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Effects of nitrogen supply on the photosynthetic capacity of the hybrid citrus cultivar ‘Huangguogan’

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Abstract

Pot experiments were conducted to determine the effects of nitrogen (N) fertilization rate on the photosynthetic efficiency of ‘Huangguogan’ (*Citrus reticulata* × *Citrus sinensis*). We observed that plant growth increased with increasing N. Maximum values for parameters evaluated were recorded for an N addition rate of 120 g per year. Leaf chlorophyll content was positively correlated with the leaf N content. The relative reduction in photosynthetic rate (P_N) at high N (150 and 180 g) correlated with the parallel decreases in the leaf N content. Rubisco activity was positively correlated with the initial slope of the P_N/C_i response curve.indicating that N supplement improved photosynthesis by enhancing carboxylation and CO_2 diffusion, photosynthetic capacity increased with N supply up to 120 g. Thereafter, the rate of increase declined with any further increase in N supply. These results provide a reference for a rational application of nitrogen fertilizer in orchards of ‘Huangguogan’.

Additional key words: apparent CO_2/light-compensation point; diurnal respiration; maximum fluorescence; mesophyll conductance; steady-state fluorescence.

Introduction

Citrus is a commercially important genus of the Rutaceae. Many widely cultivated fruit species belong to this family (Hynniewta *et al.* 2014). ‘Huangguogan’ (*Citrus reticulata* × *Citrus sinensis*) is a new interspecific hybrid citrus cultivar in China; nonetheless, its cultivated area has been expanding rapidly in Southwestern China (Xiong *et al.* 2017).

Nitrogen is an essential nutrient for plant growth. In general, plants require more N than any other nutrient (Cruz *et al.* 2003). Several authors have proposed the need to determine the effect of N on citrus growth (Zekri and Obreza 2015, Sun *et al.* 2016). An appropriate N supply usually increases leaf N and Rubisco contents (Ookawa *et al.* 2004, Li *et al.* 2012). In contrast, N deficiency reduces chlorophyll (Chl) contents, electron transport rate (J), and the number of thylakoid components (Bondada and Syvertsen 2003).

Photosynthesis is closely associated with citrus growth, development, yield, and fruit quality (Vu 2005). Nitrogen is an important structural component of Chl and Rubisco. The contents of these two biomolecules are highly correlated with leaf N. In theory, photosynthetic rate (P_N) should increase with leaf N (Makino *et al.* 1997), but Rubisco activity is limited by CO_2 concentration at the chloroplast carboxylation sites (C_c) (Adachi *et al.* 2013), and a reduction in Rubisco lowers the P_N (Marcus *et al.* 2008). Although early studies suggested that C_c is limited mainly by stomatal conductance (g_s), it was recently reported that mesophyll conductance (g_m) has as strong effect on C_c as g_s (Evans *et al.* 2009, Xiong *et al.* 2015). Additionally, effects of N on g_m have been demonstrated (Buckley and Warren 2014, Xiong *et al.* 2015, Barbour and Kaiser 2016). Nutritional stress influences g_m as well as g_s; consequently, it also affects P_N (Yamori *et al.* 2011). The differential

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Abbreviations: C_a – atmospheric CO_2 concentration; $C_{a\text{-}c}$ – ambient CO_2 concentration in the cuvette; C_c – CO_2 concentration at the carboxylation sites inside the chloroplast; CE – carboxylation efficiency; $CE_{PN/Cc}$ – initial slope of the P_N/C_c response curve; $CE_{PN/Ci}$ – initial slope of the P_N/C_i response curve; Chl – chlorophyll; C_i – intercellular CO_2 concentration; C_i^* – apparent CO_2 light-compensation point; F_s – steady-state fluorescence; F_m' – maximum fluorescence; g_m – mesophyll conductance; g_s – stomatal conductance; J – rate of electron transport; J_{max} – RuBP regeneration supported by electron transport; P_N – photosynthetic rate; R_D – day respiration; RuBP – ribulose-1,5-bisphosphate; TPU – triose phosphate use; V_c – carboxylation rate; V_{cmax} – maximum carboxylation rate limited by Rubisco; V_o – oxygenation rate; α – apparent quantum yield; Φ – ratio of V_o to V_c; $Φ_{PSII}$ – actual photochemical efficiency of PSII; $Γ^*$ – CO_2-compensation point in the absence of respiration.

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responses of stomatal morphology to N application rate have also been studied (Yan *et al.* 2012). Further, it has been shown that plant N content is highly and positively correlated with P_N, and that it regulates stomatal traits (Li *et al.* 2003), and stomatal density, so that gas diffusion resistance decreases with increasing stomatal density (Chen *et al.* 1995).

In the present study, seedlings of cultivar 'Huangguogan' were grown in a pot experiment and subjected to seven different N concentrations. The effects of N supplementation on the following parameters were evaluated: (*1*) leaf N and photosynthetic pigments; (*2*) Rubisco content and activity; and (*3*) gas-exchange parameters and Chl fluorescence.

