

## Impacts of canopy position and nitrogen on nitrogen allocation and photosynthesis of switchgrass (*Panicum virgatum L.*)

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### Summary

Switchgrass (*Panicum virgatum L.*) is an important biomass source widely considered for US energy production. Investigating nitrogen allocation and photosynthetic processes is fundamental to understanding the growth pattern of switchgrass, but the information is scarce in the literature. Nitrogen allocation varies throughout the growing season and canopy profile and depends on the level of nitrogen application. In the present study seasonal variations in leaf nitrogen concentration, leaf photosynthetic, morphological and biochemical properties were investigated across a canopy profile for switchgrass under two N treatments (0 and 224 kg ha<sup>-1</sup>). Photosynthetic properties, such as maximum rate of rubisco carboxylation ( $V_{cmax}$ ), net photosynthesis (A), carboxylation efficiency (k), stomatal conductance (gs), PSII efficiency ( $F_v'/F_m'$ ) and PSII electron transport ( $J_{PSII}$ ) all peaked in June and declined until September. The addition of nitrogen increased A and  $V_{cmax}$  at the early growing season.  $V_{cmax}$ , A, and  $J_{PSII}$  were all higher at the top than at the bottom of the canopy. N concentration for different canopy layers was increased by N fertilization and the distribution of leaf N concentration within canopy followed light gradients. N treatment increased leaf area index (LAI), but had no effect on specific leaf area (SLA). Chlorophyll and soluble sugar content were also increased by N treatment and were higher at the top than at the bottom of the canopy. Our results therefore suggest that the photosynthetic capacity of switchgrass depends on both canopy position and N fertilization. Switchgrass adapts to the light environment and nitrogen application by adjusting leaf morphological and biochemical properties and through increased LAI. These results should aid in providing valuable parameters for developing models that can accurately estimate carbon dioxide flux in terrestrial systems and biomass production in biomass crop ecosystems.

### Abbreviations

A, net photosynthesis ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ); Chl *a*+*b*, chlorophyll *a*+*b* content ( $\mu\text{g cm}^{-2}$ ); Chl *a/b*, the ratio of chlorophyll *a* to chlorophyll *b*; N<sub>m</sub>, mass-based nitrogen concentration (%); N<sub>a</sub>, area-based nitrogen concentration ( $\text{g m}^{-2}$ ); V<sub>cmax</sub>, maximum rate of rubisco activity ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ); k, carboxylation efficiency; g<sub>s</sub>, stomatal conductance; F<sub>v</sub>'/F<sub>m</sub>', PSII efficiency; q<sub>p</sub>, photochemical quenching; q, quantum yield; J<sub>PSII</sub>, PSII electron transport rate; SLA, specific leaf area ( $\text{m}^2 \text{kg}^{-1}$ ); LAI, leaf area index ( $\text{m}^2 \text{m}^{-2}$ ); Fru, fructose content ( $\mu\text{g cm}^{-2}$ ); Suc, sucrose content ( $\mu\text{g cm}^{-2}$ ); Glc, glucose content ( $\mu\text{g cm}^{-2}$ ).

## Introduction

Switchgrass, a warm-season perennial grass native to North America, has been recommended by the United States Department of Energy as a model bioenergy feedstock because of its high yield potential across a wide geographic range (MacLaughlin *et al.*, 2002). Such characteristics as ability to grow on marginal, highly erodible, and droughty soils, potential for sequestering large amounts of atmospheric carbon and providing nesting habitats for migratory animals (Sanderson *et al.*, 1996; MacLaughlin *et al.*, 2002; Roth *et al.*, 2005), make switchgrass an ideal biofuel crop. It has been shown that precipitation and nitrogen are the two principal resources limiting productivity in switchgrass (Wang *et al.*, 2010), thus efficiently utilizing these resources are fundamental when attempting to grow switchgrass profitably across large scales.

