Yield, biomass composition, and N use efficiency during establishment of four *Miscanthus* × *giganteus* genotypes as influenced by N management

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ABSTRACT

Few studies have examined N management strategies to accelerate *Miscanthus* establishment. Our objectives were: (i) determine differences in biomass yield among various *M. × giganteus* genotypes as influenced by N management during establishment; (ii) quantify the impact of genotype and N management on biomass composition; and (iii) determine how *M. × giganteus* genotype and N management influence nitrogen use efficiency (NUE). Four *M. × giganteus* genotypes were planted near Schochoh, KY and Lafayette, IN in 2010. A two-year total N application of 150 kg ha⁻¹ was applied using various combinations of 50, 75, and 100 kg ha⁻¹ y⁻¹. Control N rates included 0 and 150 kg ha⁻¹ each year. Yield, composition, and NUE of the MS and IL clones were similar. Two-year cumulative yields of Nagara were higher than the other genotypes in KY, and the IL clone in IN. There was no response of yield to N on the silt loam soil in KY, whereas high biomass yields were achieved with 50 kg ha⁻¹ y⁻¹ of N on the sandy loam soil in IN. Yields of plots provided high N in Season 1 were similar to unfertilized control plots in Season 2 suggesting little N carryover from Season 1 to 2. Biomass fiber concentrations were not influenced by N-fertilization, but high leaf retention of the Nagara lines reduced biomass cellulose and lignin concentrations. Site-specific genotypic differences in NUE were observed. Annual N applications of 50 kg ha⁻¹ is recommended to enhanced *Miscanthus* yield during establishment on the coarse-textured soils.

Keywords:

Miscanthus Keng
Clonal differences
Biomass yield
Establishment
Cellulose
Lignin

51 Nitrogen use efficiency

52 *Abbreviations:*

53 NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; GHG,
54 greenhouse gas; IL clone, Illinois clone of *Miscanthus × giganteus*; MS clone, Mississippi clone
55 of *Miscanthus × giganteus*; OP, open pollinated; C, carbon; N, nitrogen; ANUE, agronomic
56 nitrogen use efficiency; PNUE, physiological nitrogen use efficiency.

58 1. Introduction

59 The Energy Independence and Security Act of 2007 mandates that by 2022, the United States
60 will replace $136 \text{ hm}^3 \text{ y}^{-1}$ of transportation fuels with biofuels, with at least $61 \text{ hm}^3 \text{ y}^{-1}$ coming
61 from cellulosic feedstocks. To meet this goal there is a pressing need to understand sustainable
62 production of cellulose-based biofuels from dedicated energy crops. Qualification as a cellulosic
63 feedstock requires a 60% reduction in life-cycle greenhouse gas (GHG) emissions as compared
64 to current fossil fuels. Perennial warm-season grasses are expected to yield large quantities of
65 aboveground biomass with minimal inputs such as fertilizers, herbicides, and pest and pathogen
66 control measures, etc. [1,2]. Nitrogen (N) fertilizer is a key input generally necessary for
67 productivity in agriculture, but N fertilizer synthesis requires substantial energy [3] and this
68 detracts from favorable GHG emission reductions and net energy balance [4]. Therefore,
69 minimizing fertilizer N input while maintaining high biomass yield are important co-objectives.
70 In general, relatively low fertilizer N rates are considered sufficient for perennial crops because
71 they accumulate significant N reserves in below-ground organs that provide N when growth is
72 initiated in spring and also during regrowth after in-season harvest [5,6]. However, our
73 understanding of optimal N management strategies for perennial bioenergy grasses, especially
74 during the critical phase of stand establishment, is fragmented and incomplete including

influences on belowground N reserves for overwintering and spring regrowth the subsequent year during the establishment phase.

Miscanthus × *giganteus* is a perennial warm-season grass originating from Asia that is an inter-specific hybrid between *M. sinensis* and *M. sacchariflorus* [7]. Agronomic studies over the past 30 years reveal that *M. × giganteus* can be highly productive [8,9], but clonal propagation is costly and its limited genetic variation may enhance susceptibility to biotic and abiotic stresses. To overcome the limitations associated with a narrow genetic base, Baldwin [10] starting with the IL clone, undertook serial asexual propagation and selection to develop the ‘Mississippi’ (‘MS’) sterile triploid clone of *M. × giganteus*. This clone exhibited changes in expressed mRNA when compared with the IL clone [11], but both clones possess identical nuclear and plastid DNA [12]. Alternatively, Deuter [13] crossed a tetraploid *M. sacchariflorus* with a diploid *M. sinensis*, creating a new sterile triploid *M. × giganteus* genotype called ‘Nagara’ [14] that is genetically distinct from the ‘IL’ clone [12]. Surprisingly, four seeds from this cross were recovered and used to create a fertile tetraploid population collectively known as the Nagara-siblings (Nagara-sibs). These plants and their progeny offer the possibility to develop a traditional *M. × giganteus* breeding program and reduce establishment costs by seed rather than rhizome propagation. Its greater genetic diversity also provides the potential for improved long-term agronomic performance when compared to the clonally propagated IL or MS genotypes but data on relative performance at field-scale are not yet available. In addition, while it has been reported that concentrations of lignin, cellulose, and hemicellulose vary among *Miscanthus* species/genotypes [15], and that such compositional diversity can impact conversion efficiency [16], little data exist comparing the biomass composition of *M. x giganteus* genotypes during establishment.

Nitrogen fertilization of *M. × giganteus* remains controversial with some reports indicating no yield response to N fertilization [17-20], while others assert that high biomass yields require at least nominal annual N fertilizer applications [9,21-24]. Recent studies suggest that the IL clone generally requires modest (40-120 kg ha⁻¹) N fertilizer applications when grown in the Midwest US compared to annual cropping systems like maize where 150 to 200 kg ha⁻¹ of N are generally recommended [25,26]; however, *Miscanthus* was not responsive to N at some locations [9]. In experiments where *M. × giganteus* was not responsive to N, researchers have speculated that associative N₂ fixation may contribute to the N nutrition/balance of this species [27-31]. Certainly, more agronomic research is needed in order to develop robust N recommendations specific to the US Midwest that distinguish N needs of established stands from that required during establishment.