Materials and methods

Plant material and N treatments: One-year-old potted seedlings of the hybrid citrus cultivar 'Huangguogan' were grafted onto trifoliate orange [*Poncirus trifoliata* (L.) Raf] obtained from the 'Huangguogan' demonstration nursery stock in September 2015. Seedlings were grown under ambient conditions at the campus of Sichuan Agricultural University in Chengdu, China. The annual average temperature was < 20°C and the annual rainfall was 760 mm. Seedlings with uniform leaf area and N content were grown in 10-L pots in order to avoid any restrictions of root growth. The potting medium was a loamy soil (40% sandy loam, 30% silt, 20% rotten leaf soil, and 10% chicken manure). The main physicochemical properties of this medium were: 19.58 g(organic matter) kg^{-1}, 0.93 mg(total N) kg^{-1}, 95.33 mg(hydrolytic N) kg^{-1}, 52.48 mg(available P) kg^{-1}, and 54.42 mg(available K) kg^{-1}. Seedlings were acclimated to their new location for six months, during which they were subject to standard citrus management.

The experiment was laid in a completely randomized design with seven treatments (N_1–N_7) and five replicates, each consisting of one tree, for a total of 35 plants which were all fertilized with 60 g of phosphorus [$CaP_2H_4O_8$; $P_2O_5 \geq 12\%$] and 120 g of potassium [K_2SO_4; $K_2O \geq 50.0\%$]. Nitrogen [$CO(NH_2)_2$; $N \geq 46.67\%$] was applied at 0 (N_1), 30 (N_2), 60 (N_3), 90 (N_4), 120 (N_5), 150 (N_6), and 180 (N_7) g per year. Fertilizers were applied at germination (G), physiological fruit dropping (P), young fruit expansion (Y), and color change (C). The G:P:Y:C ratios were 40:10:40:10% for the $CO(NH_2)_2$ application model, 30:10:40:20% for the $CaP_2H_4O_8$ application model, and 20:30:40:10% for the K_2SO_4 application model (Table 1). All trees were frequently irrigated to ensure nonlimiting water availability. They received normal horticultural care for pest and disease control. The whole experiment was conducted for one year under natural conditions. Five plants per the experimental group were marked and used to measure photosynthetic parameters and Chl fluorescence. The third to fifth healthy functional leaves on the southern side of the crown were sampled to determine the Chl content and stomatal opening. Intermediate leaves were excised and ground under liquid nitrogen to determine photosynthesis-related enzyme activities.

Gas exchange and fluorescence: All measurements were taken with a *Li-Cor 6400* portable photosynthesis system (*Li-Cor Inc.*, Lincoln, NE, USA) on mature current-year leaves from five plants per treatment over 3 d in March 2017. The rates of light-saturated photosynthesis were measured from 09:00 to 11:30 h. The leaf chamber conditions were as follows: PPFD of 1,600 μmol m^{-2} s^{-1}, relative humidity of 60 ± 3%, CO_2 concentration in the cuvette ($C_{a\text{-}c}$) of 400 ± 2 μmol(CO_2) mol^{-1}, and leaf temperature of 29 ± 0.2°C. Data were recorded after steady state was reached (~ 15 min). The sampled third to fifth healthy functional leaves on the southern side of the crown were labeled and their leaf areas calculated based on the labeled areas. Subsequent gas-exchange measurements were conducted on these labeled leaves. After gas-exchange measurements, light-response curves were plotted. Leaf temperature, relative humidity, and $C_{a\text{-}c}$ were maintained as described above. Before measurements, sampled leaves were acclimated in the leaf chamber at PPFD of 1,600 μmol m^{-2} s^{-1} for 10–15 min for full photosynthetic activation; PPFD was then controlled at 2,300; 2,000; 1,800; 1,600; 1,400; 1,200; 1,000; 800, 600, 400, 200, 150, 100, 75, 50, and 0 μmol m^{-2} s^{-1}. The corresponding apparent quantum yields (α) were calculated as the slopes of the light-response curves when PPFD in the leaf cuvette was controlled at 150, 100, 75, and 50 μmol m^{-2} s^{-1}.