While it is known that nitrogen addition typically increases plant growth (Lebauer & Treseder, 2008), much less is known about the mechanisms on how nitrogen addition affects plant growth, physiology, and patterns of nitrogen allocation within leaves and among canopy profiles. The response of switchgrass yield to added nitrogen varies widely, including no response (Christian *et al.*, 2001) or a positive response (Brejda *et al.*, 2000; Muir *et al.*, 2001). The differences in the yield response to N could reflect differences in N availability, climate, site history, or cultivars chosen (Parrish & Fike, 2005), as well as the complexity of plant N allocation patterns. Nitrogen allocation varies both vertically in the plant canopy in response to changes in light availability (Rosati *et al.*, 2000) and also changes across the growing season (Heaton *et al.*, 2009). It has been reported that N allocation in a canopy followed the gradient of light distribution, with higher N allocated in the top canopy (Field, 1983; Reich & Walters, 1994). In addition, acclimation to light has been shown to affect nitrogen allocation within leaves (Rosati *et al.*, 1999) in order to preserve a balance between enzymatic (i.e. Rubisco) and light-harvesting (i.e. chlorophyll) capabilities (Givnish, 1988; Warren & Adams, 2001). Foliar chlorophyll concentration tends to increase with decreasing growth irradiance, while N in Rubisco usually decreases with decreasing irradiance (Evans, 1989; LeRoux *et al.*, 2001; Turnbull *et al.*, 2007). These results however, have mostly been tested on the fully expanded sunny leaves from different species or sites and/or a wide range of growth forms (e.g. herbs, shrubs, conifers, broadleaf trees) (Meir *et al.*, 2002), rather than within species on leaves within a single canopy.

Light availability and nitrogen concentration are two main factors affecting net photosynthesis of leaves (Field & Mooney, 1986). Few data, however, are available for switchgrass on the ontological changes in the correlation between leaf N concentration and light availability at different canopy levels and their relationship to photosynthetic processes. Successfully up-scaling photosynthesis from the leaf to the canopy level also requires understanding the rate-determining factors in leaf photosynthesis (Laisk *et al.*, 2005). Systematically measuring photosynthesis across growing seasons and among canopy is therefore critical for validating growth models and for elucidating the physiological basis for observed differences in productivity (Dohleman *et al.*, 2009).

The variability in foliage characteristics in canopy and time presents a challenge for understanding plant growth pattern and for modeling physiological processes and growth of whole canopies and stands (Sellers *et al.*, 1992). The relationship between photosynthesis, SLA,  $N_a$ ,  $N_m$  and irradiance has not been extensively studied and almost nothing is known about whether anatomy or biochemistry plays the leading role in their photosynthetic performance at different canopy layers for switchgrass. Therefore, we studied the acclimation of leaves of switchgrass to within-canopy light levels and the effect of nitrogen fertilization on these relationships. Specifically, we hypothesize: (1) growth and  $V_{max}$  are increased by fertilization; (2) foliage N will be higher at the top than at the bottom of the canopy and SLA will be higher at the bottom than at the top canopy; (3) distribution of N within chloroplasts is affected by irradiance, such that more N will be allocated to thylakoid proteins in the lower canopy; and (4) acclimation to irradiance varies with N nutrition, with more N allocated in the top layer at N-applied plots than at the control plots.

## Methods

### Study site

Four year-old switchgrass [*Panicum virgatum* L. cv. Cave-in-Rock] stands were grown in an agricultural study site in Savoy, IL ( $40^{\circ}10'20''N$ ,  $88^{\circ}11'40''W$ , 228 m above sea level). The soil at the site is a Flanagan series silt loam (Fine, smectitic, mesic Aquic Argiudolls). The experiment design was split plot arrangements in randomized complete block with four replications. Subplots ( $4.6\text{ m} \times 2.1\text{ m}$ ) were blocked by N fertility levels (0 and  $224\text{ kg N ha}^{-1}$ ) and N in the form of urea was applied on 12 May 2009. Plots were harvested with a plot harvester (Model Cibus S, Wintersteiger, Ried, Austria) in December 2009, by cutting a 1.22 m swath through the middle of the plots. A subsample was collected from each plot to determine dry biomass. Throughout the growing season, one plant was randomly selected within each plot of each treatment on day 168, 205, 240, and 261 and fully expanded leaves were sampled from top (0–0.5 m from the top) and bottom layers (0.5 m lower from the top where leaves are not senescent) of the canopy.

