show any response to fertilization under drought conditions. Additionally, the LDA showed clear separation in cell wall monomer composition due to fertilization and cultivar treatments in ambient conditions, but not under drought conditions. Drought also minimized differences in cell wall composition between the two cultivars in our study. Our study was done on mature switchgrass stands and the finding that drought stress can override benefits of N-fertilization is consistent with other switchgrass studies [22, 23]. However, one study [53] has shown N-fertilization to be more important in dry vs. wet conditions during the switchgrass establishment phase. Interactions between fertilizer and drought may be more important to consider during crop establishment years [54].

We recognize that additional variables may have also affected plant cell wall composition in our study. For example, we harvested plants in October following protocols for the region, but some studies have shown that cell wall composition can change over the winter if harvest is delayed until spring [e.g., 53]. It is also possible that the minor reduction in light availability (~19%) caused by the rainout shelters may have influenced plant responses [55], though light levels tends to be less important than variability in precipitation or temperature in terms of influences on plant cell wall composition [56].

In conclusion, our results show that drought negates any benefits of N-fertilization on switchgrass biomass quality. Our results also support prior findings that switchgrass is a relatively drought-tolerant species that will be a viable bioenergy feedstock even in a changing climate [57, 58]. Although our experimental drought treatments were relatively moderate, they were applied continuously over two growing seasons. Future work should evaluate whether thresholds exist for switchgrass responses to extreme droughts which are predicted to increase in the future [59, 60]. Also, although our experiment was not conducted on marginal lands per se, the lack of N-fertilization effects suggests that there are no obvious benefits in terms of biomass quality or quantity under drought conditions, and so may not warrant the economic costs associated with large-scale applications.

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Figure Legends

Figure 1. Effects of N-fertilization on above-ground biomass of two mature switchgrass cultivars in a) ambient precipitation and b) drought subplots in the two years of the rainout shelter experiment. There were no significant effects of fertilization in either precipitation treatment. ANOVA results: Control: Cultivar $F_{1,24} = 3.12$, p = 0.09; Fertilizer $F_{1,24} = 1.04$, p = 0.32; Fertilizer x Cultivar $F_{1,24} = 0.20$, p = 0.66; Block $F_{3,24} = 0.40$, p = 0.75; Year $F_{1,24} = 3.68$, p = 0.07. Shelter: Cultivar $F_{1,24} = 0.99$, p = 0.33; Fertilizer $F_{1,24} = 0.003$, p = 0.96; Fertilizer x Cultivar $F_{1,24} = 0.18$, p = 0.75; Block $F_{3,24} = 3.02$, p = 0.05; Year $F_{1,24} = 10.53$, p = 0.003. Error bars indicate ± 1 SE.

Figure 2. Linear discriminant analysis (LDA) of cell wall monomers in two switchgrass cultivars grown in long-term fertilized and unfertilized plots in a) ambient precipitation and b) drought subplots. Ovals represent 95% confidence interval ellipses for each group (variety and fertilizer treatment). For ambient subplots, the misclassification rate was 19%. For drought subplots, misclassification rate was 69%. See Table A.2 for discriminant function loadings.

Figure 3. Effects of fertilization on major cell wall components of two cultivars of switchgrass grown in (a-c) ambient precipitation and (d-f) drought subplots. Error bars indicate ± 1 SE. Asterisks indicate differences due to fertilization ($p<0.05$). There were no significant differences between cultivars. See Table A.3 for MANOVA statistics.