Simultaneous P_N/C_i curve and Chl fluorescence measurements were conducted on light-adjusted leaves using a *Li-Cor 6400* infrared gas analyzer (*Li-Cor Inc.*, Lincoln, NE, USA). Leaf temperature, relative humidity, and PPFD were controlled as described above. Before measurements, leaves were placed in the leaf chamber under PPFD of 1,600 μmol m^{-2} s^{-1}, CO_2 was maintained at 400 μmol mol^{-1} by running a CO_2 mixer for 10–15 min; CO_2 was controlled at 400, 300, 200, 100, 50, 400, 600, 800; 1,000; 1,300; 1,600; and 2,000 μmol(CO_2) mol^{-1}. Gas exchange, steady-state fluorescence (F_s), and maximum fluorescence (F_m') were recorded after reaching the steady state. F_m' at each CO_2 concentration was measured with a 0.8-s saturating light pulse [~ 8,000 μmol(photon) m^{-2} s^{-1}]. F_s and F_m' at 1,600 μmol(photon) m^{-2} s^{-1} and 400 μmol(CO_2) mol^{-1} were used to calculate $(F_m' - F_s)/F_m'$. Carboxylation efficiency (CE) for each N treatment was calculated as the initial slope of the P_N/C_i curve when CO_2 was < 200 μmol(CO_2) mol^{-1}.

Leakage was inevitable while the leaves were attached to the leaf chamber. Therefore, the same measurements were used to minimize the effects of leakage on P_N and C_i as previously described (Flexas and Ribas-Carbó 2007, Li *et al.* 2009).

Diurnal respiration (R_D) and apparent CO_2 light-compensation point (C_i^*): R_D and C_i^* were determined between 0:00 and 04:00 h by the Laĭsk method (Laĭsk 1977), using the same leaves used for measurements of P_N/C_i and light-response curves (Guo *et al.* 2007). Briefly, P_N/C_i curves were plotted over the linear portion of the dose-response curve [25, 50, 100, and 150 μmol(CO_2) mol^{-1}] under three PPFD levels (150, 300,

Table 1. Amount of fertilizer applied during growth of 'Huangguogan' citrus plants. N_1, N_2, N_3, N_4, N_5, N_6, and N_7 stand for 0, 30, 60, 90, 120, 150, and 180 g(N) year^{-1}, respectively. Fertilizers were applied at germination (G), physiological fruit dropping (P), young fruit expansion (Y), and color change (C).

Treatment	Period	$CO(NH_2)_2$ ($N \geq 46.67\%$) [g per plant]	$CaP_2H_4O_8$ ($P_2O_5 \geq 12\%$) [g per plant]	K_2SO_4 ($K_2O \geq 50.0\%$) [g per plant]
N_1	G	0	18	24
	P	0	6	36
	Y	0	24	48
	C	0	12	12
N_2	G	12	18	24
	P	3	6	36
	Y	12	24	48
	C	3	12	12
N_3	G	24	18	24
	P	6	6	36
	Y	24	24	48
	C	6	12	12
N_4	G	36	18	24
	P	9	6	36
	Y	36	24	48
	C	9	12	12
N_5	G	48	18	24
	P	12	6	36
	Y	48	24	48
	C	12	12	12
N_6	G	60	18	24
	P	15	6	36
	Y	60	24	48
	C	15	12	12
N_7	G	72	18	24
	P	18	6	36
	Y	72	24	48
	C	18	12	12

and 600 μmol m^{-2} s^{-1}). The curves intersected where P_N was the same for all PPFD tested. Therefore, P_N represented R_D and C_i represented C_i^*.

Rubisco activity and content, Chl and leaf N content: Leaf samples were ground to a fine powder in liquid nitrogen and Rubisco activity and content were measured with a *Plant RuBisCO activity ELISA Kit* and a *Plant RuBisCO content ELISA Kit*, respectively (*Shanghai BOYE Biology Science & Technology Co. Ltd.*, Shanghai, China), according to the instructions of the manufacturer.

Acetone and anhydrous alcohol were mixed in a 1:1 volumetric ratio. Leaves were cut into 1-mm pieces and combined. Then, 0.5-g samples were placed in test tubes containing 25 mL of extraction mixture, sealed, and extracted in darkness for 24 h until the leaves were totally bleached. A UV spectrophotometer (*UV-1600*, *Shimadzu Corp.*, Kyoto, Japan) was used to measure the absorbances of Chl *a*, Chl *b*, and carotenoids at 665, 649, and 470 nm, respectively. Pigment concentrations were calculated with the Arnon formula (Esteban *et al.* 2017).

Total N was determined by the Kjeldahl method. Whole labeled leaves were oven-dried for 30 min at 105°C and then to constant mass at 70°C. The leaves were digested with H_2SO_4-H_2O_2 at 260–270°C. An automatic Kjeldahl nitrogen determination apparatus (*ATN-300*, *Shanghai Drawell Scientific Instrument Co. Ltd.*, Shanghai, China) was used to determine total N.

Stomatal morphology: Thin layers of nail polish were brushed onto the leaf blade surfaces. When the nail polish was dry, transparent adhesive tape was used to remove it from the leaf blade. It was then inspected under an *Olympus BX51* microscope (*Guangzhou Ming-Mei Technology Co. Ltd.*, Guangdong, China). Stomatal densities were determined at 400× magnification. Stomatal size and opening were observed at 1000× magnification. Stomatal size was determined with a microscope stage micrometer (Jiang *et al.* 2011).