### LAI measurement

Leaf area index (LAI) and the proportion of photosynthetically active radiation (PAR, 400–700 nm) intercepted by the canopy were measured at four dates during the growing season: day 168, 205, 240, and 261. The measurements were taken by measuring the PAR outside the crop canopy using an external sensor (Model LI-190, LI-COR Biosciences) connected to a linear ceptometer (Model PAR-80, Decagon Devices, Inc.) which was used to measure the amount of PAR not intercepted by the crop canopy. These measurements were taken at two depths within the crop canopy, between 10:00 and 14:00 hours on mostly sunny days when the minimum PAR was at least  $1400\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ . Light interception was determined by calculating the proportion of PAR intercepted by the crop canopy. Leaf area index was estimated for each subsample using the observations of radiation interception beneath and outside the canopy, and zenith angle and leaf angle distribution (Deblonde *et al.*, 1994).

### Gas exchange measurements

Shoots from different canopy layers were sampled before dawn and returned to the lab partially submerged in water and put in the dark before measurement. Gas exchange and chlorophyll fluorescence were measured on leaves with a portable infrared gas analyzer (LI-COR 6400LCF; LI-COR, Lincoln, NE).  $\text{CO}_2$  response ( $A-C_i$ ) curves and light response ( $A-Q$ ) curves were measured following the method described in Wang *et al.* (2011, in review). Post-PSII electron transport ( $J_{\text{PSII}}$ ), PSII efficiency ( $F_v'/F_m'$ ) and photochemical quenching ( $q_p$ ) in light-adapted leaves were also measured using a Licor 6400-40 Leaf Chamber Fluorometer.  $A-C_i$  and  $A-Q$  curves were fitted to a coupled photosynthesis-stomatal conductance model by Collatz *et al.* (1992). The initial slope and rate saturated region of the  $A-C_i$  curves were used to estimate carboxylation efficiency ( $k$ ) and maximum Rubisco activity ( $V_{\text{max}}$ ) (Miguez *et al.*, 2009). The initial slope of the  $A-Q$  curves was used to estimate quantum efficiency ( $q$ ) (Miguez *et al.*, 2009).

### Leaf harvest, specific leaf area (SLA), chlorophyll and C, N measurements

Immediately following gas-exchange measurements, ten  $0.5\text{ cm}^2$  leaf punches from each canopy layer were taken and oven-dried at  $65^{\circ}\text{C}$  for 2 wk for measurement of SLA and two  $0.5\text{ cm}^2$  leaf punches were taken for chlorophyll measurements. N and C concentration were measured with a Perkin Elmer CHN Analyzer (Model 2400). Chlorophyll was extracted with 80% ethanol (Richardson *et al.*, 2002) and measured at absorbance of 645 and 663 nm (Varian Cary 300 spectrophotometer, Varian). Chlorophyll *a* and *b* concentrations were calculated with the equations of Wellburn (1994) and expressed as  $\mu\text{g cm}^{-2}$  leaf area.

### Statistical analysis

Fixed effects of date, fertilization (N), and canopy position (CA) and their interactions on the morphological, biochemical and physiological parameters were tested by ANOVA (PROC GLM,

SAS 9.1, [SAS Institute Cary, NC, USA]). Post-hoc Tukey HSD tests were made on specific contrasts to examine significant treatment effects among groups.

## Results

Yield at fertilized plots (8.5 tons  $\text{ha}^{-1}$ , n=4) was not significantly higher than at the control plots (7.9 tons  $\text{ha}^{-1}$ , n=4). LAI was 5.1 and 4.2 at fertilized and control plots in late July, respectively. LAI was not measured late into growing season because of lodging problems. Specific leaf area was not affected by fertilization and the canopy position (Fig 1. Table 1).