Rapid establishment of *M. × giganteus* and other perennial grasses used for biomass is important from both economic and environmental perspectives [32]. Strullu et al. [33] recently concluded that, while water availability always impacted productivity of *M. × giganteus*, N was a key factor limiting stand establishment. Miguez et al. [8] used a meta-analysis approach to summarize the N response of the IL clone of *M. × giganteus* from planting through Year 7 and concluded that N did not have an important effect for the first three growing seasons, but enhanced yield modestly thereafter. However, their analysis compared N at 0 and 100 kg ha⁻¹ fertilizer rates, and did not examine N management options such as timing or placement. Others [34] applied a wider range of N fertilizer rates during establishment (0, 100, 200 kg ha⁻¹), but did not sample plants in the first season, nor vary N application strategies. As a result N recommendations during *Miscanthus* establishment remain unclear and range from following European guidelines where no fertilizer is applied during the first 2 years of establishment [35]

to NPK and lime applications at planting following regionally relevant best management practices for maize [36]. Fertilizer N recommendations are further complicated by the fact that established *M. × giganteus* plants accumulate N in belowground storage organs in autumn that can be remobilized to support shoot N needs in spring and after biomass harvest [21]. Thus, we hypothesize that rhizome N storage resulting from application of high N rates in Season 1 might supplement or even supplant N required for rapid shoot growth in treatments receiving moderate or no N in Season 2 of establishment. However, with poorly developed rhizome and root systems the rate and timing of N fertilization of recently planted *M. × giganteus* may differ in important ways from that of well-established plants.

Minimizing N fertilizer input, while still accelerating plant establishment remains an important agronomic goal because this strategy enhances N use efficiency (NUE) and improves system net energy balance because synthesis/application of N fertilizer is energy intensive [24]. Multiple ways exist to calculate NUE [37-40]. Reported NUE values for *M. × giganteus* have been limited to established stands, and the range in NUE values in these studies have been large [41]. It is not known how N fertilizer rate interacts with timing of N application (annual versus alternate year N applications) to achieve the co-objectives of high NUE and rapid establishment.

The overall objectives of this study were to understand the genotype × N management interactions during establishment of *M. × giganteus* genotypes. Specifically, our objectives were: (i) determine differences in biomass yield among various *M. × giganteus* genotypes as influenced by N management during establishment; (ii) quantify the impact of genotype and N management on biomass composition; and (iii) determine how *M. × giganteus* genotype and N management influence nitrogen use efficiency (NUE).

2. Materials and methods

2.1 Experimental sites and crop management

For this study two locations were selected. The first was established in Schochoh, KY (36.760006N,-86.768904W) on an Elk silt loam soil (fine-silty, mixed, active, mesic Ultic Hapludalfs). The second location was planted on a Billett sandy loam soil (coarse-loamy, mixed, superactive, mesic Mollic Hapludalfs) near Lafayette, IN (40.484096N,-86.815827W) [42]. Weather data (Fig. 1) for the years of KY study and the 50-year average were obtained from the Bowling Green-Warren County Regional Airport 50 km to the northeast of the study site. Weather data for the years of the IN study was obtained from the FFR Cooperative weather station in Lafayette IN co-located with the experimental site. The 50-year data record for the IN location was obtained from the Purdue University Airport located 15 km southwest of the study site.

The four *M. × giganteus* germplasms used include the ‘IL’ (also known as ‘1993-1780’), [7], ‘MS’ (also known as ‘MSU MFLI’ and ‘Freedom’; [10]), and ‘Nagara’ (also known as ‘MBS 7001’ and ‘M116’) [13] sterile triploid clones, and a fertile, tetraploid, open-pollinated (OP) Nagara-sib population. The ‘OP-Nagara’ consists of seed collected from a polycross from four fertile tetraploid hybrids of a tetraploid *M. sacchariflorus* and diploid *M. sinensis* plants, as described by Sacks et al. [43].

To standardize propagule size across genotypes at planting, plants were started in a commercial greenhouse (Speedling Inc., Ruskin, FL) either by vegetative propagation or by seed, depending on genotype, in 72-cell EPS trays size: 34 cm W × 67 cm L × 76 cm D (Speedling Inc., Ruskin, FL). Plants were grown for 2 to 4 months in the greenhouse (timing differed by location, not genotype), and shoots were trimmed to 10 cm - 15 cm before transplanting. Maize was the preceding crop at both locations. Prior to planting, tillage and an application of

glyphosate [N-(phosphonomethyl) glycine] used according to the label, were applied to control winter-annual weeds. Both sites were planted in 2010 using a Holland Rotary One Transplanter (Holland Transplanter Co., Holland MI) at a population density of 19,760 ha⁻¹. Plots were four 9.14 m rows spaced 0.91 m between rows. Plants within a row were spaced every 0.54 m for a total of 17 plants per row. Immediately after transplanting, Harness Xtra® Acetochlor [2-chloro-N-ethoxymethyl-N-(2-ethyl-6-methylphenyl) acetamidine] and Atrazine, [2-chloro-4-(ethylamino)-6-(isopropylamino) s-triazine] were applied according to the label and annually each year thereafter in early spring as a pre-emerge herbicide. Dimethylamine salt of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) was applied at labeled rates when needed to control broadleaf weeds. At the KY location irrigation was applied when needed by center pivot during the duration of the study. The IN location was rain fed. The center two rows at each location were harvested for yield and biomass quality analysis.

Previous results [44] have shown April and May as the optimum transplanting window at these locations to maximize first and second year biomass yields. Timely transplanting occurred at the KY location in 2010 so the N treatments were applied 2010 and 2011 (Season 1 and 2 at this location, respectively). Excessive rainfall (Fig. 1) that delayed planting in IN until late June of 2010 followed by limited precipitation August through October (Fig. 1) resulted in plants that were too small to fertilize and harvest; therefore, N treatments at the IN site began in 2011 (Season 1) and concluded in 2012 (Season 2). Table 1 provides a timeline of planting, harvesting, and fertilization practices for both. Throughout this paper Season 1 and Season 2 refer to the first and second season of N treatment, respectively, rather than time after planting. Details of N fertilizer managements are summarized in Table 2. Briefly, Treatment 1 was an unfertilized control. Treatments 2 to 6 received a 2-year total of 150 kg ha⁻¹ of N applied at

various rates over two years. For example, 50-100 kg ha⁻¹ refers to 50 kg ha⁻¹ applied in Season 1 and 100 kg ha⁻¹ in Season 2. Treatment 7 received 150 kg ha⁻¹ both years and serves as a high N-rate control. Nitrogen was applied by hand as AgrotainTM-coated urea.