Models: The actual photochemical efficiency of PSII (Φ_{PSII}) was calculated as follows:

$$\Phi_{PSII} = (F_m' - F_s)/F_m \tag{1}$$

The electron transport rate (J) was calculated as follows:

$$J = \Phi_{PSII} \times PPFD \times \alpha \times \beta \tag{2}$$

where α is the leaf absorbance and β is the partitioning of the absorbed quanta between PSI and PSII. In this study, α and β were assumed to be 0.85 and 0.5, respectively (Albertsson 2001).

V_{cmax} was $8 \times 2.5 \times 10^6/550000$ (= 36.36) times the Rubisco content (g m^{-2}). J_{cmax} was determined from J_{max} as follows (Farquhar *et al.* 1980):

$$J_{cmax} = J_{max}/(4 + 4\Phi) \tag{3}$$

where Φ is the ratio of the oxygenation rate (V_o) to the carboxylation rate (V_c). Φ was determined according to Sharkey (1988):

$$\Phi = V_o/V_c = 2\Gamma^*/C_c \tag{4}$$

C_c and g_m were determined according to the variable J method (Harley *et al.* 1992) as follows:

$$C_c = \frac{\Gamma^*(J + 8(P_N + R_D))}{J - 4(P_N + R_D)} \tag{5}$$

$$g_m = P_N/(C_i - C_c) \tag{6}$$

Γ^* (CO_2-compensation point in the absence of respiration) was calculated as follows (Harley *et al.* 1992):

$$\Gamma^* = C_i^* + R_D/g_m \tag{7}$$

P_N was calculated as follows (Farquhar *et al.* 1980):

$$P_N = \frac{V_{cmax} C_c}{C_c + K_c(1 + O/K_o)}\left(1 - \frac{\Gamma^*}{C_c}\right) - R_D \tag{8}$$

where K_c and K_o are the Michaelis-Menten constants for CO_2 and O_2, and O is atmospheric O_2 concentration.

Statistical analysis: Simple linear regressions were run in *Microsoft Excel 2010*. One-way analysis of variance (*ANOVA*) was run in *SPSS v. 23.0* (*IBM Corp.*, Armonk, NY, USA) to assess the significance of the differences between treatments for each parameter. Means were compared by the least significant difference (*LSD*) multiple comparison test ($P<0.05$).

Results

Effects of N on 'Huangguogan' leaf biochemistry parameters: Relative to the control (N_1), leaf N progressively increased in N_2, N_3, N_4, and N_5, but declined in N_6 and N_7 (Fig. 1*B*). Similarly, individual leaf area progressively increased in N_2, N_3, and N_4, but decreased in N_5, N_6, and N_7 (Fig. 1*A*). Concomitantly, the Rubisco content and activity progressively increased in N_2, N_3, N_4, and N_5 but were reduced in the N_6 and N_7 (Fig. 1*G*,*H*). Similarly, the carotenoid content and Chl *a*, Chl *b*, and total Chl were enhanced in N_2, N_3, N_4, and N_5, but decreased in N_5, N_6, and N_7 (Fig. 1*C–F*). In all cases, differences between treatments were significant. Overall performance of the measured indices was best in plants under N_5, compared to controls (N_1). The Chl content was 15.6% higher in N_5 than that in N_1. The leaf N content was 177% higher in N_5 than that in N_1, while Rubisco content in N_5 was only 37.4% higher than that in N_1.

Effects of N on leaf gas-exchange parameters: Carboxylation efficiency (CE) increased with increasing N supply until saturation, but then decreased at higher (excessive) N fertilization doses. Differences between N treatments in terms of CE and α were significant (Fig. 2*A–C*). Intercellular CO_2 dose-response curves showed that the maximum light-saturated CO_2 assimilation rate (P_{Nmax}), increased with N supply up to N_5, where it peaked. P_{Nmax} were ~ 22.61, 21.23, 26.54, 26.55, 28.29, 23.58, and 19.70 μmol(CO_2) m^{-2} s^{-1} for N_1, N_2, N_3, N_4, N_5, N_6, and N_7, respectively (Fig. 3*A*). The initial slope of the P_N/C_i response curve, $CE_{PN/Ci}$, initially increased but then decreased with increasing N supply. According to the estimated chloroplast CO_2 dose-response curves, there were significant differences between N concentrations in terms of the initial slope of the P_N/C_c response curve, $CE_{PN/Cc}$ (Fig. 2*A*,*B*). Similarly, there were significant differences between the N treatments in terms of V_{cmax}, J_{max}, J_{cmax}, and TPU, all of which increased with increasing N up to peak values under N_5. Thereafter, they all decreased with further increasing N supply (Fig. 2*D–G*).