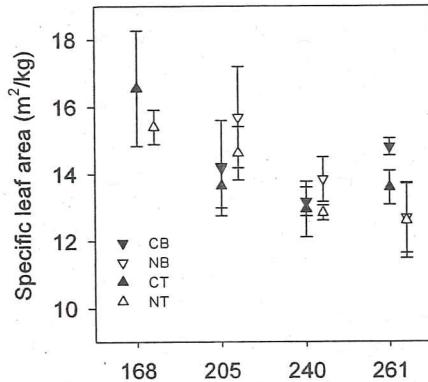


Fig. 1. Effects of nitrogen ( $\nabla \Delta$  - with nitrogen;  $\nabla \blacktriangle$  - without nitrogen) and canopy position ( $\nabla \blacktriangledown$  - bottom canopy;  $\Delta \blacktriangle$  - top canopy) on SLA throughout the growing season. Values are means  $\pm 1$  SE; n=4.

$F_v'/F_m'$ ,  $p$ ,  $J_{PSII}$  and  $q$  decreased over the course of the growing season (Fig. 2). Fertilization had no significant effect on  $F_v'/F_m'$ ,  $p$ ,  $J_{PSII}$  and  $q$ . Leaf photosynthetic traits varied appreciably along the vertical gradient from the top to the bottom of the canopy.  $F_v'/F_m'$  was significantly higher at the top canopies than at the lower canopies.

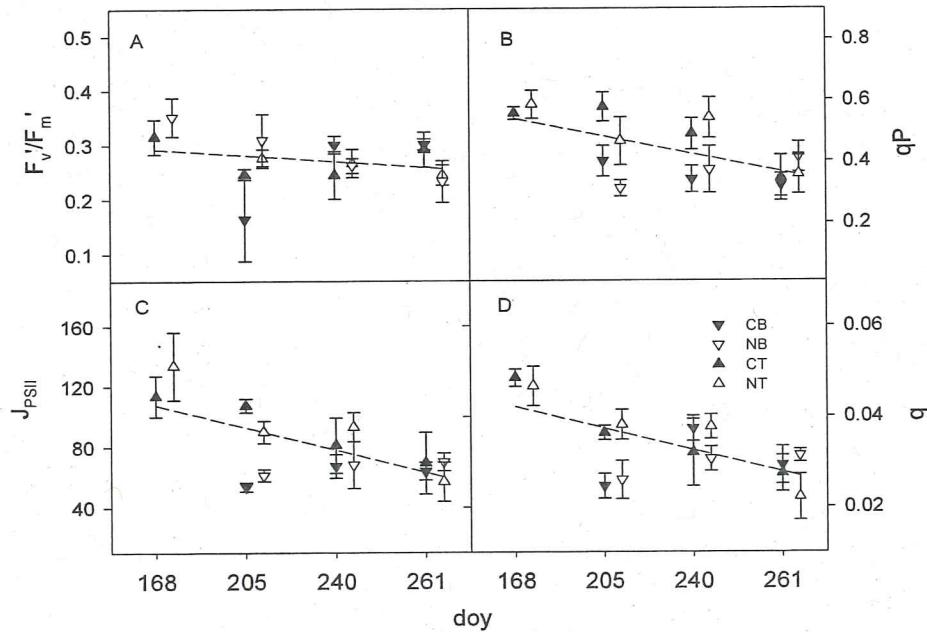


Fig 2. Effects of nitrogen ( $\nabla \Delta$  - with nitrogen;  $\nabla \blacktriangle$  - without nitrogen) and canopy position ( $\nabla \blacktriangledown$  - bottom canopy;  $\Delta \blacktriangle$  - top canopy) on  $F_v'/F_m'$ ,  $p$ ,  $J_{PSII}$  and  $q$  throughout the growing season. Values are means  $\pm 1$  SE; n=4.