After the killing frost, but prior to biomass harvest in Season 2, plants were visually scored for leaf blade retention. Plots were given a score between 1 and 5 with 1 ≤ 20% retention, 2 between 20 to 40% retention, 3 between 40 to 60% retention, 4 between 60 to 80% retention, and 5 between 80 to 100% leaf retention. Preliminary results indicate that leaf sheath retention did not vary among genotypes and heretofore was not included in this rating scheme. Ratings were obtained prior to biomass harvest in January 2012 (KY) and in November 2012 (IN) (Table 1).

Plots were harvested with a tractor-mounted Kemper C 1200 forage harvester silage head and a Wintersteiger Cibus TRM (Wintersteiger Inc., Salt Lake City UT) that targeted a 15 – 20 cm cut height and excluded the leaf litter layer. The KY plots were harvested in January of 2011 and 2012 for the 2010 and 2011 growing season, respectively. The IN plots were harvested in January 2012 for the 2011 growing season and November 2012 for the 2012 growing season (Table 1). Subsamples of aboveground biomass (0.3 to 0.8 kg wet weight) were placed in either paper or cloth bags and dried in forced-air ovens at 60 °C until constant weight was attained. Dried samples were weighed and percent dry matter (DM) mass fraction calculated. The DM was used to adjust field fresh weight data and harvested area to biomass yield on a dry weight basis. Season 1 mean yields for the 0 and 150 N treatments have a total of eight replications each because of identical plot treatment (0-0, 0-150 for the 0 N treatment; 150-0, 150-150 for the 150 N treatment) in Season 1. Differential N treatment in Season 2 subsequently resulted in the normal four replicates of each of the five treatments. Cumulative yields were summed over

Seasons 1 and 2. Representative subsamples were ground to initially pass a 6-mm screen (Wiley Mill, Thomas Scientific, Swedesboro, NJ), then re-ground to pass a 1-mm screen using a cyclone sample mill (Udy Corp., Fort Collins, CO) for laboratory analysis.

Stunting of certain plots, believed to be associated with nematode injury, was observed at the KY location in 2011 and this effect was quantified for all plots using a visual rating scale where 0 (plants unaffected) to 5 (all plants dead). These data were used as a covariant in the statistical analysis.

2.2 Laboratory analysis

Fiber concentration was determined using the detergent-fiber analysis system [45]. Samples were analyzed in duplicate for neutral detergent fiber (NDF) and acid detergent fiber (ADF) using an ANKOM 2000 Fiber Analyzer (ANKOM Corp., Fairport NY USA). Acid detergent lignin (ADL) was determined using a Daisy II wheel incubator (ANKOM Corp., Fairport NY USA). Samples (500 mg) in fiber bags were sequentially extracted with NDF and ADF solutions, then placed in Daisy II incubator jars and covered with 72% volume fraction H_2SO_4 . The samples were rotated in the incubator for 3 h at approximately 25 °C. After rotation, samples were washed four times with reverse-osmosis (RO) water, and then boiled in RO water for 10 min to remove remaining H_2SO_4 . After each NDF, ADF, and ADL extraction, residues were rinsed in acetone for 10 min, dried at 102°C overnight, and weighed. Hemicellulose concentration was calculated by subtracting the ADF concentration from the NDF concentration. The cellulose concentration was calculated by subtracting the ADL concentration from the ADF concentration [46]. Total ash was determined by placing 1 g of plant tissue into pre-weighed crucibles, combusting the samples in a muffle furnace at 550°C for 4 h, and weighing the remaining residue.

Aboveground biomass was also analyzed for total C and N concentrations using a flash combustion elemental analyzer (Flash EA 1112 Series, Thermo Fisher Scientific, Netherlands). Agronomic NUE (biomass kg⁻¹ N fertilizer applied) was calculated for plots within a replicate as the change in biomass per unit of N applied relative to the 0 N control plots ($\text{Biomass N Rate } X - \text{Biomass N Rate } 0 / (N \text{ Rate } X - N \text{ Rate } 0)$ where N Rate X = 50, 75, 100 or 150 kg ha⁻¹. The physiological nitrogen use efficiency is calculated as the ratio of DM yield and N accumulation per hectare [38,47].

2.3 Statistical analysis

Both locations had four replications with treatments arranged in a randomized complete-block design (RCBD). Data were analyzed separately by location and year because N managements extended over 2 years (Table 2) and because of the delayed planting in IN. Data were analyzed using PROC GLM in SAS 9.4 (SAS inst. Inc., Cary, NC, USA). Cumulative yield data from Seasons 1 and 2 were summed and analyzed for each location separately. Stunting scores were used as a covariate for data from the KY location using ANCOVA. Where F-tests were significant, Tukey's test was used to separate means [48]. Correlation analysis among leaf blade retention, yield, and biomass composition traits was performed for Season 2 data using PROC Corr in SAS 9.4.

3. Results

3.1. Weather

Temperatures were slightly above average during the spring of 2010 and 2011, but were consistently warmer than the 50-year average for 2012 from January through August at Lafayette, IN (Fig. 1). Shoot growth in spring 2012 initiated in March because of the exceptionally warm temperatures. However, a freeze in early April 2012 at the IN location

damaged aboveground tissue, and shoot regrowth was initiated a second time from the belowground rhizomes about a week later. Annual precipitation was normal in 2010, but about 25% above normal in 2011 at both locations. Precipitation at the IN location was 41% below normal from January to July 2012.

3.2. Biomass yield

At the KY location stunting believed to be associated with Lance nematode (*Hoplolaimus galeatus*) was observed in a section of this experiment that reduced plant growth and development (Personal communication, Christian Hillnhütter, Data management supervisor DuPont Crop Protection, email, 2015-February-17). Stunting scores (Table 3) were used as a covariant in all yield analyses at this location in order to adjust for this unforeseen effect. Averaged across N managements, *M. × giganteus* genotypes differed in biomass yield, but responses were affected by year and location. At the KY location, Nagara had the greatest biomass yield in Season 1 (8.6 Mg ha⁻¹), while the IL clone had the lowest yield at this location in Season 1 (Table 4). The OP Nagara had yields intermediate between Nagara and the IL clone. Averaged over genotypes, Season 2 yields were more than 2-fold greater than those in Season 1 and averaged 12.8 Mg ha⁻¹. In Season 2 the IL and MS clones had the greatest yield (avg. 13.7 Mg ha⁻¹), while the OP Nagara had the lowest yield (11.4 Mg ha⁻¹). Cumulative two-year yields at the KY location were 16.8 to 21.0 Mg ha⁻¹ for OP-Nagara and Nagara, respectively (data not shown). At the IN location, yield differences were detected in Season 1 and for the two-year cumulative yields (Table 4). In Season 1 the IL clone had lower yield than the Nagara and the OP-Nagara lines. Cumulative yields in IN differed (at $P = 0.07$) among lines with Nagara having higher biomass yield than the IL clone (data not shown).