Effects of leaf N content on R_D, Γ*, C_c, and g_m: The value of P_N was significantly higher for N_5 than for any other treatment. R_D, g_s, and g_m also increased with N supply and reached maxima under N_5 (Fig. 3*A–D*). These indices all showed similar variation patterns in response to increasing N as those described above for other variables. In contrast, C_i, C_c, C_i^*, and Γ^* all tended to decrease with increasing N supply up to N_5 (Fig. 3*E–H*).

Responses of gas exchange to rapid changes in CO_2 concentration: Fast intercellular CO_2-response (P_N/C_i) curves were analyzed for the seven N treatments under evaluation here (Fig. 4*C*). $CE_{PN/Ci}$ increased with N, reached a maximum at N_5, and decreased thereafter (Fig. 2*A*). The values for P_N were 8.8, 13.61, and 6.54 μmol(CO_2) m^{-2} s^{-1} for N_1, N_5, and N_7, respectively (Fig. 1*I*). Both, g_s and g_m decreased with increasing C_i under N supplementation (Fig. 4*A*,*B*).

Effects of different N concentrations on photosynthetic light-response curves: In all N treatments, P_N rapidly increased to a maximum rate with increasing PPFD. However, as PPFD continued to increase, P_N was stabilized under N_2, N_3, N_4, and N_5, but then rapidly decreased under N_1, N_6, and N_7. Moreover, the PPFD levels inducing maximum P_N significantly differed between the N treatments (Fig. 5).