Table 1. Degrees of freedom (df) and F-statistics from ANOVA on the fixed effect of date, fertilization (N), canopy position (CA) and their interactions on the morphological, biochemical and physiological parameters

Factors	SLA	$F_v'/F_m$	p	$J_{psu}$	q	$g_s$	A	k	$V_{max}$	N <sub>m</sub>	N <sub>a</sub>	Chl	Chl a+b	Suc	Fru	Glc
Date	3,42	3,42	3,42	3,42	3,42	3,42	3,42	3,39	3,39	3,39	3,42	3,42	3,99	3,99	3,99	3,99
N	6.8*	3.2*	2.9*	7.7*	13.1*	18.3*	23.5*	27.5*	21.7*	6.0*	10.0*	6.2*	26.5*	28.4*	9.4*	19.4*
CA	1,42	1,42	1,42	1,42	1,42	1,42	1,42	1,39	1,39	1,39	1,42	1,42	1,99	1,99	1,99	1,99
Date*N	3,42	3,42	3,42	3,42	3,42	3,42	3,42	3,39	3,39	3,39	3,42	3,42	3,99	3,99	3,99	3,99
Date*CA	2,42	2,42	2,42	2,42	2,42	2,42	2,42	2,39	2,39	2,39	2,42	2,42	2,99	2,99	2,99	2,99
N*CA	1,42	1,42	1,42	1,42	1,42	1,42	1,42	1,39	1,39	1,39	1,42	1,42	1,99	1,99	1,99	1,99
Date*N*CA	2,42	2,42	2,42	2,42	2,42	2,42	2,42	2,39	2,39	2,39	2,42	2,42	2,99	2,99	2,99	2,99

\* denotes significance at  $P < 0.05$ . See text for abbreviations.

A,  $g_s$ ,  $V_{cmax}$  and k decreased throughout the growing season (Fig. 3). A at the top layer decreased from about  $25 \mu\text{mol m}^{-2} \text{s}^{-1}$  to  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$  from the early to the late growing season. A  $C_{max}$  and  $V_{cmax}$  at the top layer were significantly higher than at the lower canopies (Table 1). Fertilization had a significantly positive effect on A,  $g_s$ , and k.

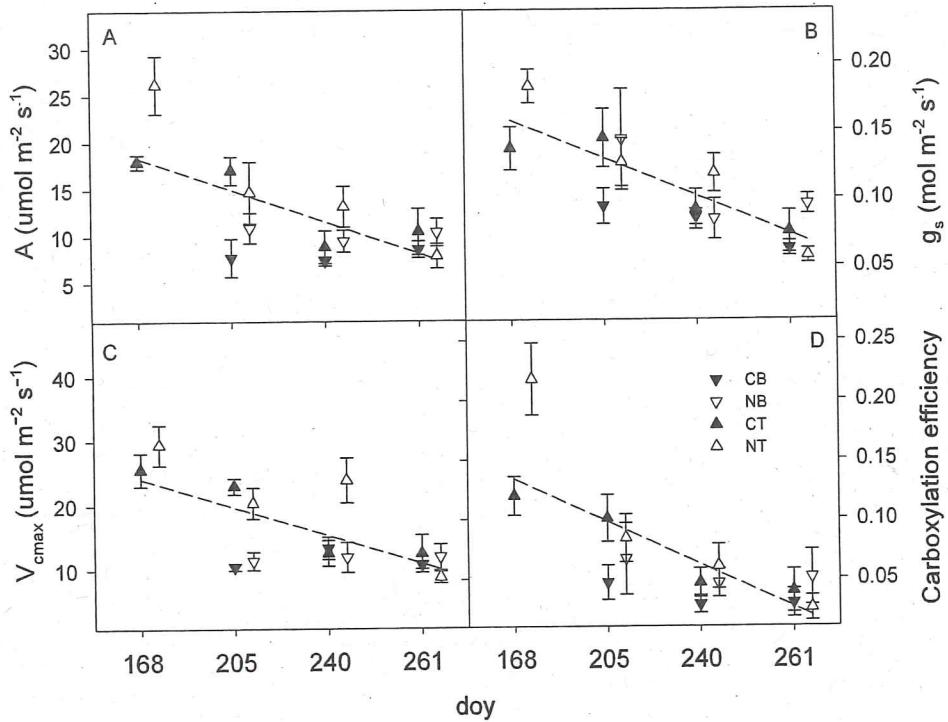


Fig 3. Effects of nitrogen ( $\nabla \Delta$  - with nitrogen;  $\nabla \blacktriangle$  - without nitrogen) and canopy position ( $\nabla \blacktriangledown$  - bottom canopy;  $\Delta \blacktriangle$  - top canopy) on  $g_s$ ,  $A_{max}$ , k and  $V_{cmax}$  throughout the growing season. Values are means  $\pm 1$  SE; n=4.

