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Effects of rhizobia inoculation and nitrogen fertilization on photosynthetic physiology of soybean

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Abstract

Plant growth, contents of photosynthetic pigments, photosynthetic gas exchange, and chlorophyll (Chl) fluorescence in soybean [*Glycine max* (L.) Merr. cv. Heinong37] were investigated after it was inoculated with *Sinorhizobium fredii* USDA191 or treated with 5 mM $(NH_4)_2SO_4$ (N5) and 30 mM $(NH_4)_2SO_4$ (N30), respectively. In the plants following N5 fertilization, not only plant biomass, leaf area, and Chl content, but also net photosynthetic rate (P_N), stomatal conductance (g_s), carboxylation efficiency (CE), maximum photochemical efficiency (F_v/F_m) of photosystem 2 (PS2), and quantum yield of PS2 (Φ_{PS2}) were markedly improved as compared with the control plants. There were also positive effects on plant growth and plant photosynthesis after rhizobia inoculation, but the effects were much less than those of N5 fertilization. For N30 plants there were no significant positive effects on plant growth and photosynthetic capacity. Plant biomass, P_N, and g_s were similar to those of N-limited (control) plants. Φ_{PS2} and photochemical quenching (q_P) were obviously declined while content of carotenoids and non-photochemical quenching (q_N) were significantly enhanced in N30 treated plants. This indicated that excess N supply may cause some negative effects on soybean plants.

Additional key words: biological nitrogen fixation; biomass; carboxylation efficiency; carotenoids; chlorophyll fluorescence; *Glycine max*; intercellular CO_2 concentration; leaf area; stomatal conductance; quantum yield.

Introduction

Nitrogen (N) is the mineral nutrient that plants require in greatest quantity and is the nutrient that most often limits plant growth and crop yield. Nitrogen supply affects a wide range of physiological processes in higher plants (Cechin and Fumis 2004, Gough *et al.* 2004, Gutierrez-Boem *et al.* 2004). There are numerous studies suggesting that photosynthetic capacities of higher plants change dramatically in response to different N supply (Sims *et al.* 1998, Bondada and Syvertsen 2003, Huang *et al.* 2004, Marchese *et al.* 2005) but the underlying mechanisms of photosynthetic acclimation are still not well understood. Moreover, previous studies which investigated the effects of N supply on plant CO_2 assimilation provide confusing results concerning the relationship between leaf N status and CO_2 assimilation. Research on apple leaves indicated that total ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) activity increased linearly with increasing leaf N content while RuBPCO activation state decreased with increasing leaf N (Cheng and Fuchigami 2000). One study with tobacco leaves reported that at high leaf N there was no acclimation of photosynthetic rate to elevated CO_2, only a small decrease of RuBPCO and no decrease of other Calvin cycle enzymes while at low leaf N there was a marked acclimation of photosynthetic rate and a general decrease of Calvin cycle enzyme activities in elevated CO_2 (Geiger *et al.* 1999). Robinson and Burkey (1997) reported that N supply has little impact on plant CO_2 assimilation.

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Abbreviations: Car – carotenoid; C_i – intercellular CO_2 concentration; CE – carboxylation efficiency; Chl – chlorophyll; F_0 – minimal fluorescence in dark-adapted state; F_0' – minimal fluorescence in light-adapted state; F_m – maximum fluorescence in dark-adapted state; F_m' – maximum fluorescence in light-adapted state; F_s – steady-state fluorescence in light-adapted state; F_v – variable fluorescence in dark-adapted state; F_v/F_m – maximum photochemical efficiency; g_s – stomatal conductance; P_N – net photosynthetic rate; P_{Nmax} – photosynthetic capacity at saturation irradiance; PPFD – photosynthetic photon flux density; q_N – non-photochemical quenching; q_P – photochemical quenching; Φ_{PS2} – quantum yield of PS2.

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Nitrogen can be put into agricultural systems in the form of N-fertilizer or be derived from atmospheric N_2 *via* biological N fixation. Although there are some studies on the different responses to rhizobia inoculation and N fertilizer in leguminous plants (Seneviratne *et al.* 2000, Lanier *et al.* 2005), their results are limited to growth response. Whether different source of N supply (organic N by symbiotic N fixation and chemical N by fertilizer) will have different effects on plant photosynthesis or not is unclear because photosynthetic responses to these two forms of N supply are seldom compared in leguminous plants. As N nutrition is closely associated with plant growth and development, it is potentially of great importance for understanding the different photosynthetic responses of soybean to symbiotic N fixation and N fertilization and explaining the underlying mechanisms that control plant growth.

