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Nitrogen enhanced photosynthesis of *Miscanthus* by increasing stomatal conductance and phospho*enol*pyruvate carboxylase concentration

X.-P. FENG[*], Y. CHEN[*], Y.-H. QI[*], C.-L. YU[*], B.-S. ZHENG[**,+], M. BRANCOURT-HULMEL[***], and D.-A. JIANG[*,+]

State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou 310058, China[*]
School of Forestry & Biotechnology, Zhejiang A & F University, Linan 311300, China[**]
INRA/USTL UMR SADV 1281, Estrées-Mons BP 50135, 80203 Péronne Cedex, France[***]

Abstract

Miscanthus is one of the most promising bioenergy crops with high photosynthetic nitrogen-use efficiency (PNUE). It is unclear how nitrogen (N) influences the photosynthesis in *Miscanthus*. Among three *Miscanthus* genotypes, the net photosynthetic rate (P_N) under the different light intensity and CO_2 concentration was measured at three levels of N: 0, 100, and 200 kg ha^{-1}. The concentrations of chlorophyll, soluble protein, phospho*enol*pyruvate carboxylase (PEPC), ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit, leaf anatomy and carbon isotope discrimination (Δ) in the leaf were analyzed to probe the response of photosynthesis in *Miscanthus* genotypes to N levels. P_N in all genotypes rose significantly as N application increased. The initial slope of response curves of P_N to C_i was promoted by N application in all genotypes. Both stomatal conductance and C_i were increased with increased N supply, indicating that stomatal factors played an important role in increasing P_N. At a given C_i, P_N in all genotypes was enhanced by N, implying that nonstomatal factors might also play an important role in increasing P_N. *Miscanthus* markedly regulated N investment into PEPC rather than the Rubisco large subunit under higher N conditions. Bundle sheath leakiness of CO_2 was constant at about 0.35 for all N levels. Therefore, N enhanced the photosynthesis of *Miscanthus* mainly by increasing stomatal conductance and PEPC concentration.

Additional key words: bundle sheath leakiness of CO_2; leaf mass per unit area; nitrogen-use efficiency; Rubisco.

Introduction

With rising atmospheric carbon dioxide (CO_2) concentration and decreasing energy security, there is an increasing demand for renewable energy (McKendry 2002, Le Quéré *et al*. 2009). In terms of environmental benefits, it is helpful to explore biomass production by plants through photosynthesis converting solar energy to chemical material. *Miscanthus* is an annual low-input bioenergy crop, with high potential yield and high adaptability to various growing conditions (Remlein-Starosta 2007, Clifton-Brown *et al*. 2008). As it is common for a potential bioenergy crop, *Miscanthus* uses the NADP-malic enzyme (NADP-ME) pathway of C_4 photosynthesis (Wang *et al*. 2008). Generally, C_4 plants have higher PNUE compared to C_3 plants as they have a CO_2

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[+]Corresponding authors; De-An Jiang: tel: 0086 (0)571 88206461, fax: 0086 (0)571 88206461, e-mail: dajiang@zju.edu.cn; Bing-Song Zheng: tel: 0086 (0)571 63741626, fax: 0086 (0)571 63740809, e-mail: bszheng@zafu.edu.cn
Abbreviations: Chl – chlorophyll; LMA – leaf mass per unit area; C_i – intercellular CO_2 concentration; LNC – leaf nitrogen content per area; N – nitrogen; g_s – stomatal conductance; PEPC – phospho*enol*pyruvate carboxylase; P_N – net photosynthetic rate; PNUE – photosynthetic nitrogen-use efficiency; PPFD – photosynthetic photon flux density; Rubisco – ribulose-1,5-bisphosphate carboxylase/oxygenase; rbcL – Rubisco large subunit; WUE – water-use efficiency; Φ – bundle sheath leakiness of CO_2; Δ – carbon isotope discrimination.
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concentrating mechanism (Still *et al*. 2003). The efficiency of the C_4 pathway is viewed as a result of leaf anatomy and biochemistry (Ghannoum *et al.* 2000, Pengelly *et al*. 2010).

In C_4 photosynthesis, the initial procedure is the fixation of atmospheric CO_2 by PEPC in the mesophyll cells, and then C_4 acids diffuse to inner bundle sheath cells (BS), where the acids decarboxylate and CO_2 is assimilated by Rubisco (Sage 2004). However, not all CO_2 can be fixed by Rubisco; some leaks back to intercellular spaces or mesophyll cells, where it can be refixed by PEPC – the energy cost of this process reduces the efficiency of C_4 photosynthesis (Hatch *et al*. 1995). The fraction of CO_2 leakage from BS cells refixed by PEPC is defined as CO_2 leakiness (Φ) (Cousins *et al*. 2006). *Amaranthus cruentus*, a C_4 dicot, showed an increased Φ with decreased photosynthetic photon flux density (PPFD) and this contributed to a decreased CO_2 assimilation rate (Tazoe *et al*. 2008), but was independent of leaf N (Tazoe *et al*. 2006). Sugarcane leaf tissue carbon isotope discrimination (Δ) increased linearly with the decreasing quantum yield for CO_2 uptake under N-deficiency (Ranjith *et al*. 1995, Meinzer and Zhu 1998).