When averaged across *M. × giganteus* lines there was no effect of N management on biomass yield at the KY location in either year or total two-year cumulative yields (Table 5). However, Season 1 yields in IN were lower with the 0 kg ha⁻¹ N treatment when compared to plots receiving any N fertilizer rate. In Season 2 biomass yield of plots not receiving N that season (0-0 and 150-0 kg ha⁻¹ treatments) were low yielding, while the 0-150 and 75-75 kg ha⁻¹ treatments had the greatest yields. Cumulative yields in IN were similar among all treatments receiving N irrespective of N rate and/or application timing, and all were greater than that of the 0 N control treatment. The genotype x N management interaction for biomass yield was not significant at the KY site. There was a significant genotype x N management interaction ($P < 0.10$) in Season 1 at the IN location associated with the response of the OP-Nagara having a lower yield response to N when compared to the other genotypes. However, this change in magnitude of N responsiveness and not change in ranking suggested that in these years and locations all four *M. x giganteus* genotypes generally responded similarly to these N managements.

3.3. Carbon and nitrogen concentrations and NUE

The genotype × N treatment interaction for biomass C concentration was not significant, therefore, main effects of genotype and N treatment are reported. Biomass C concentrations were similar among genotypes (475 g kg⁻¹) in Season 1 at the KY location. In IN and during Season 2 at KY the IL and MS clones had higher biomass C concentrations (476 vs. 482, 472 vs. 477, and 473 vs. 479 g kg⁻¹ respectively) than Nagara and OP-Nagara. Averaged over genotypes, N treatment did not alter biomass C concentration at either location in any year. The C concentrations for KY averaged 465 and 476 g kg⁻¹, while for IN they averaged 479 and 474 g kg⁻¹ for Seasons 1 and 2, respectively (data not shown).

There was a significant genotype $\times$ N treatment interaction for biomass N concentration in Season 1 at the IN location due to the greater N concentration in OP-Nagara compared to the other genotypes. However, the genotype $\times$ N treatment interaction for biomass N concentration was not significant at the KY location nor Season 2 in IN. Therefore, only the main effects of genotype and N treatments are discussed. Averaged over N treatments and regardless of location and year, the OP-Nagara biomass always had higher N concentrations than the IL and MS clones (Table 4). The Nagara genotype had higher biomass N concentrations than the IL genotype in Season 1 in IN, and the IL and MS clones in Season 2 at the KY location. Biomass N concentrations of the IL and MS genotypes were similar at both locations each year.

Averaged over genotypes, biomass N concentration was highly influenced by N management (Table 5). The 0 kg ha⁻¹ treatment had a lower biomass N concentration than all other N treatments except the 50 kg ha⁻¹ treatment in Season 1 at KY. The 150 kg ha⁻¹ treatment had the greatest N concentration at both locations in Season 1. In Season 2 at the KY site biomass N concentrations of all N treatments were similar except the 50-100 and 0-0 kg ha⁻¹ treatments where biomass N concentration was lower than the 150-150 kg ha⁻¹ treatment. The 150-150 kg ha⁻¹ treatment also had the highest biomass N concentration in Season 2 in IN, with values similar to the 0-150 kg ha⁻¹ treatment. At the IN site in Season 2 biomass of the 150-0 kg ha⁻¹ treatment contained only 5.3 g kg⁻¹ in spite of having been fertilized with N at 150 kg ha⁻¹ in Season 1. This was the second lowest N concentration observed in this environment and suggests little N carryover in soil or mobilization from below- ground storage organs from Season 1 to biomass in Season 2 occurred despite the very high initial N fertilizer application. Genotype and N treatment main effects for biomass C:N ratios were significant with the primary cause being

differences in tissue N concentration as tissue C concentration differences were small (C:N data not shown).

Agronomic N use efficiency (ANUE, kg kg^{-1} fertilizer applied) differed among genotypes and N rates, and there was no genotype $\times$ N rate interaction for ANUE. The ANUE of genotypes, averaged over N rates, was similar in Season 1 at Kentucky (Table 4), whereas the Nagara had higher ANUE in Season 2 at this location than the other genotypes producing 31 kg kg^{-1} fertilizer applied. The ANUE of the OP Nagara was two- to four-fold greater than the other genotypes in Season 1 in IN. In Season 2 at this location the OP Nagara produced twice as much biomass per unit of N as Nagara (34 vs 15.1 kg kg^{-1} , respectively) with ANUE values of the other genotypes intermediate.

Averaged over genotypes ANUE also was influenced by N fertilizer management in Season 2 in KY and in both years in IN (Table 5). The lowest rate of applied N (50 kg ha^{-1}) in Season 2 in KY resulted in higher ANUE (nearly 28 kg kg^{-1}) than plots receiving 75 or 150 kg ha^{-1} of N ($<12 \text{ kg kg}^{-1}$). In Season 1 in IN the ANUE of the plots provided 50 and 75 kg ha^{-1} averaged about 24 kg kg^{-1} and this was higher than plots receiving N at 150 kg ha^{-1} that averaged only 7.6 kg kg^{-1} . In Season 2 in IN the plots fertilized with 50 and 75 kg ha^{-1} had higher ANUE ($>33 \text{ kg kg}^{-1}$ N fertilizer) than the plots fertilized with 150 kg ha^{-1} for both Seasons 1 and 2 (12.8 kg kg^{-1}), and plots fertilized with 100 kg ha^{-1} in Season 2 (19.2 kg kg^{-1}).