To better understand the changes of growth and photosynthetic properties in soybean plants after rhizobia inoculation and N fertilization, we determined plant photosynthetic gas exchange, chlorophyll (Chl) fluorescence, and growth parameters of soybean.

Materials and methods

Plants: Seeds of soybean [*Glycine max* (L.) Merr. cv. Heinong37] were provided by the Soybean Institute, Heilongjiang Academy of Agricultural Sciences. Seeds were sterilized for 10 min in 95 % ethanol, followed by 10 min in 0.1 % $HgCl_2$, then thoroughly washed with sterile distilled water, and germinated in aseptic Petri dish in dark. After germination, seedlings were transferred to glass tubes (40 cm in height, 10 cm in diameter) containing sterilized mixture of vermiculite : sand of 1 : 1 by volume. Soybean plants were then subjected to four treatments: (*1*) watered with N-free medium of Fahraeus (1957) as control; (*2*) inoculated with 10^8 cells per tube of *Sinorhizobium fredii* USDA191; (*3*) supplied with 5 mM $(NH_4)_2SO_4$ which was dissolved in N-free Fahraeus solution (N5); (*4*) supplied with 30 mM $(NH_4)_2$ SO_4 which were dissolved in N-free Fahraeus solution (N30). Plants were thinned to two plants per tube after emergence. They were grown in a growth chamber with temperatures of 28/22 °C (day/night) under irradiation of 250 µmol m^{-2} s^{-1}.

Gas exchange measurements were performed using an open flow gas exchange system (*Li-6400*; *Licor*, Lincoln, NB, USA) on the middle leaflet of the third fully expanded trifoliate leaves from the top of each plant after 5 weeks of growth. Stomatal conductance (g_s), intercellular CO_2 concentration (C_i), and net photosynthetic rate (P_N) were determined at CO_2 concentration of 370 µmol mol^{-1} and saturating incident photosynthetic photon flux density (PPFD) of 1 000 µmol m^{-2} s^{-1}. The PPFD- and CO_2-response curves were performed by means of the auto-programs given by *Li-6400*. A gradient of PPFD was set as follows: 1 600, 800, 400, 300, 200, 100, 75, 50, 25, and 0 µmol m^{-2} s^{-1} and the series of reference CO_2 concentrations was 400, 50, 100, 150, 200, 300, 400, 600, and 800 µmol mol^{-1}. CO_2 carboxylation efficiency (CE) was estimated by fitting maximum likelihood regressions to the initial slope and plateau of the CO_2-response curves. Curve fittings and calculations of all parameters were performed using *Photosynthesis Assistant* version *1.1* software (*Dundee Scientific*, Dundee, UK).

Chl fluorescence was measured with a portable, pulse amplitude modulated fluorometer (*PAM-2000*; *Walz*, Effeltrich, Germany) at room temperature (25 °C). After 30 min adaptation, the minimal fluorescence (F_0) was measured under a weak modulated radiation (<0.1 µmol m^{-2} s^{-1}), and maximum fluorescence (F_m) was measured after a saturating pulse (8 000 µmol m^{-2} s^{-1}) of 0.8 s. The maximum efficiency of photosystem 2 (PS2) photochemistry was determined as $F_v/F_m = (F_m - F_0)/F_m$. An “actinic light” (200 µmol m^{-2} s^{-1}) was then applied to achieve steady-state fluorescence (F_s). After that, a saturating pulse was applied to obtain maximum fluorescence of light-adapted leaves (F_m’). At the end of measurement, a weak 3-s far-red flash was used to determine the minimal fluorescence of light-adapted leaves (F_0’). The fluorescence parameters, F_v/F_m (Butler 1978), Φ_{PS2} (Genty *et al.* 1989), and q_P and q_N (van Kooten and Snel 1990) were automatically calculated using the data acquisition software *DA-2000* (*Walz*, Effeltrich, Germany).

Photosynthetic pigments: Leaf discs were removed from the 3rd leaves and extracted with 95 % ethanol in dark for 48 h until they were blanched. The concentrations of Chl *a*, Chl *b*, and carotenoids (Cars) were determined according to Lichtenthaler and Wellburn (1983).

Leaf area and plant biomass: Leaf area measurements were carried out using the same leaves used in the gas exchange measurements by the method of copy-weighing described in Chason (1991). Plants were dried in an oven at 65 ºC for 48 h for dry mass determination.

Statistical analysis: All measurements were carried out on replicate samples taken from four individual plants per treatment, and were presented as the mean and SE. Test of significance for the effects of nitrogen on leaf were performed using *SPSS 10.0* software (*SPSS*, Chicago, IL, USA). Statistically significant effects were assumed when $p<0.05$.