Quantitative responses of CO_2 assimilation rate to leaf N concentration have been reported (Weng and Hsu 2001, Tazoe *et al.* 2006). Increasing N supply led to proportional increases in grain yield, plant biomass, leaf area index, photosynthesis, and Rubisco and PEPC activities for two field-grown maize (*Zea mays* L.) hybrids and then PEPC served as a reservoir for excess N accumulation in leaves (Uribelarrea *et al.* 2009). Maize has a high PNUE because of the greater N investment in the thylakoid components and the CO_2-concentration mechanism (Makino *et al.* 2003). NADP-ME C_4 grasses allocate 30% of leaf N to thylakoids, 41% to soluble protein, and only one-seventh of which is Rubisco (Ghannoum *et al.* 2005). Maranvill and Madhavan (2002) reported that PEPC and enzymes associated with phospho*enol*pyruvate synthesis may be significant factors to maintain relatively high photosynthesis of sorghum under N stress.

Miscanthus exhibits large yield increases in response to N supply under appropriate growth conditions (Ercoli *et al.* 1999, Cosentino *et al.* 2007). However, there has been little research on the effect of N levels on photosynthesis of *Miscanthus* (Weng and Hsu 2001), and none lucubrates how N influences the physiology and biochemistry of photosynthesis. Most *Miscanthus* research has focused on one species, *M.* × *giganteus*, which is a sterile, triploid hybrid of diploid *M. sinensis* and tetraploid *M. sacchariflorus* (Greef and Deuter 1993). Hence, to comprehensively investigate the responses of photosynthesis of *Miscanthus* to N use, the photosynthetic activities of three *Miscanthus* genotypes (*M.* × *giganteus* and its two parental species, *M. sinensis* and *M. sacchariflorus*) were compared at three levels of N application. The aims of this study were to investigate the relationship between photosynthesis and N application among *Miscanthus* genotypes and physiological or biochemical factors that influence the photosynthetic rate.

Materials and methods

Plants and growth conditions: The experiments were conducted in a greenhouse of Zhejiang University, Hangzhou, China in May 2010, using a completely randomized block design under conditions of temperature 19–30°C, relative humidity 58–90% and photosynthetic photon flux 200–1,200 μmol(photon) m^{-2} s^{-1}. The soil used in the experiments was air-dried, from which debris, weeds, and gravel were removed before use. Soil nutritional properties were (all mg kg^{-1}) 30 of N, 0.6 of phosphorus (P) and 25 of potassium (K). Each plastic pot with diameter of 30 cm contained 10 kg of prepared soil and received 1.2 g of K and 0.2 g of P. N levels were 0, 100 and 200 kg ha^{-1} of N; with 0 kg ha^{-1} being the N-deficient control (CK). N was applied as urea. There were three replications for all N treatments. Rhizomes of three *Miscanthus* genotypes (*M.* × *giganteus*, *M. sinensis* and *M. sacchariflorus*) were collected from a field in Linan, China and propagated in a greenhouse. Each plastic pot was planted with one rhizome. After the rhizomes were established one and half month old, a homogenous population of plants with the 8th leaf old was used to measure all parameters of the full expanding top leaf of main column. Five plants were used for each replication.

Measurements of N content and PNUE: The total N content was measured by the micro-Kjeldahl method (Stuart 1936). The N contents of leaves of *Miscanthus* were calculated based on data from N determinations. The leaf N content per unit leaf area (LNC) and PNUE were calculated according to the method reported by Ethier *et al*. (2006).

Measurement of leaf gas exchange and leaf area: P_N was measured by using a portable photosynthesis system (*LI 6400, LI-COR*, Lincoln, NE, USA) with red/blue LED light source with a 6-cm^2 clamp-on leaf chamber. Fully-expanded youngest leaves of 3–5 plants per treatment were used for measurements. Samplings were conducted after gas-exchange parameters reached the steady state. A P_N set range of PPFD of 0–2,000 μmol m^{-2} s^{-1} was used by the open gas-exchange system. The response of P_N to step increases of intercellular CO_2 (C_i) was measured. Water-use efficiency (WUE) was calculated from P_N divided by transpiration rate, both recorded at the same time. All measurements were made in a temperature-controlled room [32°C, 12-h d, 800–1,000 μmol(photon) m^{-2} s^{-1} and relative humidity 50%]. The subsamples were used to determine the leaf mass (oven-dried) per unit leaf

area (LMA). Leaf area was determined by a digital image analyzer (*ci-203, CID Inc*., USA). Once P_N was obtained, the illuminated leaf disks were freeze-clamped at liquid N_2 temperature and stored at –80°C for soluble protein contents, enzyme contents and chlorophyll (Chl) assays.

Analysis of carbon isotope discrimination and Φ: The leaf samples were dried at 80°C for 48 h and then ground. The carbon isotope ratio of CO_2 was analyzed by a mass spectrometer (*MAT251*, Finnigan, USA). The difference in ratio of ^{13}C- to ^{12}C-CO_2, δ^{13} was calculated using Eq. 1:

$$\delta^{13} = (RP/RST - 1) \times 1{,}000, \quad (1)$$

where RP and RST represent $^{13}C/^{12}C$ ratio of leaf sample and standard Pee Dee Belemnite, respectively.

