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Why Nitrogen Use Efficiency Decreases Under High Nitrogen Supply in Rice (*Oryza sativa* L.) Seedlings

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Abstract Hydroponic experiments were conducted in a greenhouse to examine the effects of different nitrogen (N) supply (low, 20 mg L^{-1}; intermediate, 40 mg L^{-1}; and high, 100 mg L^{-1}) on the growth, nitrogen use efficiency, and photosynthetic characteristics of rice seedlings (*Oryza sativa* L., cv. “Shanyou 63” hybrid *indica.* China). Leaf gas exchange was conducted to identify the photosynthetic-limiting factors in plants with high N supply. The results showed that (1) shoot biomass, leaf area, and tiller numbers per plant under low N were lower than under intermediate and high N supplies. No significant differences were observed between plants supplied with intermediate and high N. (2) About a 35% increase in leaf N content in plants fed by high N resulted in about a 15% increase in carboxylation efficiency (CE) and photosynthetic rate. (3) The noncorresponding increases in photosynthetic rate in rice seedlings fed by high N relative to low N resulted from Rubisco activity and/or CE. (4) The decreased Rubisco activity was induced by a relatively insufficient CO_2 supply under high N supply. These results indicated that insufficient CO_2 supply under high N supply accounted for the decreased Rubisco activity and the noncorresponding increases in photosynthetic rate to leaf N content, and as a result, decreased (photosynthetic) nitrogen use efficiency.

Keywords Rice (*Oryza sativa* L.) · Nitrogen use efficiency · Photosynthesis · Rubisco

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Introduction

In modern agricultural production systems, nitrogen plays an important role in yield improvement because of the fundamental role of nitrogen (N) in protein formation. The requirement for N in higher plants is greater than that of other macro- and microelements (Cruz and others 2003). Nitrogen increases plant height, chlorophyll content, soluble protein levels, and ultimately the potential yield of crops (Ding and others 2005; Walker and others 2006; Good and others 2007). However, excess N is frequently supplied to a field to obtain a high yield, causing numerous negative effects such as lower N use efficiency (NUE), water pollution (Hasegawa 2003; Mancus 2007), and contribution to the greenhouse effect (Yu and Patrick 2003; Li and others 2006). Compared to the 1930s, increases in crop yield do not match increases in nitrogenous fertilizer application. Fourfold increases in crop yield were obtained in the 1980s accompanied by 11-fold increases in applied N fertilizers (Odum 1989), and further increasing the application of N fertilizer correspondingly decreases N fertilizer use efficiency. Annual rice yield per kg N fertilizer after application of 56 kg N ha^{-1} decreased from 56.95 kg to 15.69 kg with the application of 280 kg N ha^{-1}. Compared to 168 kg N ha^{-1}, there was no increase in seed yield with the application of 280 kg N ha^{-1} (Good and others 2007), confirming that increases in nitrogenous fertilizers do not always increase grain yield (Samonte and others 2006). In particular, high N supply in low phosphorus-(P) or potassium-(K) containing soil will reinforce these nutrient deficiencies and will further reduce plant growth (Güsewell 2005).

Low NUE is partially caused by limited N absorption. When nitrogenous fertilizers are applied in excess, N is lost from the root zone rather than being absorbed effectively

(Youngdahl and others 1982). Secondly, although leaf N content increases with high N supply, photosynthetic nitrogen use efficiency (PNUE, determined by photosynthetic rate per unit leaf organic N content) is lower in high-N-content leaves of certain species (Sage and Pearcy 1987). The proportion of total leaf N in the thylakoids remains constant with increasing leaf N content, whereas leaf soluble N increases (Evans 1989; Makino and others 1997). Increases in leaf N only slightly increase quantum photosynthetic yield (Evans and Terashima 1987; Evans 1989; Khamis and others 1990; Cruz and others 2003). The question of why increases in nitrogenous fertilizer application do not correspond to increases in crop yield should be looked at based on the physiological role of nitrogen in photosynthesis.

Chloroplastic CO_2 concentration (C_c) is not saturated for photosynthesis in C_3 plant leaves under the current atmospheric CO_2 concentration (Bernacchi and others 2002; Leegood and others 2004). The carboxylation capacity of Rubisco, which is the central CO_2-fixing enzyme in the Calvin cycle, is the ultimate limiting factor in leaf photosynthesis. In the present study we hypothesized that under high N supply, the predominant factor limiting leaf photosynthetic rate is Rubisco activity and decreased Rubisco activity is related to limited CO_2 conductance.