Results

Photosynthetic pigments: As compared to the control, the contents of Chl in soybean plants were significantly increased following rhizobia inoculation and N fertilization (Table 1). The different increases of Chl *a* and *b* contents led to the differences of Chl *a*/*b* ratio between treatments (Table 1). For plants grown under N5, Chl (*a* + *b*) was the highest in four treatments while Chl *a*/*b* was the lowest (Table 1). Plants grown under N30 treatment showed the highest Car content (Table 1).

Table 1. Pigment contents, plant biomass, leaf area, and gas exchange of soybean following inoculation of rhizobia and nitrogen fertilization. Means ± SE ($n = 4$). Parameters within each row followed by different letters were significantly different at $p<0.05$.

Parameters	Control	Inoculation	N5	N30
Chl $a+b$ [g kg^{-1}(FM)]	1.152±0.088c	1.777±0.040b	2.235±0.111a	1.509±0.025d
Chl a/b	4.01±0.19a	2.97±0.08b	2.24±0.24c	3.44±0.04b
Car [g kg^{-1}FW)	0.307±0.018b	0.355±0.002a,b	0.349±0.022a,b	0.378±0.006a
Plant biomass [g DM]	0.39±0.02b	0.41±0.01b	0.75±0.03a	0.40±0.04b
Leaf area [cm^2]	11.98±0.31c	13.39±0.14b	17.42±1.11a	13.46±0.41b
P_N [μmol m^{-2} s^{-1}]	5.05±0.38c	7.20±0.83a,b	8.22±0.63a	6.20±0.56b,c
C_i [μmol mol^{-1}]	292.75±7.05a	276.40±13.50a	235.67±5.36b	273.33±10.87a
g_s [mmol m^{-2} s^{-1}]	0.13±0.02b	0.17±0.04a,b	0.24±0.03a	0.15±0.04a,b
P_{Nmax} [μmol m^{-2} s^{-1}]	6.08±0.26c	7.82±0.11b	9.76±0.74a	6.47±0.26c
CE [mol m^{-2} s^{-1}]	0.025±0.002d	0.040±0.001b	0.047±0.002a	0.033±0.004c

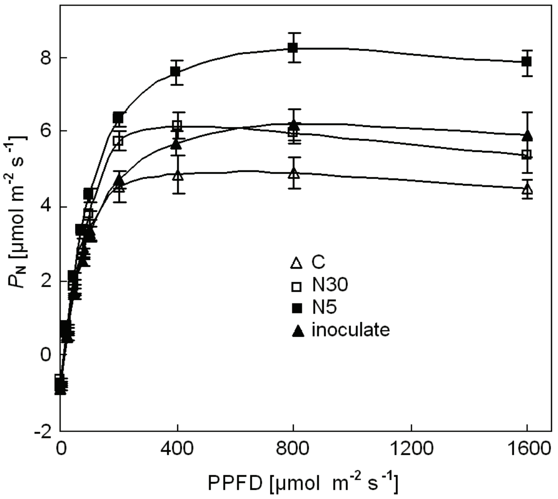

Fig. 1. Photosynthetic photon flux density (PPFD) response curves of soybean leaves following rhizobia inoculation and fertilization. Means ± SE of four replicates. *Vertical bars* represent SE.

Biomass production and leaf area: Soybean plants following N5 fertilization had 92.3 % more dry mass than controls. There were also increases in plant biomass following rhizobia inoculation and N30 fertilization, but these increases were not statistically significant (Table 1).

Leaf area was significantly increased following inoculation and fertilization. There was 45.4 % greater leaf area in N5 treated plants than in controls at harvest (Table 1).

Photosynthetic characteristics: P_N was significantly affected by rhizobia inoculation and N5 fertilization. Compared with the control plants, P_N of inoculated leaves and N5 treated leaves increased by 42.6 and 62.8 %, respectively (Table 1). There was also an increase in P_N in plants following N30 fertilization; however, this increase was not statistically significant (Table 1).

Soybean following N5 fertilization had higher g_s and lower C_i (Table 1). Although there was also increase in g_s and decrease in C_i in soybean following inoculation of rhizobia and N30 fertilization, these changes were not statistically significant (Table 1). Analysis of PPFD-response curves (Fig. 1) showed that P_{Nmax} values increased significantly after rhizobia inoculation and N5 fertilization (Table 1). And analysis of CO_2-response curves showed that CE also increased significantly by 60, 88, and 32 %, respectively, after inoculation, N5, and N30 fertilization (Table 1).