Carbon isotope discrimination (Δ) was calculated using Eq. 2 (Henderson *et al*. 1992, von Caemmerer *et al*. 1997):

$$\Delta = (\delta a - \delta p)/(1 + \delta p) \times 1{,}000, \quad (2)$$
$$\Delta = a + [(b4 + \Phi\,(b3–s)–a)\, p_i]/\, p_a, \quad (3)$$

where δa and δp express the isotopic compositions of air and plant material, respectively; a (4.4‰) is the fractionation associated with diffusion (Farquhar 1983); b4 (–5.7‰) is the combined fractionation of PEP carboxylation; b3 (29‰) is the fractionation by Rubisco, and s (1.8‰) is the fractionation during CO_2 leakage from BS cells. Furthermore, p_i and p_a are the CO_2 partial pressures between intercellular space and ambient air, respectively, which were taken from gas-exchange measurements in the greenhouse. Eq. 3 can be rearranged as an explicit expression of Φ, which was estimated by the equation derived by Farquhar (1983):

$$\Phi = [\Delta - a + (a - b4)\, p_i/p_a]/[(b3 - s)\, p_i/p_a] \quad (4)$$

Chl, soluble protein, PEPC and Rubisco: Frozen leaf tissue was rapidly homogenized in the modified extraction buffer following the procedure by Tazoe (2006). The extraction buffer contained 50 mM HEPES-KOH at pH 7.0, 1 mM EDTA-2Na, 10 mM $MgCl_2$, 0.1 % (v/v) Triton X-100, 5% (v/v) glycerol, 1% (w/v) polyvinylpyrrolidone, 5 mM dithiothreitol, 1 mM benzenemethanesulfonyl fluoride, 2 mM iodoacetic acid and the Complete Protease Inhibitor (*Roche Applied Science*, USA).

A part of the homogenate was used to determine Chl according to the method developed by Arnon (1949) and Chl *a*/*b* ratios were calculated by the method Porra *et al*. (1989). The other homogenate was centrifuged at 15,000 × *g* for 5 min at 4°C. The supernatant was used to determine soluble protein with the Bradford method (Makino *et al*. 2003). Both Chl and soluble protein contents were expressed in the basis of leaf area.

Western blotting was carried out following the procedure by Tazoe (2006). The total soluble proteins representing equal amounts of leaf area were electrophoresed on 10% (w/v) SDS-PAGE gels and blotted onto the nitrocellulose. PEPC and Rubisco large subunit proteins were probed with rabbit polyclones of maize PEPC (*Abcam*, Hong Kong) and rice (*Oryza sativa* L.) Rubisco large subunit (rbcL) previously made in our laboratory (Wang *et al*. 2009) , respectively. The blots were incubated with second antibody goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate (*Bio-Rad*, Richmond, USA). The quantification band intensities of PEPC and rbcL in SDS-PAGE were analyzed by Quantity One (*Quantity One^{TM} Version 4.5, Bio-Rad*).

Statistical analysis: Data are presented as mean ± SD. Statistical comparisons were made by a one-way analysis of variance (*ANOVA*). The differences between means were established using *Duncan*'s multiple tests and the correlation analysis was conducted with Excel. All experiments were performed at least three times.

Results

Effects of different N levels on P_N and PNUE: P_N in all genotypes rose significantly as N application increased (Fig. 1*A–C*), especially in high-light conditions. With increased application of N from 0 to 100 kg ha^{-1}, and then to 200 kg ha^{-1}, P_N under PPFD of 1,500 μmol(photon) m^{-2} s^{-1} increased progressively in *M. sinensis* by 9.8 and 16.6%, respectively; and correspondingly in *M. sacchariflorus* by 16.9 and 25.8%, and in *M.* × *giganteus* by 13.4 and 29.8%. The *M.* × *giganteus* was more affected by N application, with the highest P_N under 200 kg ha^{-1} of N in high-light conditions (Fig. 1*C*). The response curves of P_N to C_i illustrated that *Miscanthus* had a low CO_2 saturation point, as typical of the C_4 photosynthetic pathway. The initial slope of the curve, representing carboxylation efficiency, was increased by N application in all genotypes (Fig. 1*D–F*), so that P_N at the same CO_2 concentration was always higher with increased N application (Fig. 1*A–C*).

As a consequence of the increase of P_N with changes in LNC (Fig. 2*A*), increased N application did not significantly change PNUE in *M. sinensis*, but did significantly reduce PNUE in *M. sacchariflorus* and *M.* × *giganteus* (Fig. 2*B*). We analyzed the relationships between P_N and LNC (Fig. 3*A*), and between PNUE and LNC (Fig. 3*B*) and none had significant difference between genotypes (Fig. 3). The three genotypes showed the same tendency of both P_N and PNUE that common equations can be expressed, in which P_N increased, but PNUE declined with increasing LNC in *Miscanthus*.

Similarly to P_N, the stomatal conductance (g_s) and transpiration were also enhanced by N application in each genotype (Fig. 4*A–F*). Both g_s (Fig. 4*A–C*) and C_i (Fig. 1*D–F*) were increased with increased N supply, indicating that stomatal factors played an important role