Effects of N on stomatal size and density in leaves of

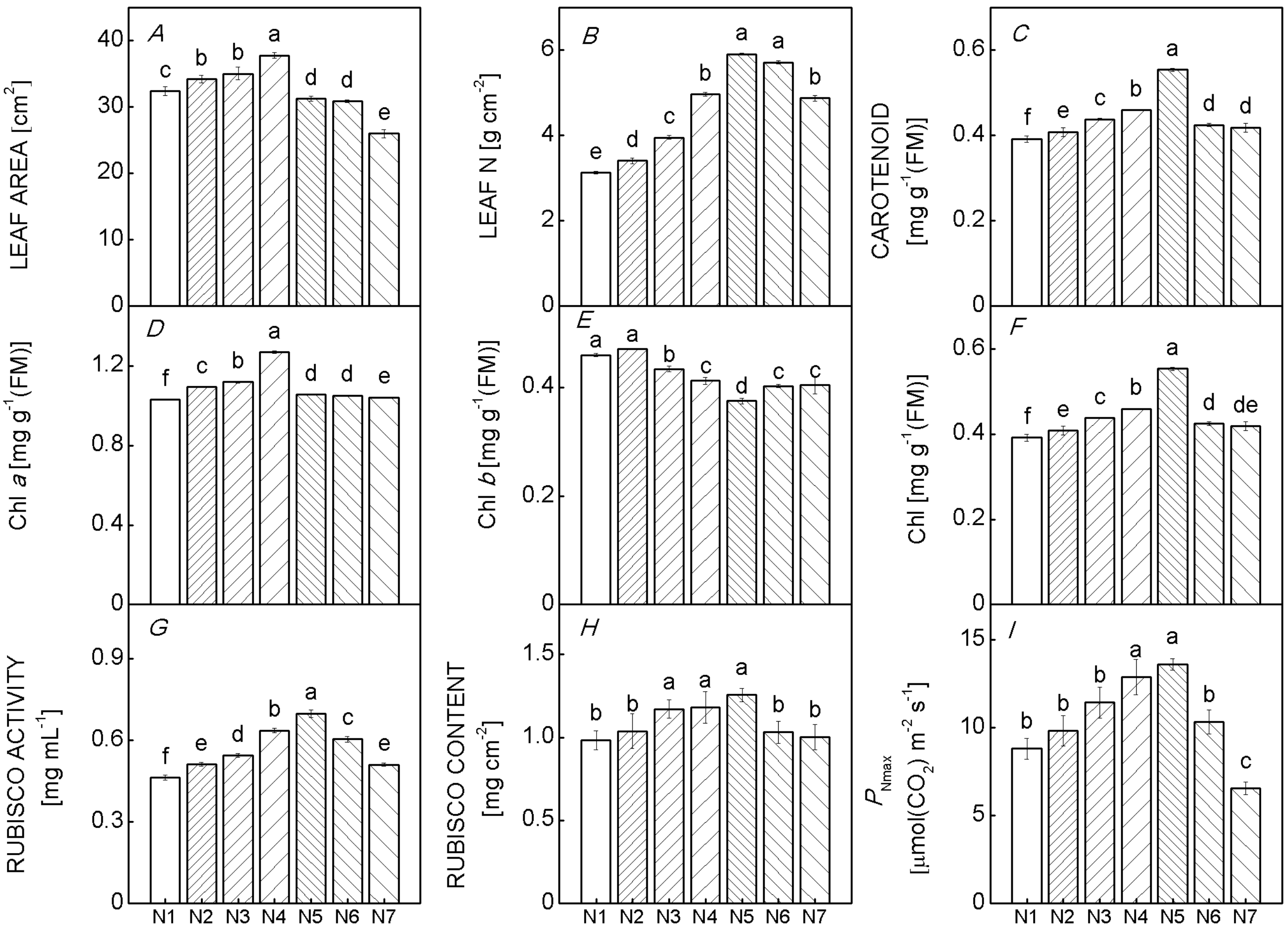

Fig. 1. Effects of different N levels on individual leaf area (*A*), leaf N content (*B*), carotenoid content (*C*), leaf Chl *a* (*D*), Chl *b* (*E*), and total Chl content (*F*), Rubisco activity (*G*), Rubisco content (*H*), and maximum light-saturated CO_2 assimilation rate (P_{Nmax}) (*I*) of ‘Huangguogan’ citrus plants. Data are means ± SD of five replications. Significant differences ($P<5\%$) between treatments are indicated by *different letters*. N_1, N_2, N_3, N_4, N_5, N_6, and N_7 stand for 0, 30, 60, 90, 120, 150, and 180 g(N) year^{-1}, respectively.

cultivar ‘Huangguogan’: Stomatal density significantly decreased, while stomatal length slightly increased and stomatal width slightly decreased with increasing N supply (Table 2; Fig. 1S, *supplement*).

Discussion

Photosynthetic rate in leaves of hybrid citrus cultivar ‘Huangguogan’ is related to N supplement and leaf N content: Positive correlations between leaf N or N fertilization rate and Chl content have been documented for numerous plant species (Chang and Robison 2003, Mauromicale *et al.* 2006, Nageswara Rao *et al.* 2010). In this experiment, we observed that plant growth increased with increasing N fertilization. The beneficial effects of N supply increase include the increased leaf N content, leaf area, and Chl content. Nevertheless, these three parameters tended to decrease, relative to controls, at the higher N concentrations tested (150 and 180 g). Leaf Chl content was positively correlated with the leaf N content. There is a clear linear relationship between leaf N content and light-saturated photosynthetic rate (Yamori *et al.* 2011, Barbet-Massin *et al.* 2015, Xiong *et al.* 2015). Photosynthetic pigment contents are important indicators of senescence (Brown *et al.* 1991). Here, we found that P_N progressively increased in plants under N_2, N_3, N_4, and N_5, but decreased in plants under N_6 and N_7, compared with the control plants (N_1). The relative reduction in P_N at high N (150 and 180 g) might be correlated with the parallel decreases in the leaf N content and photosynthetic pigment biosynthesis.

In C_3 plants, P_N is limited by Rubisco carboxylation capacity and/or C_c (von Caemmerer and Evans 2010). Here, we recorded that Rubisco content and activity changed in parallel with P_N at increasing N application rates. It was proposed that Rubisco activity in leaves with a high N content was greater than that in leaves with low N content (Li *et al.* 2012). Trends in the changes of C_i and C_c were consistent with these findings; indeed, C_i is the product of the cooperation of C_a, g_s, g_m, and P_N. Increases in C_a, g_s, and g_m and decreases in P_N may increase C_i. On the other hand, C_a remained stable in the present study. Therefore, C_i was determined from g_s and P_N. Only when g_m is small enough, it significantly affects C_i. We found