Chl fluorescence parameters: Soybean plants showed F_v/F_m around 0.83 following rhizobia inoculation and N30 fertilization, a slight decrease in N-limited plants (control), and a marked increase following N5 fertilization (Fig. 2*A*). Φ_{PS2} was also enhanced in inoculated and N5 fertilized plants, but it decreased in plants following N30 fertilization (Fig. 2*B*). There was no significant change in q_P for plants following inoculation and N5 fertilization other than a marked decrease in N30 leaves (Fig. 2*C*). Inoculated and N5 fertilized plants showed a clear reduction of q_N while N30 plants showed an increase in q_N (Fig. 2*D*) as compared with control plants.

Discussion

N supply and plant growth: Soybean plants can utilize both symbiotic N fixed in root nodules and mineral N absorbed from soil to meet their relatively high N requirement. In agricultural management, N fertilization as one way of supplying N is commonly applied to enhance soybean productivity. Previous literature showed that optimum N supply could stimulate plant growth (Gan *et al.* 1997, 2003, Gough *et al.* 2004). Our experiments showed that 5 mM $(NH_4)_2SO_4$ supply to soybean resulted in maximum plant growth with the largest leaf area, the greatest Chl contents, and the highest plant biomass among all the four treatments (Table 1). However, excess supply of N fertilizer to soybean did not bring maximal plant growth as we expected. In our study, although N30 treatment supplied much more N to soybean than N5 did, the plant growth was not as good as in N5 plants. Compared with N-limited plants (control), the increases in leaf area and Chl (*a*+*b*) content in N30 plants were only 12.4 and 31.0 %, respectively, and no significantly higher plant biomass was detected (Table 1).

There were also increases in leaf area, contents of photosynthetic pigments, and biomass in soybean following inoculation of rhizobia (Table 1). But these increases were not comparable to those in N5 plants. Symbiotic N fixation is much slower in plant early stage and the nitrogenase activity reaches a peak only some time after flowering, whereupon it drops dramatically during the early seed-filling period (Brun 1978). The total N fixed in root nodules can not satisfy the increasing N demand in soybean especially at its later growth stages (Imsande 1998). In our study, symbiotic N fixation alone was insufficient for maximum plant growth. These results indicated that maximum soybean seed yields in agricultural practice could be achieved only by the optimum use of both symbiotic N fixation through the nodules and N absorption from soil.

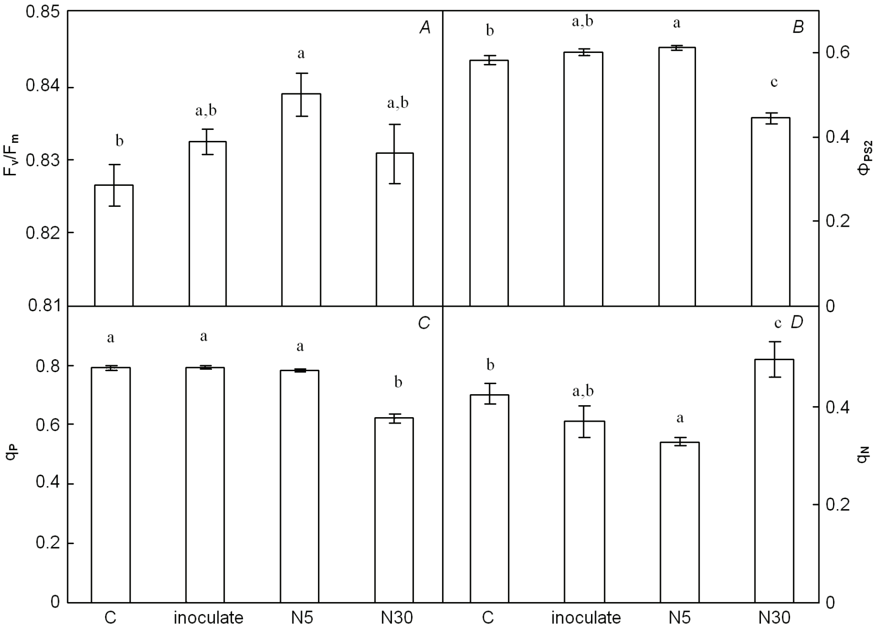

Fig. 2. Responses of chlorophyll fluorescence parameters [*A*: F_v/F_m; *B*: Φ_{PS2}; *C*: q_p; *D*: q_N] to inoculation by rhizobia and fertilization. Means ± SE of four replicates. *Vertical bars* represent SE. *Values with different letters* are significantly different ($p<0.05$). C = control.