$N_a$  and  $N_m$  decreased appreciably throughout the growing season. Fertilization increased  $N_m$  (Fig 4, Table 1). Both  $N_a$  and  $N_m$  decreased continuously throughout the canopy from upper to lower canopy positions (Fig. 4).

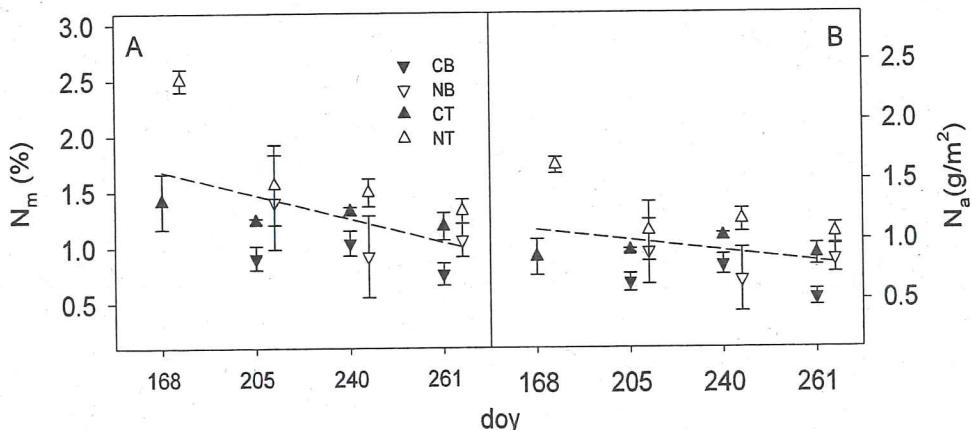


Fig 4. Effects of nitrogen ( $\nabla \Delta$  - with nitrogen;  $\nabla \blacktriangle$  - without nitrogen) and canopy position ( $\nabla \blacktriangledown$  - bottom canopy;  $\Delta \blacktriangle$  - top canopy) on  $N_a$  and  $N_m$  throughout the growing season. Values are means  $\pm 1$  SE; n=4.

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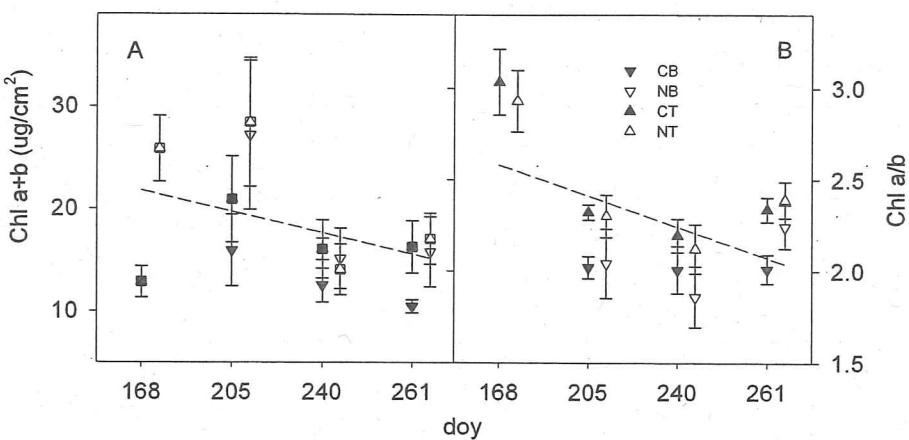


Fig 5. Effects of nitrogen ( $\nabla\Delta$  - with nitrogen;  $\nabla\blacktriangle$  - without nitrogen) and canopy position ( $\nabla\blacktriangledown$  - bottom canopy;  $\Delta\blacktriangle$  - top canopy) on Chl  $a$  and Chl  $a/b$  throughout the growing season. Values are means  $\pm 1$  SE;  $n=4$ .