Materials and Methods

Plant Materials and Growing Conditions

After germination on moist filter paper, rice seeds (*Oryza sativa* L., cv. "Shanyou 63" hybrid *indica* China) were transferred to a 2.0-mmol l^{-1} $CaSO_4$ solution for germination at 30 ± 5°C. After 3 days rice seedlings were transplanted to 6-l rectangular containers (30 × 20 × 10 cm) and transferred into quarter-strength nutrient solution (for composition, see below). Three days later the seedlings were transferred to half-strength nutrient solution. Five days later the seedlings were supplied with full-strength nutrient solution for 1 week. The seedlings were supplied with low N (20 mg l^{-1}), intermediate N (40 mg l^{-1}), or high N (100 mg l^{-1}) nutrient solution. The N sources were equal moles of $(NH_4)_2SO_4$ and $Ca(NO_3)_2$. The composition of the other nutrients in the solutions was as follows: macronutrients (mg l^{-1}): 10 P as KH_2PO_4, 40 K as K_2SO_4 and KH_2PO_4, 40 Mg as $MgSO_4$; micronutrients (mg l^{-1}): 2.0 Fe as Fe-EDTA, 0.5 Mn as $MnCl_2{\cdot}4H_2O$, 0.05 Mo as $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.2 B as H_3BO_3, 0.01 Zn as $ZnSO_4{\cdot}7H_2O$, 0.01 Cu as $CuSO_4{\cdot}5H_2O$, and 2.8 Si as $Na_2SiO_3{\cdot}9H_2O$. The Ca in the low N and intermediate N treatments was compensated for by adding $CaCl_2$. A nitrification inhibitor (dicyandiamide) was added to each nutrient solution to prevent ammonium oxidation. Nutrient solutions were changed every 2 days, and pH was adjusted to 5.50 ± 0.05 every day with 0.1 mol l^{-1} HCl and 0.1 mol l^{-1} NaOH. Each treatment had five replicates in a completely randomized design. The placement of different treatments was randomized to avoid edge effects in the greenhouse.

Plants were grown in a greenhouse with 25/18°C day/night temperatures. Light was supplied by SON-T AGRO 400-W bulbs, which maintained a minimum light intensity of 1,000 μmol photons m^{-2} s^{-1} (PAR) at leaf-level and a 14-h photoperiod.

Gas Exchange Measurements

Forty days after the treatments started, the light-saturated photosynthetic rate and chlorophyll fluorescence of newly expanded leaves were simultaneously measured from 09:00 to 15:00 with a Li-Cor 6400 infrared gas analyzer. Leaf temperature during the measurements was maintained at 25°C with a photosynthetic photon flux density (PPFD) of 1,500 μmol photons m^{-2} s^{-1}. Ambient CO_2 concentration in the cuvette ($C_{a\text{–}c}$) was adjusted to the atmospheric CO_2 concentration (C_a) (380 μmol CO_2 mol^{-1}), and relative humidity was maintained at 40%. Ten minutes later, $C_{a\text{–}c}$ was controlled across a series of 1,000, 800, 600, 400, 200, 150, 100, and 50 μmol CO_2 mol^{-1} gas-exchange measurements. Data were recorded after equilibration to a steady state. Carboxylation efficiency (CE) was calculated as the initial slope of the A/C_i curves when $C_{a\text{–}c}$ was below 200 μmol CO_2 mol^{-1}. The measured leaves were labeled and leaf areas were calculated.

Chlorophyll content of the labeled leaf segments was determined with a SPAD meter. Following the gas exchange measurements, the labeled leaves were harvested and oven-dried at 105°C for 30 min and then at 70°C to constant weight.

Determination of Leaf Rubisco, Nitrate, Total N, and Organic N Contents

The Rubisco content of newly expanded leaves was determined according to the method of Makino and others (1985, 1986) with minor modifications. Briefly, newly expanded leaves were sampled, immersed in liquid N, and stored at −70°C. Newly expanded leaves (0.5 g) were ground with a buffer solution containing 50 mmol l^{-1} Tris-HCl (pH 8.0), 5 mmol l^{-1} *β*-mercaptoethanol, and glycerol 12.5% (v/v), and the extracts were centrifuged for 15 min at 1,500 *g* and 2°C. The supernatant was mixed with a dissolving solution containing 2% (w/v) SDS, 4% (v/v)