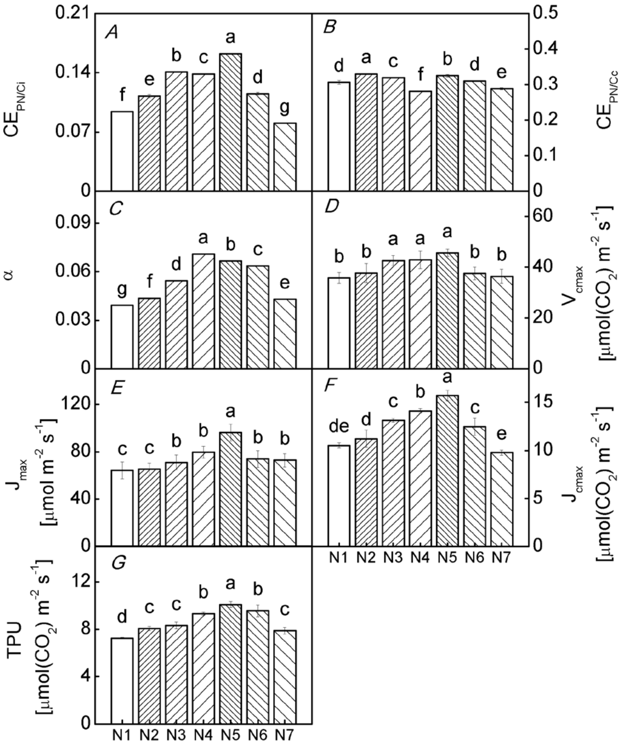

Fig. 2. Effects of different N levels on carboxylation efficiency calculated from P_N/C_i curves (CE$_{PN/Ci}$) (*A*) and P_N/C_c curves (CE$_{PN/Cc}$) (*B*), apparent quantum yield (α) (*C*), maximum carboxylation rate limited by Rubisco (V$_{cmax}$) (*D*), electron transport rate (J$_{max}$) (*E*), RuBP regeneration (J$_{cmax}$) (*F*), and triose phos-phate use (TPU) (*G*) of 'Huangguogan' citrus plants. Data are means ± SD of three replicates. Significant differences ($P<5\%$) between treatments are indicated by *different letters*. N_1, N_2, N_3, N_4, N_5, N_6, and N_7 stand for 0, 30, 60, 90, 120, 150, and 180 g(N) year^{-1}, respectively.

that with increasing nitrogen application, P_N and g_s initially increased and then decreased, whereas the response of C_i was the reverse one. Thus, relative to N_1, the decrease in C_i caused by an increase in P_N was more significant than the increase in C_i caused by the increase in g_s. Consequently, decreases in C_i were noted for N_2, N_3, and N_4. In contrast, the decrease in C_i caused by a decrease in g_s was more significant than the increase in C_i caused by a decrease in P_N. Therefore, C_i increased under N_5, N_6, and N_7.

Stomatal conductance affects photosynthesis by changing C_i (Arp 1991). We found that the N application rate increased g_s up to a threshold after which it first leveled and then decreased. The stomatal size decreased with increasing stomatal density (Franks and Beerling 2009). On the other hand, g_s is correlated with the stomatal size, density, and extent of aperture. In the present study, stomatal density decreased significantly with increasing N, while stomatal volume changed only slightly. Therefore, changes in g_s may be due to alterations in stomatal morphology caused by nitrogen content. Thus, the decline in photosynthesis caused by excessive N supply may, to a certain extent, be the consequence of reduced g_s.

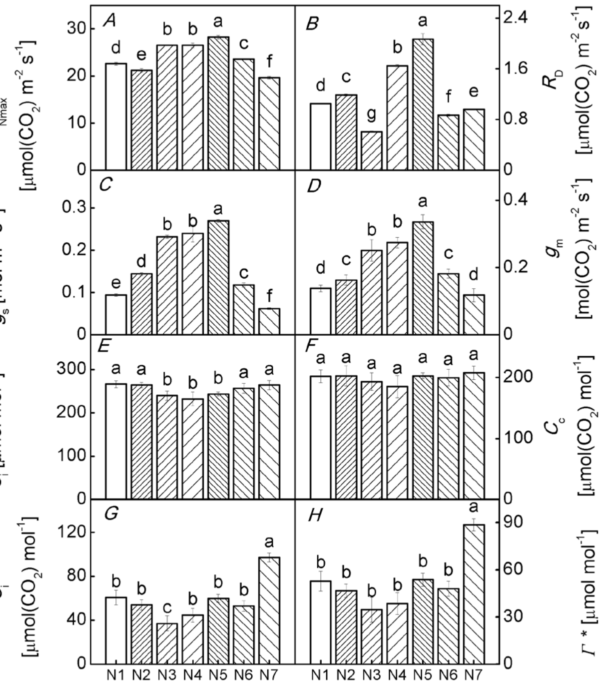

Fig. 3. Effects of different N levels on light-saturated CO_2 assimilation (P_{Nmax}) (*A*), day respiration rate (R_D) (*B*), stomatal conductance (g_s) (*C*), mesophyll conductance (g_m) (*D*), intercellular CO_2 concentration (C_i) (*E*), chloroplastic CO_2 concentration (C_c) (*F*), CO_2-compensation point related to C_i (C_i^*) (*G*), and CO_2 compensation point in the absence of respiration (Γ*) (*H*). Data are means ± SD of five replicates for C_c and g_m, and of three replicates for P_{Nmax}, R_D, g_s, C_i, C_i*, and Γ*. Significant differences ($P<5\%$) between treatments are indicated by *different letters*. N_1, N_2, N_3, N_4, N_5, N_6, and N_7 stand for 0, 30, 60, 90, 120, 150, and 180 g(N) year^{-1}, respectively.

N supplement improved photosynthesis by enhancing carboxylation and CO_2 diffusion: Rubisco content was reported excessively high under relatively large N supply (Adams 2004). It is also well known that photosynthetic capacity may decline in response to a reduction in Rubisco activity (Wong 1979). Here, we observed significant differences between N treatments in terms of CE$_{PN/Ci}$ and α; further, Rubisco activity was positively correlated with CE$_{PN/Ci}$. Indeed, there were significant differences between N application rates in terms of CE$_{PN/Ci}$ and CE$_{PN/Cc}$ (Fig. 2); in general, as N increased, CE$_{PN/Ci}$ and CE$_{PN/Cc}$ tended to increase first and decrease thereafter. Concomitantly, increase in total Rubisco activity was lower than that in leaf N, with increasing N supply (Cheng and Fuchigami 2000). We found that Rubisco activity and leaf N rapidly decreased, while Rubisco content decreased much more gradually as N supply increased to the highest doses tested (150 and 180 g).