Fertilization increased Chl  $a+b$  content. The value of Chl  $a/b$  was higher at the top canopy but was not affected by fertilization (Fig. 5, Table 1). Sugar content decreased throughout the growing season. N fertilization had a positive effect on the Glc and Fru content, but had no effect on Suc (Fig. 6).

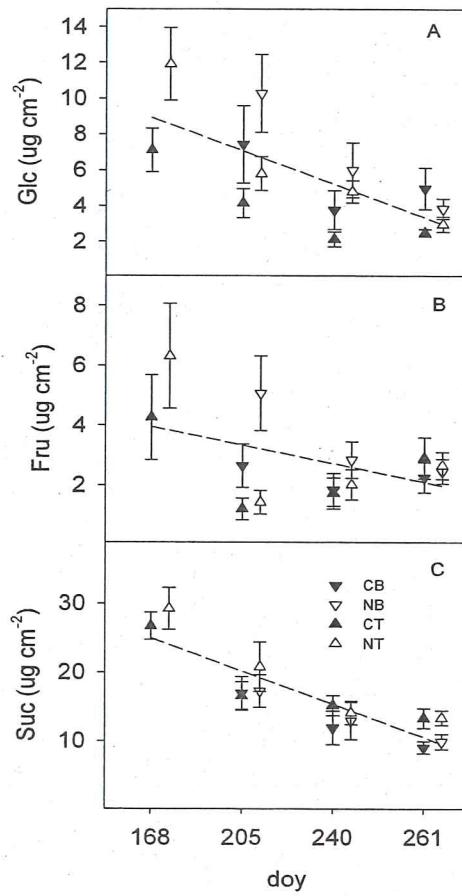


Fig 6. Effects of nitrogen ( $\nabla\Delta$  - with nitrogen;  $\nabla\blacktriangle$  - without nitrogen) and canopy position ( $\nabla\blacktriangledown$  - bottom canopy;  $\Delta\blacktriangle$  - top canopy) on Glu, Fru and Suc throughout the growing season. Values are means  $\pm 1$  SE;  $n=4$ .

## Discussion

Patterns of variation in leaf structure, nitrogen concentration and photosynthetic capacity within a single canopy for switchgrass was directly tested in this study. The results provide not only fundamentals in the ecophysiology of switchgrass but also parameters for model development.

In biomass feedstock production, the use of nitrogen fertilizer must be optimized to balance the economics, energy, and environmental costs of fertilizer use with the resulting gains in yield (Wang *et al.*, 2010). As confirmed on C<sub>3</sub> species (MacDonald *et al.*, 1986; Drecer *et al.*, 2000), the effect of N on plant growth is generally due to both a positive effect on photosynthesis and leaf growth (Gastal & Lemaire, 2002). It has been pointed out previously (Sinclair & Horie, 1989) that there is competition between allocating N to increase photosynthesis of existing leaves and allocating N to develop additional leaf area. For switchgrass, even though LAI was increased by nitrogen application, yield production was not stimulated by nitrogen. Switchgrass in this study flowered in late July and might allocate more assimilate into below ground and reproductive organs, which could account for this discrepancy between LAI increases and unchanged production. Another reason for this lack of N effect for switchgrass might be due to the high rate of nitrogen treatment indicated by another parallel study (Lee *et al.*, in prep), in which the yield of switchgrass was increased by nitrogen at the rate of 56, 112 and 168 kg ha<sup>-1</sup>, but at the rate of 224 kg N ha<sup>-1</sup>, which is the rate used in this study, yield started to decline. Significant lodging problem at high nitrogen plots observed in this study could also contribute to the insignificant N effect.