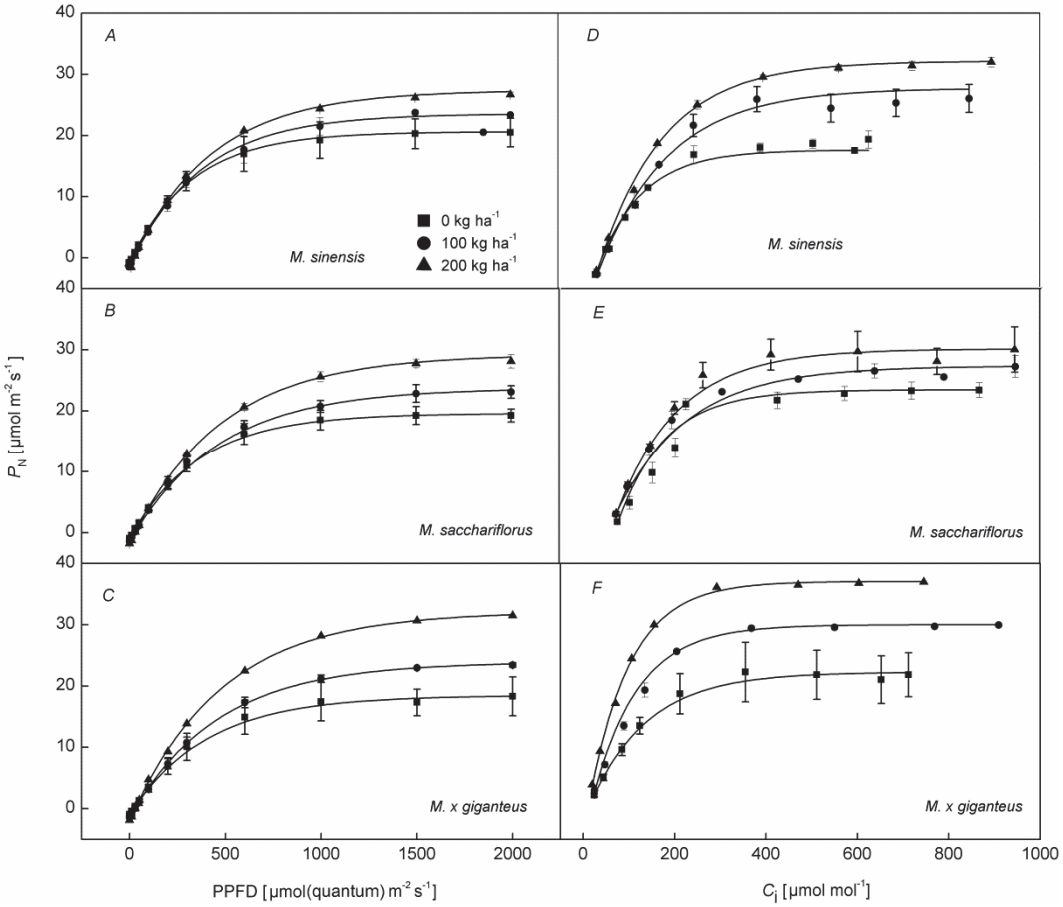

Fig. 1. The net photosynthetic rate (P_N) *vs*. photosynthetic photon flux density (PPFD) (*A–C*) and intercellular CO_2 concentration (C_i) (*D–F*) of three *Miscanthus* genotypes at three N levels. P_N–PPFD curves were measured with values of an ambient CO_2 concentration of 400 μmol mol^{-1}, when P_N/C_i curves were taken at the PPFD of 1,500 μmol(photon) m^{-2} s^{-1}. Values are presented as means ± SD (n = 3).

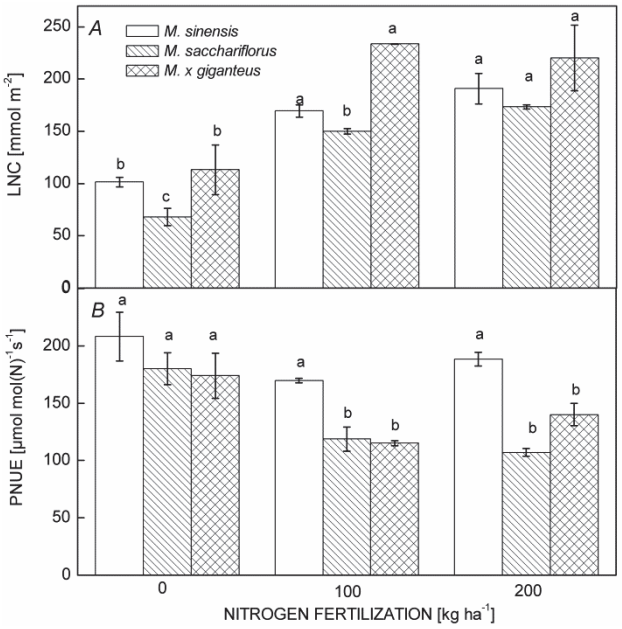

Fig. 2. The effect of N levels on leaf N content per area (LNC) (*A*), and photosynthetic nitrogen-use efficiency (*B*) of three *Miscanthus* genotypes. Values are presented as means ± SD (n = 3).

in increasing P_N. However, WUE of each genotype with increasing N did not differ significantly (Fig. 4*G–I*). At a given C_i, P_N in all genotypes enhanced with increasing N. This phenomenon implied that nonstomatal factors might also play an important role in increasing P_N with increased N application. Such circumstances may correspond to increased carboxylation efficiency relevant to PEPC or/and Rubisco.

Effects of different N levels on Chl and soluble protein: To investigate how N levels enhanced P_N, we determined the contents of Chl and soluble protein in leaves of *Miscanthus*. N supply increased the total Chl content of each genotype, but in *M. sinensis* did not increase significantly at different N levels. In *M. sacchariflorus* and *M.* × *giganteus*, there were significant differences only between CK and 100 or 200 kg(N) ha^{-1} levels