Differences in physiological N use efficiency (PNUE, kg kg^{-1} of tissue N in biomass) were observed between genotypes when data were averaged over N treatment. The OP-Nagara had the lowest PNUE values, while the IL and MS clones had high PNUE in all environments tested (Table 4). The Nagara genotype had higher PNUE than the OP-Nagara in Season 1 in KY, and both years in IN, but PNUE of this genotype was lower than the IL and MS clones in KY in

Season 2. Depending on the environment, PNUE of the OP-Nagara ranged from 126 to 216 kg kg⁻¹, while the range in PNUE of the IL clone was 144 to 297 kg kg⁻¹.

Averaged across genotypes, N rate effects on PNUE were significant in all environments (Table 5). The PNUE for the 0 and 0-0 kg ha⁻¹ treatments was significantly greater than that of 150 and 150-150 kg ha⁻¹ treatments. In Season 1 PNUE for the 0 and 150 kg ha⁻¹ treatments were 157 vs 132 kg kg⁻¹ at KY and 303 vs 216 kg kg⁻¹ at IN, respectively, while during Season 2 PNUE values for the 0-0 and 150-150 treatments were 282 vs 224 kg kg⁻¹ at KY and 226 vs 146 kg kg⁻¹ at IN. In Season 1 and 2 in IN the intermediate N treatments resulted in PNUE values between that of the 0 and 150 kg ha⁻¹ treatments, whereas at the KY location many of the intermediate N treatments had PNUE values similar to the 150 kg ha⁻¹ treatment. The genotype × N treatment interaction for PNUE was significant at KY in Season 2 and at IN in Season 1. At the IN location this was associated with PNUE of the OP-Nagara producing less biomass per unit of biomass N compared to the other genotypes.

3.4. Biomass composition

There were few genotype × N treatment interactions for the fiber components. Therefore, the main effects of genotype and N management will be presented. Hemicellulose concentrations were similar among genotypes in Season 1 at both sites (Table 6). In Season 2 hemicellulose concentrations at the KY site were similar in the IL and MS clones, and both were higher than hemicellulose concentrations in the Nagara clone and the OP-Nagara. In Season 2 in IN hemicellulose concentrations were highest for the IL and MS clones, lowest for the OP-Nagara, with Nagara being intermediate. Genotypic differences in cellulose concentration were observed in every site-year. Cellulose concentrations of the IL and MS clones were similar, and always greater than the OP-Nagara. Cellulose concentrations of the Nagara clone were lower than the IL

and MS clones in three of four site-years (exception IN Season 2) and were greater than the OP-Nagara in all site-years except KY in Season 2. Acid detergent lignin (ADL) concentrations also were significantly different among genotypes in every site-year. The IL and MS clones always had higher ADL concentrations than Nagara genotypes. The Nagara clone had lower ADL than the OP-Nagara in both years at the KY site, while both years at the IN site the OP-Nagara had lower ADL than the Nagara clone. Total ash concentrations of genotypes did not differ in KY in Season 1 and IN in Season 2. The IL and MS clones had lower ash concentrations than the OP-Nagara in the other two environments, and Nagara had intermediate ash concentrations in KY in Season 2.

Even though some differences were detected among fiber constituents there was no consistent pattern across years or locations as a result of the N treatments. Averaged over genotypes, N treatment had no effect on the fiber composition during both years at the KY site (data not shown). At the IN location, total ash was influenced by N fertilization in Season 1 while ADL was affected in Season 2. Total ash concentrations ranged from 20.6 to 23.6 g kg⁻¹ with ash concentrations of the 0 kg ha⁻¹ treatment lower than that of the 50, 100 and 150 kg ha⁻¹ treatments. The ADL concentration for the 0-0 kg ha⁻¹ treatment was lower than the 0-150 kg ha⁻¹ treatment (72.1 vs 77.4 g kg⁻¹) in Season 2.

3.5. Leaf retention and correlations to biomass composition

In Season 1 genotypic differences in leaf retention were observed, but not quantified. Leaf retention scores were taken on all plots in Season 2. The genotype × N treatment interaction for leaf retention was not significant at either location. In addition, averaged across genotypes, N treatment had no effect on the leaf retention (data not shown). Differences in leaf retention among the genotypes were significant at both locations (Table 6). The IL and MS clones had

similar retention scores, and both of these genotypes had lower leaf retention than the Nagara and OP-Nagara genotypes. In KY, leaf retention of OP-Nagara was significantly higher than that of the Nagara clone; a difference not observed at the IN location. The trend for higher leaf retention at the IN site, when compared to the KY site, could be due to harvesting/scoring plants in November (IN) instead of January (KY) by which time additional biomass weathering would be expected to cause accelerated leaf loss.

There were significant correlations between the leaf blade retention scores and various quality parameters at both locations. At both sites, positive correlations were observed between leaf blade retention scores and concentrations of hemicellulose and ash (Table 7). Likewise, at both locations consistent negative correlations were observed between leaf blade retention score and concentrations of C, ADL and cellulose. Biomass yield did not correlate with leaf blade retention score. The correlation of both N concentration and NUE with leaf blade retention score dependent on location. At the KY site, N and NUE were highly correlated ($r = 0.72$, $P < .0001$ and $r = -0.74$, $P < .0001$) with leaf retention score, while only a low correlation between N and leaf retention score ($r = 0.17$, $P < 0.07$) was observed at the IN location.

4. Discussion

Few reports exist on N management of *M. × giganteus* genotypes during establishment and its effect on yield, composition, and NUE. Our findings that N responsiveness of these *M. × giganteus* genotypes during establishment is site-specific, occurring on the light-textured, sandy loam soil at the IN site, but not at the heavier soil at the KY site agrees with previous studies with established stands where N responsiveness is often inconsistent and site-specific. For example, some studies reported yield increased with N fertilization [9,22,24,34,49,50], while other studies revealed either no yield response to applied N [17-19]. In addition, significant

genotype x N rate interactions have been reported for well-established *M. × giganteus* stands, with some genotypes responding more to applied N than others [19]. We did not observe a significant genotype x N rate interaction at either location; biomass yield was greatest during establishment at the N-responsive IN site when 75 kg ha⁻¹ or more N was applied (Table 5). The site-specific yield response of well-established *M. × giganteus* to N fertilizer has been associated with differences in soil organic matter with higher N responses reported on low organic matter soils [23]. Understanding mineralization of N from soil organic matter may inform where a positive response to fertilizer N might be achieved as *M. × giganteus* is deployed as a biomass crop.