Contrary to what had been found in other species (Ellsworth & Reich, 1993; Evans & Poorter, 2001), specific leaf area (SLA) did not change through the canopy profiles. It has been suggested that in fertilized plots, plants tend to have thinner or less dense leaves (Knops & Reinhart, 2000). However, we observed no effect of nitrogen on SLA, which suggested that partitioning of leaf mass was independent of N fertilization and canopy position for switchgrass. N<sub>a</sub> and N<sub>m</sub> were both higher at the top than at the lower canopy, which is consistent with the light-nitrogen hypothesis (Ellsworth & Reich, 1993; Rosati *et al.*, 1999; Frak *et al.*, 2002). However, N fertilization resulted in a continuous increase only in N<sub>m</sub> (not N<sub>a</sub>) relative to the control plots. These results suggest N<sub>m</sub> should be paid more attention than N<sub>a</sub> when trying to maximize the carbon gain of switchgrass.

We proposed that more nitrogen would be allocated to the top canopy at the nitrogen-applied plot, considering that competition for light increases at high nitrogen when the competition for soil nitrogen decreases. However, in this study, nitrogen fertilization did not result in preferential allocation of N<sub>m</sub> (N<sub>m</sub> × CA in Table 1), indicating that N allocation among leaves in a canopy is fixed regardless of N availability and does not contribute to adaptation to irradiance. There is generally a trade-off between the capacities for light and carbon capture (Niinemets, 2006). Leaf chlorophyll *a+b* was higher at the top canopy than at the bottom canopy at the early stages of the growing season; however, in the late growing season, leaf chlorophyll *a+b* was not different among canopy profiles, suggesting that relatively more nitrogen was allocated to the bottom canopy when the light level at the bottom canopy became low. Chlorophyll *a/b* increased from the base to the top canopy. The relative abundance of Chl *b* will contribute to light harvesting in weak light. Net photosynthesis (A) averaged about 25 ummol m<sup>-2</sup> s<sup>-1</sup> for switchgrass in the early growing season, which is similar to what was reported in Dohleman *et al.* (2009). As found in other studies (Henskens *et al.*, 2001, Close *et al.*, 2004), A declined throughout the growing season and from the top to the lower canopy. The concentration of soluble sugars also decreased from the early to the late stages of the growing season and across the canopy profiles, which confirmed the variation of photosynthesis through canopy and time. Variation in A could be attributed to many factors, including g<sub>s</sub>, F<sub>v</sub>'/F<sub>m</sub>', <sub>q</sub>p, J<sub>PSII</sub>, k and V<sub>cmax</sub>. We found that the efficiency of PSII (F<sub>v</sub>'/F<sub>m</sub>') decreased throughout the canopy. The photosynthetic acclimation within the canopy was also biochemical; i.e. V<sub>cmax</sub> also increased within canopy height.

Even though  $N_m$  was increased by N fertilization throughout the growing season, positive effect of N fertilization on A and k (carboxylation efficiency) was only apparent in the early growing season.  $V_{cmax}$  was increased by N fertilization at the early part of the growing season (day 161) and after flowering (day 240). This variability in the photosynthetic response to nitrogen concentration may have several causes. First, not all N in the leaf is allocated to photosynthetic machinery, and the inorganic N content in leaves may have been built up (Evans & Poorter, 2001; Lawlor, 2002). The fraction of non-photosynthetic N has been shown to increase significantly with decreasing irradiation for *Chenopodium album* (Hikosaka & Terashima, 1996) and spinach (Evans, 1989) and to decrease for *Betula pendula* (Eichelmann *et al.*, 2005). Secondly, the proportion of N allocated to Rubisco may not increase, as shown by the carboxylation efficiency, which was not altered by nitrogen fertilization.

In conclusion, N fertilization did not stimulate the yield of switchgrass and only had little effect on A and  $V_{cmax}$  in the early growing season. Trends in the distribution of  $N_m$  within the canopy were consistent with the light-N hypothesis. Photosynthetically, the acclimation to light was both biochemical (increasing  $V_{cmax}$ , k and chlorophyll with increasing light) and physiological (increasing  $g_s$  with increasing light).

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