β-mercaptoethanol, and 10% (v/v) glycerol, and the mixture was boiled in water for 5 min for protein electrophoresis. The electrophoretic buffer system was an SDS-PAGE discontinuous buffer system with a 12.5% (w/v) separation gel and a 4% (w/v) concentration gel. Afterward, the gel was washed with deionized water several times, dyed in 0.25% Coomassie Blue stain for 12 h, and decolored until the background was colorless. Large subunits and relevant small subunits were placed into a 10-ml cuvette with 2 ml formamide and washed in a 50°C water bath at room temperature for 8 h. The washed solution was measured with a colorimeter at a wavelength of 595 nm using the background hue as a blank and bovine serum albumin (BSA) as the standard.

Nitrate from newly expanded leaves was extracted in boiling water for 30 min and reacted with salicylic acid, and the color was determined at a wavelength of 410 nm. After all measurements were finished, plants were harvested and separated into root, stem, and leaf fractions. All samples were oven-dried at 105°C for 30 min and then at 70°C to constant weight. The whole labeled leaves were digested with $H_2SO_4 \cdot H_2O_2$ at 260–270°C, and total leaf N content was determined with an Auto Analyzer 3 digital colorimeter (Bran + Luebbe). Leaf organic N was subtracted from total leaf N to obtain leaf nitrate.

Statistics

A one-way ANOVA was applied to assess differences among treatments using SAS 9.0 statistical software. Significant differences ($P < 0.05$) between treatments were indicated by different letters.

Results

Effects of Different N Levels on Rice Seedling Growth

Forty days after the treatments were started there were increases in shoot dry mass, leaf area, and tiller numbers per plant in rice seedlings treated with intermediate and low N (Table 1). However, no significant differences in these parameters were observed between the intermediate and high N supply (Table 1). There was no difference in root dry matter among the three nitrogen treatments, which resulted in a lower root/shoot ratio in the intermediate- and high-N-supply groups than in the low-N-supply group.

Effects of Different N Levels on N Content and Chlorophyll Content of Rice Plants

The N content of roots, stems, and leaves and the chlorophyll content of newly expanded leaves increased with increasing N supply (Table 2). Compared with rice seedlings fed an intermediate level of N, N content of roots, stems, and leaves and chlorophyll content in plants fed low N decreased by 17%, 34%, 9%, and 8%, respectively. Compared with the intermediate N group, these parameters increased by 64%, 72%, 23% and 12%, respectively, in rice plants fed high N (Table 2).

Effects of Different N Levels on Gas Exchange of Newly Expanded Leaves

The net photosynthetic rate (P_n) and CE of rice plants fed high N were higher than those fed low N, whereas there

Table 1 Effects of different N supply on dry mass (g plant^{-1}), leaf area (cm^2 plant^{-1}), and number of tillers per plant of rice plants

Treatments	Root	Shoot	Leaf area	No. of tillers per plant
Low-N	0.73 ± 0.06 a	3.06 ± 0.37 b	768 ± 53 b	5.40 ± 0.65 b
Middle-N	0.65 ± 0.08 a	4.22 ± 0.43 a	967 ± 119 a	7.60 ± 1.29 a
High-N	0.67 ± 0.08 a	4.75 ± 0.69 a	1116 ± 149 a	8.50 ± 1.41 a

Rice plants were supplied with three different concentrations of nitrogen solution (low, 20 mg l^{-1}; intermediate, 40 mg l^{-1}; and high, 100 mg l^{-1}). Data are given as means ± SE of four replications. Significant differences ($P < 5\%$) among treatments are indicated by different letters

Table 2 Effects of different N supply on N content (mg g^{-1}) in root, stem, and leaf, and chlorophyll content (SPAD value) of rice plants

Treatment	Root	Stem	Leaf	Chlorophyll content
Low-N	18.2 ± 2.3 b	18.3 ± 2.5 c	49.3 ± 1.9 c	34.7 ± 1.6 c
Middle-N	21.9 ± 3.5 b	27.7 ± 3.5 b	54.3 ± 4.5 b	37.7 ± 0.8 b
High-N	35.9 ± 2.6 a	47.7 ± 2.4 a	66.5 ± 0.1 a	42.4 ± 1.5 a

Rice plants were supplied with three different concentrations of nitrogen solution (low, 20 mg l^{-1}; intermediate, 40 mg l^{-1}; and high, 100 mg l^{-1}). Data are given as means ± SE of four replications. Significant differences ($P < 5\%$) among treatments are indicated by different letters