Appropriate N management should consider both the rate of N fertilizer and the timing of N application during establishment. Based on reports of N carryover in rhizomes and other storage organs from the year of application to subsequent years [51] we hypothesized that a single large (150 kg ha⁻¹) application of N in Season 1 might meet the N nutrition needs of *M. × giganteus* for two consecutive years. However, plots in IN fertilized with 150-0 had significantly lower biomass yields in Season 2 than plots receiving annual applications of 75 kg ha⁻¹ (Table 5). In addition, Season 1 biomass N concentrations in IN were higher and both NUE values lower in the 150-0 treatment than the other N treatments suggesting inefficient use of high N applications. We have previously reported net N remobilization from rhizomes to regrowing shoots [52], and additional work is underway to quantify and characterize the N pools in belowground storage organs available for year-to-year N carryover and remobilization in these genotypes.

Few studies have compared how N impacts biomass yield and composition of *M. × giganteus* genotypes during establishment. Although the OP-Nagara was slightly less responsive than the other genotypes to N fertilization in Season 1 at the IN site ($P=0.10$), these genotypes

generally responded similarly to N management during establishment. Cumulative yields of Nagara were the highest of the four genotypes, and significantly greater than the IL clone at both locations (data not shown). This observation agrees with previous results with established stands [53,54] where Nagara had higher biomass yield than the IL clone. In these previous studies, genotypic differences were associated with larger crown diameter at the soil surface, and suggests this trait may serve as a selection criterion that could accelerate establishment as this species is deployed as a biofuel crop on marginal soils.

Genotype had a much greater impact on biomass composition than N treatment, and there were few genotype x N interactions for composition traits. Additionally, even though Season 2 in IN was harvested in November, while the other site-locations were harvested in January to March, no substantial biomass quality differences were observed. Large variation in cell wall composition has been reported among genotypes of both *M. sinensis* and *M. sacchariflorus*, the progenitor species of *M. × giganteus* [15,55]. Biomass composition of the IL and MS clones were essentially identical in all four site-year combinations (Table 6). This result is not surprising given that the MS clone was derived from the IL clone [10], and that these clones possess identical nuclear and plastid DNA [12]. However, composition of the Nagara clone and the OP-Nagara differed from the IL and MS clones for nearly every compositional trait in every site-year. Nagara types were lower in cellulose and ADL both years and, in Season 2, had higher hemicellulose concentrations when compared to the IL and MS clones. Previous work has demonstrated that cellulose and lignin are found in greater concentrations in stem tissue than leaf tissue of *Miscanthus*, while the opposite is true for hemicellulose [16,56,57]. Genotypic differences in leaf blade retention resulting in higher leaf:stem ratios is likely one underlying factor contributing to varied biomass composition in our study. The negative association among

cellulose, hemicellulose, and ADL and leafiness is frequently observed with perennial forage grasses used for livestock feed [58]. Genetic differences in leaf blade retention and its relationship to plant senescence has previously been reported among *M. × giganteus* genotypes and related *Miscanthus* species [59-61] and represents an opportunity to enhance yield and improve biomass bio-conversion by exploiting composition differences that result from leaf blade retention.

Rathke et al. [62] reported that N fertilization accounted for 40–60% of the total energy input for maize production; therefore, information on NUE is critical to our understanding of net energy balance of bioenergy production systems. Both ANUE and PNUE exhibited significant genotype and N rate effects (Tables 4 and 5). Our ANUE values during the first two seasons of establishment generally agree with values previously reported for established stands of the IL clone [8,9,24]. In most cases, ANUE of these *Miscanthus* genotypes declined substantially as N fertilizer application increased, and was generally lowest for plots provided 150 kg ha⁻¹. Pedroso et al. [34] also reported a substantial (>50%) decrease in ANUE of *Miscanthus* in two of three years as N fertilizer applications increased from 100 to 200 kg ha⁻¹ and concluded that maximum ANUE occurred when 100 kg ha⁻¹ of N was applied. However, these authors did not use lower rates of N fertilizer so they could not determine if, like us, higher ANUE is achieved at even lower N fertilizer rates (50 or 75 kg ha⁻¹). The ANUE in Season 1 in KY was not influenced by N fertilizer rate or *Miscanthus* genotype. Site-specific ANUE responses ranging from 5 to over 50 kg kg⁻¹ have been reported recently for the IL clone [9]. Several locations in this study (Havana, Urbana and Fairfield IL) had ANUE values that overlapped with 0 indicating that, depending on location and environment, the IL clone may not respond to N fertilization. At the KY site Nagara had the highest ANUE in Season 2, while the OP-Nagara had higher ANUE than

Nagara both years in IN. While little is known about genetic variation in ANUE in *Miscanthus*, genotypic and site-specific differences in ANUE have been observed for many crop species, and the underlying causes for this variation has recently reviewed [63].

Because *Miscanthus* can meet shoot N needs using both N transferred from stored reserves in rhizomes and fertilizer N from soil [21,52], we also characterized NUE as the ratio of aboveground biomass to total biomass N uptake (PNUE) [38]. Our PNUE values generally agree with values previously reported for or calculated using data from established plots of the IL clone fertilized with similar N rates and ranged from 135 to 220 kg kg⁻¹ [24,59]. Higher PNUE have been reported for *Miscanthus* including values approaching 900 kg kg⁻¹ [41,49,50]. However, these extremely high PNUE values generally occurred in unfertilized plots harvested in the spring following the growing season after N had leached from biomass overwinter.

The PNUE of *Miscanthus* generally declined with N fertilization (Table 5) as N uptake rate exceeded biomass accumulation with increasing N fertilization. This asynchrony of N uptake and growth rate leading to high tissue N concentrations is often referred to as luxury consumption, and occurs in many species [64,65] including major crop plants like rice [66] and maize [67]. While luxury consumption by *Miscanthus* may be viewed as wasteful, it represents a survival strategy because it permits nutrient accumulation in excess of immediate growth requirements during nutrient flushes that can later be used to support growth after soil nutrient reserves are exhausted [64]. We are currently evaluating the N accumulation in belowground tissues of these *Miscanthus* lines used in this study to help inform N reserve accumulation of this species.