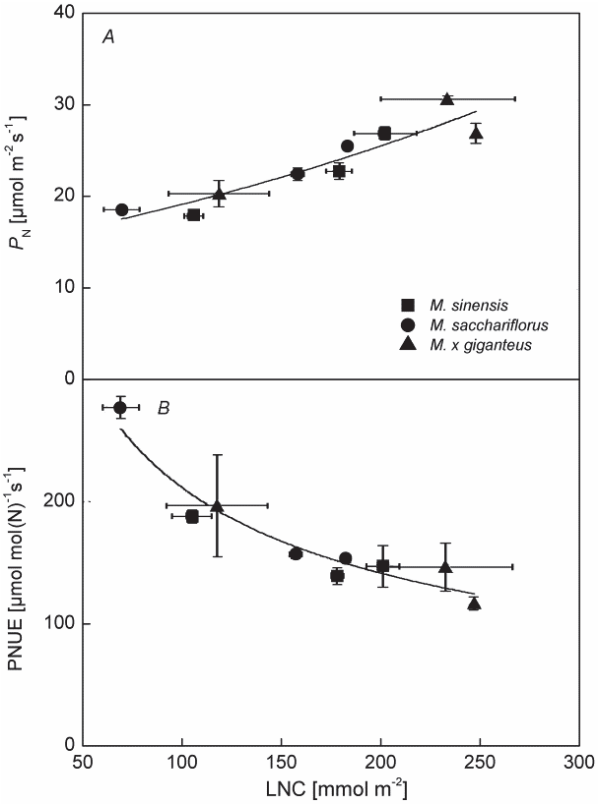

Fig. 3. The relationships between P_N and leaf N content per area (LNC, *A*) and between photosynthetic nitrogen-use efficiency (PNUE) and LNC (*B*) in three *Miscanthus* genotypes grown at three N levels. Each point represents the means of three replications at least. The equation between P_N and LNC is $y = 14.076\ e^{0.0031X}$ ($R^2 = 0.88$, $P<0.01$) and that between PNUE and LNC is $y = 2349.7\ x^{-0.545}$ ($R^2 = 0.91$, $P<0.01$) ($n = 9$).

(Fig. 5*A*). Leaf soluble protein varied according to genotype and N supply: both in *M. sinensis* and *M. sacchariflorus* it had notably greater content under 200 kg(N) ha^{-1} in comparison with CK and 100 kg(N) ha^{-1}; while in *M.* × *giganteus* there were significant differences among all N levels (Fig. 5*B*). The relative analysis indicated extremely significant logarithmically positive correlation between Chl contents and LNC (Fig. 5*C*), while extremely significant exponentially positive correlation between soluble protein contents and LNC. This result indicated that additional N led *Miscanthus* to investigate more N into soluble protein.

Effects of different N levels on Φ, Δ, and LMA: Bundle sheath leakiness of CO_2 (Φ) has been considered as a potential bottleneck associated with environmental factors in the photosynthetic performance of C_4 plants (Kromdijk *et al*. 2008). To investigate whether or not N application changed in Φ, an explicit expression of Φ was used to assess variation in photosynthetic metabolism of N application in *Miscanthus*. The results showed no differences in either N level or genotype (Table 1), indicating that the balance of photosynthetic metabolism in BS and mesophyll cells of *Miscanthus* were not changed. There was no significant difference in Δ among the three N levels (Table 1), but there were significant differences among the three genotypes. According to Eq. 3, the variation in Δ depends on the ratio of p_i/p_a and Φ. The difference in C_i among genotypes (Fig. 1*D–F*) indicated that p_i/p_a was responsible for the values of the N-induced effect on Δ. These might be explained by the different activities of PEPC or/and Rubisco fixing CO_2 in the genotypes.

There were significant differences in leaf width for *M.* × *giganteus* and *M. sinensis* with N application, with greatest mean width for *M.* × *giganteus* (Table 1). There were significant differences in LMA for the three genotypes and prominent differences in *M.* × *giganteus* with increasing N levels, indicating the leaf grew thicker following N application.

Effects of N levels on PEPC and rbcL: Western blotting for PEPC and rbcL was conducted to determine if the differences in photosynthesis corresponded to changes in the amounts of key photosynthetic enzymes. PEPC concentrations in all genotypes were significantly enhanced with increasing N levels (Fig. 6*A,C*); *M.* × *giganteus* showed the highest quantity of PEPC at all N levels. Despite increased rbcL concentrations with increased N levels there was a significant difference only for *M. sacchariflorus* (Fig. 6*A,B*). The results suggested that PEPC might play a more important role than Rubisco in the promotion of photosynthetic rate by N application in *Miscanthus*.

Discussion

P_N increase was mainly due to greater g_s and higher PEPC concentration with increased N: The photosynthetic characteristics of *Miscanthus* varied with genotypes and N effects. There is a positive correlation between P_N and LNC for C_4 plants (Tazoe *et al*. 2006, Uribelarrea *et al*. 2009), but this correlation also depends on N supply levels (Ghannoum *et al.* 2005). Weng and Hsu (2001) found that the PPFD-saturated photosynthetic rate responded to N investment. The point of inflection of the P_N/C_i curve increased with increasing N supply level in maize (Leegood and von Caemmerer 1989), as also observed in the present study (Figs. 1, 3*A*). We have found the negative correlation of PNUE with LNC.