Table 3 Effects of different N supply on light-saturated CO_2 assimilation (A, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}), intercellular CO_2 concentration (C_i, μmol CO_2 mol^{-1}), carboxylation efficiency measured from A/C_i curves of newly expanded leaves

Treatments	A	g_s	C_i	CE
Low-N	17.9 ± 2.1 b	0.53 ± 0.14 a	290 ± 13 a	0.1126 ± 0.0045 b
Middle-N	18.6 ± 1.0 ab	0.50 ± 0.10 a	281 ± 7 ab	0.1199 ± 0.0088 ab
High-N	20.6 ± 1.6 a	0.48 ± 0.10 a	269 ± 13 b	0.1271 ± 0.0050 a

$CE_{A/Ci}$ was calculated as the initial slope of A/C_i response curves, when $C_{a–c}$ was below 200 μmol CO_2 mol^{-1}. Rice plants were supplied with three different concentrations of nitrogen solution (low, 20 mg l^{-1}; intermediate, 40 mg l^{-1}; and high, 100 mg l^{-1}). Data are given as means ± SE of five replications for A, g_s, and C_i, and of three replications for CE. Significant differences ($P < 5\%$) among treatments are indicated by different letters

were no significant differences in stomatal conductance (g_s) (Table 3). The intercellular CO_2 concentration in rice plants fed high N was lower than in those fed low N (Table 3).

Effects of N Concentration on Rubisco Activation State and PNUE of Newly Expanded Leaves of Rice Plants

PNUE (calculated as P_n/Organic N) and Rubisco activation state (calculated as CE/Rubisco content) decreased with increasing N supply in the hydroponic solutions. Compared with rice plants fed low N, the Rubisco activation state in plants fed intermediate and high N decreased by 7 and 13%, respectively, and PNUE decreased by 7 and 19%, respectively.

Discussion

Effects of Different N Supply on Plant Growth and Photosynthesis

The growth and productivity of cereal crops is positively related to the number and development of tillers (Bos and Neuteboom 1998). Low stem and leaf N content frequently restricts tiller appearance and development. Rice tillers are normally developed when stem and leaf N content are higher than 27 and 35 mg g^{-1}, respectively; they stop emerging or newly developed tillers begin to die when stem and leaf N content are individually lower than 13 or 15 mg g^{-1} (Zhong and others 2003). In the present study, tiller number was closely related to the level of supplied N and, subsequently, to leaf N content in rice seedlings (Tables 1, 2), illustrating that the absorbed N was distributed primarily to the rice shoot during the seedling stage.

Because of the fundamental role of protein in plant structure and photosynthetic apparatus, most of the absorbed N was distributed in leaves, especially in plants fed low N (Table 2). This increased distribution of absorbed N into leaves, and a larger proportion of N reutilization (Mae 1997) resulted in an increased proportion of leaf N content in low N plants (Table 2). Although leaf N content approached 50 mg g^{-1} in plants fed low N, stem N content was only 18 mg g^{-1} (Table 1), and the emergence and development of tillers was limited. Inhibited tiller emergence and development resulted in a decrease in leaf area and a lower biomass. However, there were similar biomass and tiller numbers per plant in the intermediate and high N supply plants (Table 1), although both stem and leaf N content were higher in plants fed high N (Table 2).

A Lower g_t/N Ratio Under High N Supply Decreases NUE and PNUE

A higher biomass was associated with a higher rate of photosynthesis in plants fed high N compared with the other two groups (Table 3). The enhanced photosynthesis was related to higher CO_2 utilization capacity, which was the consequence of a higher mesophyll conductance (g_m) and/or higher Rubisco catalytic capacity. This estimated higher Rubisco catalytic capacity was induced by higher Rubisco content and CE in plants fed high N (Table 3, Fig. 1).