The PNUE values of the IL and MS clones were similar in all four site-years, and consistently higher (more efficient) than that of the OP-Nagara, and higher than Nagara in KY in

Season 2 (Table 4). Genotypic differences in PNUE of up to 30% have been reported for rice [66] and maize [68]; differences that were generally consistent across N fertilizer rates and locations. While we did not measure biomass and N concentrations of leaves and stems separately, differences in leaf retention (Table 6) likely contribute, in part, to genotypic differences in PNUE. Both Nagara genotypes had higher leaf retention than the IL and MS clones, and leaves have been shown to have up to four-fold higher N concentrations than stem tissues [69]. This would elevate biomass N concentrations, N removal in biomass, and contribute to lower PNUE values for the Nagara lines. The relatively high PNUE values observed in this study indicates that, regardless of genotype and N management, *M. × giganteus* remains highly efficient at utilizing N for biomass production during establishment.

5. Conclusion

In conclusion, N applied at 50 kg ha⁻¹ applied annually resulted in high yields during *Miscanthus* establishment on the sandy loam soil in IN. The loam soil in KY was not N-responsive. Yields of plots provided high N in Season 1 (up to 150 kg ha⁻¹) were similar to unfertilized control plots in Season 2 suggesting little N carryover from Season 1 to 2. Biomass composition was not altered markedly by N management, but genotypes differed markedly in cellulose, hemicellulose and lignin and these differences were associated with genotypic variation in leaf blade retention. While differences were observed among *Miscanthus* genotypes, responses of the IL and MS clones were virtually identical for all attributes measured. The PNUE of these *Miscanthus* lines differed and approached 300 kg kg⁻¹ for the IL and MS clones. The ANUE also differed among *Miscanthus* genotypes in a site-specific manner, and was highest (ca. 30 kg kg⁻¹) for the OP-Nagara genotype in IN. All *Miscanthus* lines were N-responsive on the

sandy loam soil in IN suggesting that, irrespective of genotype, soil texture and fertility
 characterization will inform N management strategies of *Miscanthus* during establishment.

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Table 1

Timeline when trials were planted, fertilized, and harvested during the establishment seasons at the Kentucky and Indiana locations.

Management Activity	Kentucky	Indiana
Planting	May 29, 2010	June 12 & 30, 2010
First N Application	July 28, 2010	June 1, 2011
First Biomass Harvest	March 18, 2011	January 5, 2012
Second N Application	May 25, 2011	May 2, 2012
Second Biomass Harvest	January 20, 2012	November 11 & 13, 2012
Leaf Retention Score	January 20, 2012	November 10, 2012

Table 2

The fertilization scheme for the N treatments. The Kentucky site was planted and fertilized in 2010 (Season 1) and 2011 (Season 2), while the Indiana site was planted late in 2010 due to heavy rainfall and saturated soil. Therefore, fertilization and sampling was delayed until 2011 (Season 1) and 2012 (Season 2) to better match management at the Kentucky site. Values are presented as kg elemental N per hectare, applied as Agrotain™-coated urea.

N Treatments	Season	Season	Total
	1	2	
	-----	kg ha ⁻¹ -----	
1	0	0	0
2	0	150	150
3	50	100	150
4	75	75	150
5	100	50	150
6	150	0	150
7	150	150	300

Table 3

Pathogenic-induced stress in terms of stunting and dead plants of four *M. × giganteus* genotypes at the KY location in 2011. Stress ranged from zero (no stunting/stress) to 5 (dead plants). Stress was defined as aboveground stunting, dead or missing plants in mid May 2011. Stress ratings were used in covariance analysis of yield at this location.

Genotypes	Stress Rating, % of total plants					
	0	1	2	3	4	5
IL Clone	57	4	0	14	14	11
MS Clone	71	7	4	7	7	4
OP Nagara	100	0	0	0	0	0
Nagara	100	0	0	0	0	0

Table 4

Biomass yield, nitrogen (N) concentrations of biomass, nitrogen removed in biomass at harvest (product of yield and N concentration), and nitrogen use efficiencies (NUE) of four *M. × giganteus* genotypes averaged over N managements during establishment over two seasons in Kentucky and Indiana. Agronomic N use efficiency (ANUE) was calculated as the change in biomass dry matter divided by increment of N fertilizer applied over the 0 N control rate. Physiological N use efficiency (PNUE) was calculated as the ratio of dry matter yield (kg ha⁻¹) and biomass N accumulation (kg ha⁻¹). Data within a site-year column without letters indicate that the genotype F-test was not significant for that trait. Means followed by the same letter within a site-year are not significantly different ($P>0.05$). The ANUE means for Indiana in Season 2 differ at $P<0.10$.

Genotype	Yield Mg ha ⁻¹	Nitrogen g kg ⁻¹	N Removed kg ha ⁻¹	ANUE kg kg ⁻¹	PNUE kg kg ⁻¹	Yield Mg ha ⁻¹	Nitrogen g kg ⁻¹	N Removed kg ha ⁻¹	ANUE kg kg ⁻¹	PNUE kg kg ⁻¹
----- Kentucky Season 1 -----					----- Indiana Season 1 -----					
IL Clone	4.1 C	7.1 B	29.9 C	4.7	144 A	10.0 C	3.6 C	35.5 C	14.2 B	280 A
MS Clone	4.8 BC	6.7 B	32.6 C	10.4	152 A	10.1 BC	3.7 BC	37.3 C	13.9 B	272 AB
Nagara	8.6 A	7.0 B	58.9 A	6.0	147 A	10.8 AB	4.0 B	43.3 B	7.1 B	258 B
OP Nagara	5.4 B	8.0 A	43.9 B	-3.2	126 B	10.9 AB	4.8 A	52.3 A	27.9 A	216 C
----- Kentucky Season 2 -----					----- Indiana Season 2 -----					
IL Clone	13.6 AB	3.4 B	46.9 B	8.4 B	297 A	12.2	5.6 B	68.8 B	24.2 AB	182 A
MS Clone	13.8 A	3.6 B	49.1 B	8.9 B	286 A	12.1	5.4 B	65.8 B	23.8 AB	190 A
Nagara	12.4 B	4.9 A	63.3 A	31.2 A	204 B	12.6	5.4 B	69.0 B	15.1 B	192 A
OP Nagara	11.4 C	5.1 A	60.8 A	9.0 B	200 B	12.1	6.4 A	78.5 A	34.0 A	161 B

Table 5

Biomass yield, nitrogen (N) concentrations of biomass, nitrogen removed at harvest, and nitrogen use efficiencies (NUE) as influenced by N management averaged over four *M. × giganteus* genotypes during establishment over two seasons in Kentucky and Indiana. Agronomic N use efficiency (ANUE) was calculated as the change in biomass dry matter divided by increment of N fertilizer applied over the 0 N control rate. Physiological N use efficiency (PNUE) was calculated as the ratio of dry matter yield (kg ha⁻¹) and biomass N accumulation (kg ha⁻¹). Data within a site-year column without letters indicate that N rate F-test was not significant for that trait. Means followed by the same letter within a site-year are not significantly different ($P>0.05$). The ANUE means for Kentucky in Season 2 differ at $P<0.10$.