N supply and plant photosynthesis: The positive effects of N supply on plant growth mainly result from stimulation of plant photosynthesis. About 70 % of N in plant leaves exists in chloroplasts and most of it is used to synthesize photosynthetic apparatus. Nitrogen supply is extremely important for the performance of photosynthetic apparatus (Huber *et al.* 1989, Uprety and Mahalaxmi 2000). We found that plants under 5 mM $(NH_4)_2SO_4$ supply possessed the best photosynthetic capacity with the maximum P_N, g_s, P_{Nmax}, and CE accompanied by minimum C_i (Table 1). Although biological N fixation could not satisfy the high N requirement of soybean completely, the photosynthetic performance of soybean after rhizobia inoculation was much better than that of N-limited plants (control). There was 42.6 and 60.0 % increase in P_N and CE, respectively, in inoculated soybean leaves as compared with the controls (Table 1). As high proportion of N in the leaf is allocated to photosynthetic function, the increase of photosynthetic capacity in our experiments may be the result of improved N nutritional status by different way of N supply. Paul and Foyer (2001) also suggested that the process of C assimilation could be influenced by the strength of the sinks to which photosynthates are allocated. In our study, symbiotic N fixation or mineral N absorption in soybean roots cost much more photosynthates and the increased sink strength is expected to enhance P_N of the plants.

At the same time we found that photosynthetic performance of soybean following N30 treatment was similar to that of N-limited plants (control). It indicated that high N content in soybean made similar damage to plant photosynthesis as N deficiency did. When NH_4^+ uptake exceeds the assimilation capacity, NH_4^+ will accumulate in the tissue, resulting in toxic effects. The uptake of essential cations such as K^+, Mg^{2+}, and Ca^{2+} obviously decreases which contributes to nutrient imbalance in plants (Britto and Kronzucker 2002). Ammonium toxicity may also cause membrane dysfunction and uncoupling of electron transport along membrane (Krupa 2003), for NH_4^+ may function in saturating membrane lipid and function as an electron acceptor. Therefore, these changes could impair plant photosynthetic function.

Excess N supply and Chl fluorescence: We also used Chl fluorescence to examine the impact of different kinds of N supply on the short-term efficiency of the photosynthetic machinery and light reactions, since photochemical efficiency depends on plant nutrient status and environmental stress (Krause and Weis 1991). Some studies demonstrated that N-deficiency had no effect on the quantum yield of PS2 (Ciompi *et al.* 1996, Lu and Zhang 2000). Verhoeven *et al.* (1997) showed that N-deficiency reduced F_v/F_m and Φ_{PS2} in spinach. We established the tendency to decreasing F_v/F_m and Φ_{PS2} both in N-limited (control) and N30 plants as compared with N5 plants (Fig. 2*A,B*). It suggested that too large N supply can also be a stress having similar detrimental effects on F_v/F_m and Φ_{PS2} as N deficiency did. Moreover, in our experiments q_P was much lower in N30 plants than in other plants (Fig. 2*C*). This is consistent with the decreasing Φ_{PS2} that indicates impaired electron transport in N30 plants.

N supply can help improve the ratio of the open part of PS2 reaction centre and make more photon energy used to drive the photosynthetic electron transport. In our experiments, q_N, representing the fraction of excitation energy dissipated in PS2 antennae in a form of thermal energy, was much lower in N5 supplied and inoculated plants than in the control ones (Fig. 2*D*). The same tendency was reported by Pillai and Ong (1999). We also found that q_N was much higher in plant following N30 supply (Fig. 2*D*). Higher q_N is favourable to dissipate the excessive excitation energy before it reaches the reaction centres, thus photo-protecting PS2 from potential damage. It was also in agreement with the high content of Car in N30 plants (Table 1). High contents of Cars may be an important photo-protective strategy as essential components of ROS-scavenging pool and xanthophyll cycle for better survival under stress induced by excess N (Edreva 2005). From this point, contribution of too much N to the damage of PS2 is undeniable.

There is much information on the relationship between N-deficiency and plant photosynthesis (Ciompi *et al.* 1996, Verhoeven *et al.* 1997, Lu and Zhang 2000) while only few authors inform on effects of high N supply. Under controlled conditions, we observed the negative effects of high N on plant photosynthesis for the first time. Owing to the fact that farmers in China tend to apply too much N to soybean during agriculture management (Gan *et al.* 2002), agronomic technologies including symbiotic N fixation and proper fertilizer application are urgently needed to be known by farmers in China and all over the world.

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