It is well known that P_N can be affected by stomatal and nonstomatal factors (Weng and Hsu 2001). Well watered loblolly pine (*Pinus taeda* L.) showed a greater relative contribution of g_s to the limitation to P_N with increased N (Green and Mitchell 1992). In the present study, P_N was increased by both g_s and C_i, suggesting that g_s was an important limitation of photosynthetic capacity

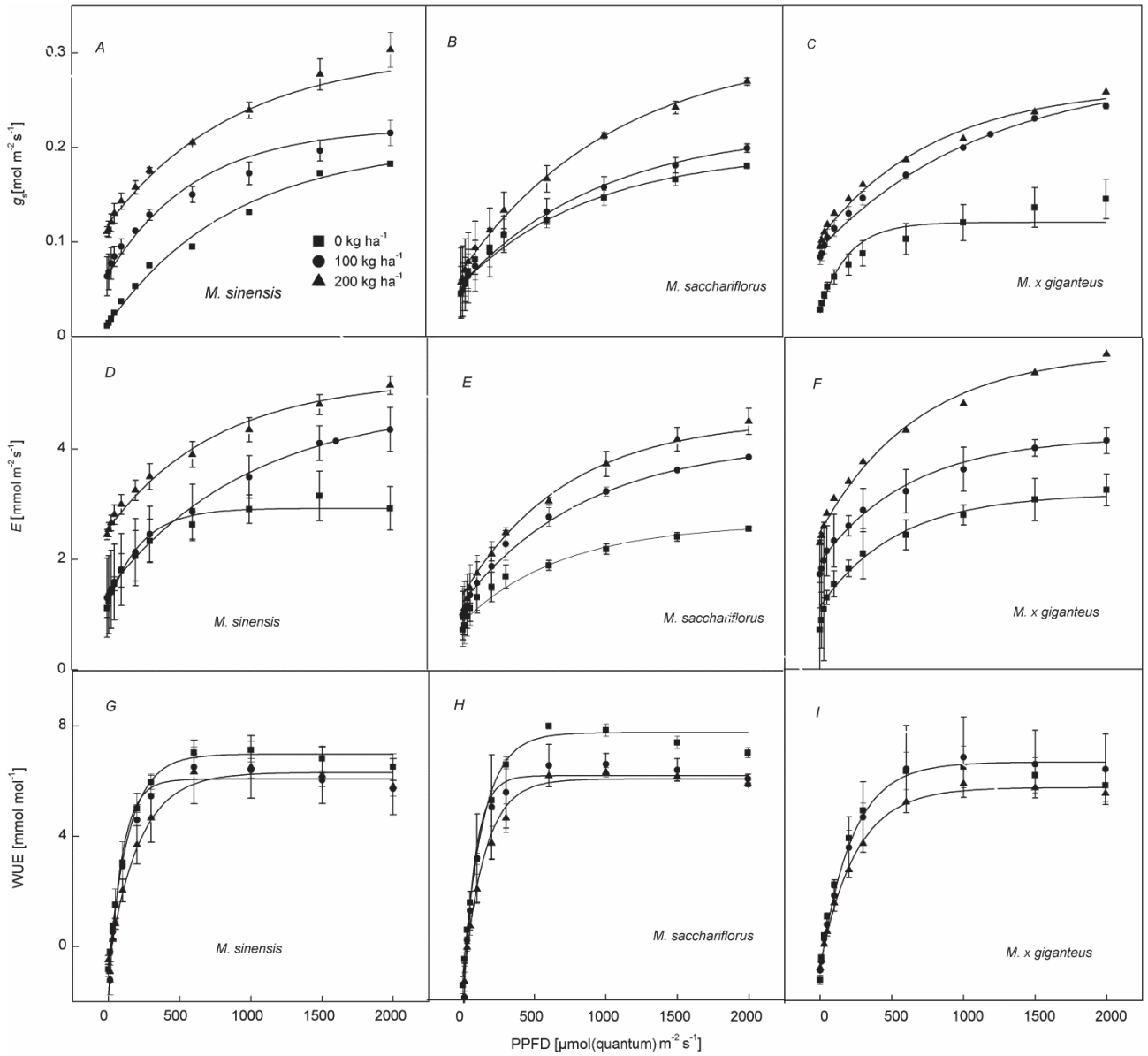

Fig. 4. Stomatal conductance (g_s) (*A–C*), transpiration (*E*) (*D–F*) and water-use efficiency (WUE, *G–I*) under different levels of incident PPFD conditions of three *Miscanthus* genotypes at three N levels. Values are presented as means ± SD (n = 3).

in *Miscanthus* at a range of N levels (Figs. 1*D–F*, 4*A–C*).

In addition to the increase in P_N at increased N applications, the leaf N contents also reflected the increased amount of soluble protein and Chl content. P_N also relies on the amount of photosynthetic apparatus, rather than LMA (Dohleman *et al.* 2009), because P_N simultaneously depends on N investment in light-harvesting complexes and the carboxylation system.

C_4 plants have a particular leaf structure for the function of CO_2 concentration (Sage 2004), which requires much less Rubisco than C_3 plants (Makino *et al.* 2003). The reduction of Rubisco content in C_4 species leads to reduced P_N (von Caemmerer *et al.* 1997). *Amaranthus cruentus* tended to invest more N in Rubisco compared to PEPC or PPDK (phosphopyruvate dikinase) (Tazoe *et al.* 2006). Nevertheless, maize showed increased PEPC with increased leaf N content, while Rubisco remained constant (Sugiyama *et al.* 1984, Makino *et al.* 2003, Uribelarrea *et al.* 2009). Sugarcane in N-deficient situation had reduced levels of the Rubisco:PEPC activity ratio which was attributed to a decline in C_3 cycle activity in the BS relative to C_4 cycle activity in mesophyll (Meinzer and Zhu 1998). However, the C_3 and C_4 cycles may be coordinately organized to carry out efficient C_4 photosynthesis (Henderson *et al.* 1992, Tazoe *et al*. 2006). The difference in photosynthesis observed in different *Miscanthus* genotypes was due to their N allocation pattern to photosynthetic enzymes. In our experiment,