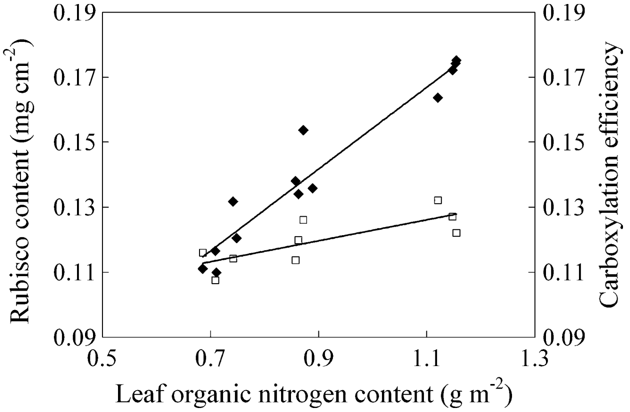

Fig. 1 The relationships between leaf organic nitrogen content and Rubisco content (*solid diamonds*), and carboxylation efficiency (*open squares*) of newly expanded leaves. The lines represent the linear regressions $y = 0.126x + 0.029$ ($R^2 = 0.929$) for Rubisco content, $y = 0.032x + 0.091$ ($R^2 = 0.616$) for carboxylation efficiency, respectively

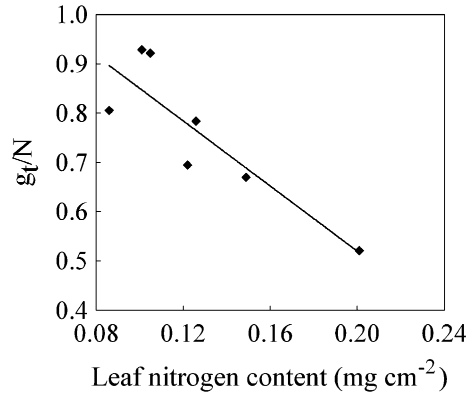

Fig. 2 The relationship between leaf N content and the ratio of total conductance to CO_2 to leaf N content (g_t/N). The line represents the linear regression $y = -3.30x + 1.18$ ($R^2 = 0.760$)

As the key CO_2-fixing enzyme in the Calvin cycle (Leegood and others 2004; Jin and others 2006), Rubisco is the most abundant protein in the plant kingdom; approximately 75% of leaf organic N is allocated to the chloroplast (Evans and Terashima 1987; Poorter and Evans 1998) and about 27% of this is in Rubisco (Evans 1989; Makino and others 1997). Close relationships between leaf N content and chlorophyll and/or Rubisco content have been demonstrated (Ookawa and others 2004), and similar results were obtained in the present experiment (Fig. 1). The catalytic effectiveness of Rubisco is extremely low under current ambient conditions (Makino and others 1997; Spreitzer and Salvucci 2002; Jin and others 2006). Firstly, Rubisco has a low kinetic affinity to CO_2; its K_m for CO_2 is 450 µM, whereas air in equilibrium with water at 25°C is about 10 µM (Jensen 2000). Secondly, due to high CO_2 diffusion resistance in the liquid phase, C_c is about 150 ppm (Flexas and others 2006), which is not saturating for Rubisco.

Based on the above-mentioned process, Rubisco operates at only 25% of its potential catalyzing capacity in C_3 plants (Sage and others 1987). The majority of Rubisco serves as N storage and exists in an inactivated form (Cheng and Fuchigami 2000; Manter and Kerrigan 2004). Although g_m is reported to be higher under high N supply (Warren 2004; Li and others 2009), the ratio of total conductance to CO_2 to leaf N content (g_t/N) decreased with increasing leaf N content (Fig. 2). A lower g_t/N ratio suggested that CO_2 supply was more severe under high N supply.

Rubisco is a bifunctional enzyme, catalyzing both carboxylation and oxygenation of RuBP (for example, Leegood and others 2004; Linka and Weber 2005). The two reactions involve competition of Rubisco catalytic sites between CO_2 and O_2, and the ratio of carboxylation to oxygenation rate is dependent on the kinetic properties of Rubisco and the partial pressure of CO_2 and O_2 at Rubisco catalytic sites (for example, Farquhar and others 1980; Wingler and others 2000; Guo and others 2007). Therefore, Rubisco activity is related to C_c (Flexas and others 2006), and a more severe CO_2 supply under high N supply will result in a decreased Rubisco activity (calculated as CE/Rubisco content in the present experiment).

The decreased Rubisco activity accounted for a lower slope of the linear relationship between leaf organic N content and CE than Rubisco content (Fig. 1). Under high N supply, the increased Rubisco functioned as N storage rather than as a catalyzing enzyme. Compared with low-N-content leaves, a 14% increase in photosynthesis in high-N-content leaves was accompanied by a 39% increase in Rubisco content or a 42% increase in leaf organic N, resulting in a lower PNUE in high-N-content leaves. The lowered PNUE under high N supply will finally result in a lower NUE.

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