	<u>Yield</u> Mg ha ⁻¹	<u>Nitrogen</u> g kg ⁻¹	<u>N Removed</u> kg ha ⁻¹	<u>ANUE</u> kg kg ⁻¹	<u>PNUE</u> kg kg ⁻¹	<u>Yield</u> Mg ha ⁻¹	<u>Nitrogen</u> g kg ⁻¹	<u>N Removed</u> kg ha ⁻¹	<u>ANUE</u> kg kg ⁻¹	<u>PNUE</u> kg kg ⁻¹
N rate (Season 1)	----- Kentucky Season 1 -----					----- Indiana Season 1 -----				
0	5.8	6.5 C	38.2	ND [†]	157 A	9.6 B	3.3 C	32.0 C	ND	303 A
50	6.0	6.9 BC	41.9	16.2	147 AB	10.4 A	3.9 B	40.1 B	23.5 A	262 B
75	5.7	7.3 AB	41.4	2.9	138 BC	11.1 A	4.0 B	44.3 B	24.6 A	257 B
100	5.3	7.4 AB	40.6	0.6	139 BC	10.8 A	4.2 B	45.3 B	15.6 AB	245 B
150	5.8	7.7 A	44.5	-0.6	132 C	10.4 A	4.8 A	50.6 A	7.6 B	216 C
N rate (Season 1-2)	----- Kentucky Season 2 -----					----- Indiana Season 2 -----				
0-0	12.2	3.6 C	44.2 C	ND	282 A	10.5 C	4.5 D	47.5 D	ND	226 A
0-150	13.1	4.4 AB	58.5 AB	8.9 B	237 BC	13.5 A	6.3 AB	84.8 A	19.8 ABC	162 CD
50-100	13.3	4.2 B	56.7 AB	18.5 AB	250 B	12.4 AB	5.9 BC	73.1 B	19.2 BC	177 BC
75-75	12.9	4.3 AB	55.3 AB	11.9 B	244 BC	13.0 A	5.6 BC	72.9 B	33.2 AB	183 BC
100-50	12.7	4.3 AB	55.5 AB	27.8 A	243 BC	12.3 AB	5.5 BC	68.1 BC	36.2 A	182 BC
150-0	12.3	4.2 AB	53.2 B	ND	248 BC	11.5 BC	5.3 C	60.7 C	ND	191 B
150-150	13.2	4.7 A	61.7 A	11.9 B	224 C	12.4 AB	7.0 A	86.6 A	12.8 C	146 D

[†]ND, not determined because N fertilizer not applied

Table 6

Concentrations of hemicellulose, cellulose, acid detergent lignin (ADL) and ash of biomass from four *M. × giganteus* genotypes averaged over nitrogen managements during establishment over two years in Kentucky and Indiana. Relative leaf retention was estimated as a visual rating ranging from 1 to 5 where 1= no leaves retained; 5= all leaves retained. Means followed by the same letter within a site-year are not significantly different ($P>0.05$).

Genotype	Hemicellulose	Cellulose	ADL	Total Ash	Leaf Retention
----- g kg ⁻¹ -----					
Kentucky Season 1 (2010)					
IL Clone	328.6	387.8 AB	72.8 AB	26.5	
MS Clone	326.3	392.6 A	75.0 A	25.8	
Nagara	321.5	381.0 B	64.8 C	24.9	
OP Nagara	325.9	364.0 C	69.8 B	26.5	
Kentucky Season 2 (2011)					
IL Clone	259.4 B	452.5 A	113.3 A	15.3 C	1.3 C
MS Clone	256.1 B	451.5 A	111.1 A	15.9 C	1.3 C
Nagara	292.5 A	415.9 B	89.2 C	21.0 B	3.2 B
OP Nagara	284.1 A	422.6 B	94.3 B	22.9 A	4.2 A
Indiana Season 1 (2011)					
IL Clone	276.4	423.8 A	98.6 A	20.4 B	
MS Clone	287.0	413.1 A	95.6 A	21.2 B	
Nagara	285.8	415.4 A	76.6 B	21.7 B	
OP Nagara	285.2	389.0 B	72.0 C	25.6 A	
Indiana Season 2 (2012)					
IL Clone	300.3 C	413.0 A	84.5 A	36.2	2.0 B
MS Clone	294.8 C	412.6 A	85.7 A	34.3	2.1 B
Nagara	316.5 B	400.6 B	66.5 B	35.2	5.0 A
OP Nagara	329.6 A	355.2 C	63.3 C	40.0	4.9 A

Table 7

Pearson's simple correlation coefficients (r-values and corresponding *P*-values) of leaf retention with yield, physiological nitrogen use efficiency (PNUE), and concentrations of carbon (C), nitrogen (N), hemicellulose, cellulose, acid detergent lignin and ash in biomass. Leaf retention data were only collected in Season 2 of the study at both locations.

	Yield	C	N	PNUE	Hemicellulose	Cellulose	ADL	Ash
Kentucky Season 2 (2011)								
Leaf Retention	0.03	-0.56	0.72	-0.74	0.43	-0.55	-0.66	0.76
<i>P</i> -value	0.77	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Indiana Season 2 (2012)								
Leaf Retention	0.06	-0.38	0.17	-0.11	0.54	-0.65	-0.90	0.27
<i>P</i> -value	0.54	<0.01	0.07	0.27	<0.01	<0.01	<0.01	<0.01

Figure Headings:

Fig. 1. Monthly averages for air temperature and precipitation at Schochoh, Kentucky and Lafayette, Indiana during the years of the experiment. The 50-year average values for mean monthly air temperature and precipitation are included as a reference.

Fig. 1. Monthly averages for air temperature and precipitation at Schochoh, KY and Lafayette, IN during the years of the experiment. The 50-year average values for mean monthly air temperature and precipitation are included as a reference.