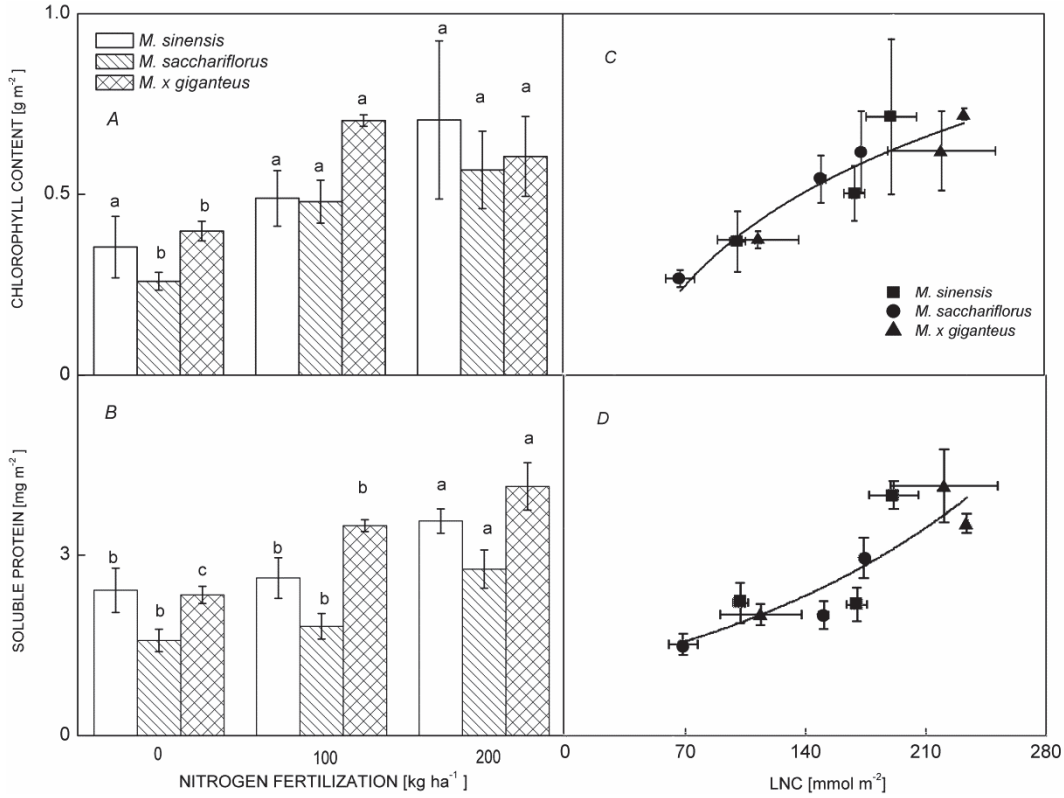

Fig. 5. Effect of N fertilization on chlorophyll content (*A*) and leaf soluble protein (*B*) in three *Miscanthus* genotypes and the relationships between chlorophyll content and LNC (*C*), and between soluble protein content and LNC (*D*) in three *Miscanthus* genotypes. Each point represents the means of three replications at least. The equation between chlorophyll content and LNC is $y = 0.38 \ln(x) - 1.4075$ ($P<0.01$, $n = 9$), and between soluble protein content and LNC is $y = 1.06\, e^{0.0056x}$ ($P<0.01$, $n = 9$).

Table 1. Effect of N levels on bundle sheath leakiness (Φ), carbon isotope discrimination (Δ) and leaf width and leaf mass per area (LMA) of three genotypes in *Miscanthus*. ($n = 3$). *Different letters* denote significant difference in N levels (*small letters*) or in genotypes (*capital letters*) ($P<0.05$).

Parameter	Species	Nitrogen fertilization			Average
		0 kg ha^{-1}	100 kg ha^{-1}	200 kg ha^{-1}	
Φ	*M. sinensis*	0.36^{a}	0.35^{a}	0.36^{a}	0.36^{A}
	M. sacchariflorus	0.35^{a}	0.36^{a}	0.36^{a}	0.36^{A}
	M. × giganteus	0.36^{a}	0.35^{a}	0.36^{a}	0.36^{A}
Δ [‰]	*M. sinensis*	4.60^{a}	4.74^{a}	4.44^{a}	4.59^{C}
	M. sacchariflorus	4.74^{a}	5.43^{a}	5.41^{a}	5.19^{B}
	M. × giganteus	5.58^{a}	5.72^{a}	5.88^{a}	5.73^{A}
Leaf width [cm]	*M. sinensis*	0.67^{b}	0.81^{ab}	0.93^{a}	0.79^{C}
	M. sacchariflorus	1.01^{a}	1.20^{a}	1.23^{a}	1.15^{B}
	M. × giganteus	1.69^{ab}	1.66^{b}	1.77^{a}	1.71^{A}
LMA [g m^{-2}]	*M. sinensis*	51.76^{b}	56.67^{a}	56.44^{a}	54.65^{B}
	M. sacchariflorus	37.55^{b}	42.78^{a}	43.29^{a}	41.21^{C}
	M. × giganteus	60.16^{b}	65.88^{ab}	68.74^{a}	64.94^{A}

although both PEPC and rbsL concentrations increased when P_N increased with increased N supply in *Miscanthus* (Fig. 6), only PEPC increased significantly in all genotypes. A strong correlation between assayed enzyme activity (PEPC and PPDK) and enzyme concentration was observed in maize (Sugiyama *et al.* 1984) and the initial slope of the P_N/C_i curves indicated *in vivo* PEPC efficiency (Ghannoum *et al.* 2000). Our result showed that the initial slope of the P_N/C_i curves increased significantly under high N supply, especially in

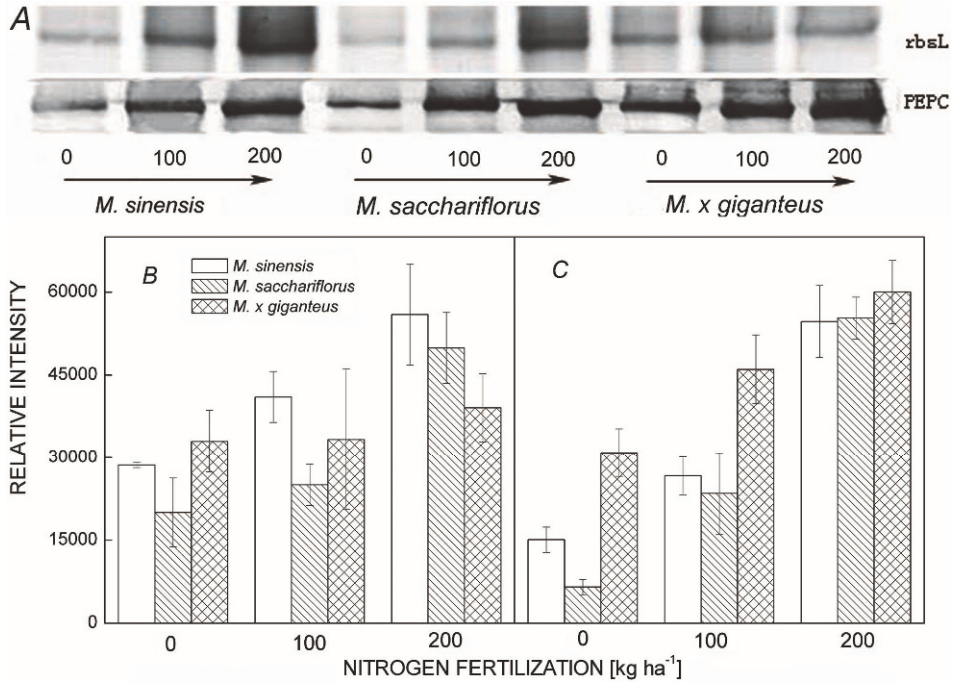

Fig. 6. Sample Western blot of phospho*enol*pyruvate carboxylase (PEPC) and large subunit of Rubisco (rbcL) exacted from *M. sinensis*, *M. sacchariflorus* and *M.* × *giganteus* leaves at three levels of N fertilization (*A*). Western blots used polyclonal primary antibodies against rice rbcL and maize PEPC. Each lane contained the same amount of soluble protein. For both rbcL (*B*) and PEPC (*C*) protein, the band intensities was quantified and plotted as columns. The values were normalized with respect to the band from the SDS-PAGE measured by the software *Quantity One* (*Bio-Rad*, USA).

M. × *giganteus* (Fig. 2*B*), meaning high PEPC activity *in vivo* after N application. Possibly the Rubisco content was also superfluous for the photosynthetic rate of *Miscanthus*. Therefore, the promotion of photosynthetic rate by N application in *Miscanthus* is mainly attributed to increased PEPC concentration.

No change in leaf Φ despite modification of LMA at different N levels: C_4 plants manipulate anatomical, biochemical and physiological changes to adapt to changes in the environment (Tazoe *et al.* 2006, 2008, Uribelarrea *et al.* 2009, Pengelly *et al.* 2010). It has been presumed that anatomical characteristics in C_4 leaves can influence Φ (Pengelly *et al.* 2010) and that the efficiency of the C_4 pathway usually relies on Φ and biochemical conversion between C_3 and C_4 cycles (Meinzer and Zhu 1998). Φ has been considered a potential bottleneck associated with environmental factors in the photosynthetic performance of C_4 plants (Kromdijk *et al.* 2008), such as low light (Pengelly *et al.* 2010), low growing temperature (Kubásek *et al.* 2007), drought and salinity stress (Zhu and Meinzer 1999). Tazoe *et al.* (2008) discovered that Φ was affected by light at PPFD < 150 μmol(photon) m^{-2} s^{-1}. Our result showed that the leaf became thicker following N treatment. However the Φ value remained constant at around 0.3 in *A. cruentus* at high light intensity, 0.2–0.3 in maize (Henderson *et al.* 1992) and in our experiment the values were stable at about 0.35 in *Miscanthus* independent of N application at PPDF of 1,500 μmol(photon) m^{-2} s^{-1}, signifying that the balance between the C_4 and C_3 cycles was not broken. Similar observations were also made by Tazoe *et al.* (2006), who reported that $\delta^{13}C$ values were independent of LNC in *A. cruentus*. Meinzer and Zhu (1998) showed in sugarcane plant a decline in quantum yield and increase in Φ upon N deficiency. In the present study, Δ did not respond to N levels, but showed genotypic diversity (Table 1), suggesting that Δ was associated primarily with p_i/p_a response to variation in *Miscanthus*. Simultaneously, the levels of photosynthetic enzymes (*e.g.* PEPC and rbsL), which responded to the carboxylation efficiency, were higher in *M.* × *giganteus*. However, Meinzer and Zhu (1998) observed that the variation in Δ in N-deficient sugarcane was more sensitive to Φ compared to p_i/p_a.. Nevertheless, which parameter more greatly affects Δ is still debated, and depends on species and environmental conditions.

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