Gaseous CO_2 diffuses through the intercellular space from the inferior stomatal cavity to the mesophyll cell wall surfaces (Flexas *et al.* 2008). Thus, CO_2 concentration at the carboxylation site is apparently lower than it is in the intercellular spaces ($C_c < C_i$) (Evans and von Caemmerer

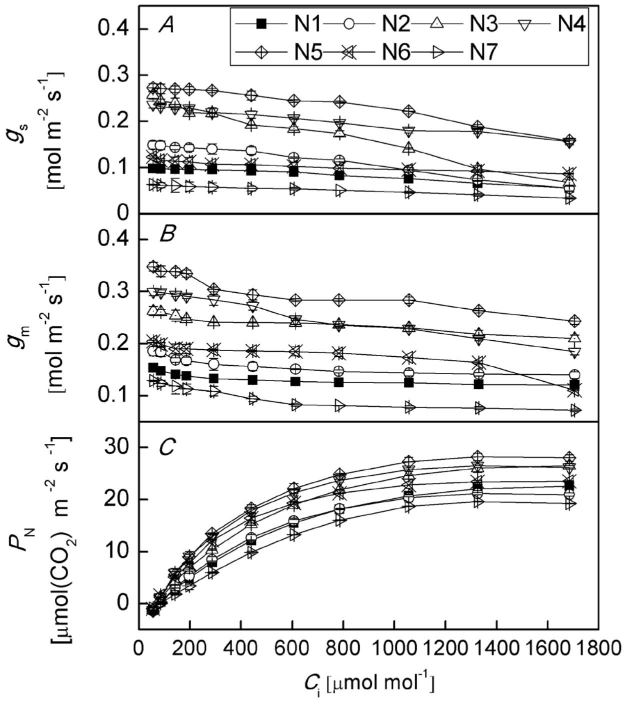

Fig. 4. Effects of N supplement on stomatal conductance (g_s) (*A*), mesophyll conductance (g_m) (*B*), and light-saturated CO$_2$ assimilation (P_N) (*C*) response to C_i. Values are means ± SE of five replicates. N_1, N_2, N_3, N_4, N_5, N_6, and N_7 stand for 0, 30, 60, 90, 120, 150, and 180 g(N) year^{-1}, respectively.

1996). It was reported that g_m varied with C_i and light level in a manner resembling the responses of stomatal conductance to CO$_2$ and light level changes (Flexas *et al.* 2007). CO$_2$ assimilation in woody plants may be limited by g_m (Harley *et al.* 1992, Lawlor 2002). The rapid response of g_m to changes in ambient CO$_2$ concentration and PPFD have been studied (Flexas *et al.* 2012). Studies have shown that g_m tends to decrease when plants grow in environments with poor N supply (Warren *et al.* 2007). There was a positive correlation between leaf N content and g_m (von Caemmerer and Evans 1991). Our results suggest that with increasing N application rate, the change trend of g_m was similar to that of leaf N content; therefore, N may play a role in the rapid response of g_m to CO$_2$ concentration. Photosynthetic capacity reflects electron transport and phosphorylation activity in plant leaves (Ye 2010). With increasing N application rate, P_N changed in the same way as g_m. Therefore, increasing N application increased the photosynthetic capacity in plants of cultivar 'Huangguogan,' but excessive N supply actually limited it. Other important indicators of photosynthetic rate are V$_{cmax}$, J$_{max}$, and TPU. These parameters help accelerate photosynthate accumulation and, by extension, influence both vegetative and reproductive growth (Wang *et al.* 2014). The Chl content changed with N concentration which, in turn, significantly altered the maximum electron transport rate (J$_{max}$) (Fig. 2). Estimated V$_{cmax}$ were 35.74, 45.67, and 36.42 µmol(CO$_2$) m^{-2} s^{-1} under N_1, N_5, and N_7, respectively. When Rubisco was fully activated, optimal Rubisco contents (Rubisco$_{opt}$) (Farquhar *et al.* 1980, Li *et al.* 2009) were 0.24, 0.37, and 0.18 g m^{-2} for N_1, N_5, and N_7, respectively. These Rubisco contents corresponded to the requirement of P_N at atmospheric CO$_2$ concentration. Rubisco$_{opt}$ accounted only for 25, 30, and 18% of the total Rubisco content under N_1, N_5, and N_7, respectively (Fig. 1). Therefore, over 50% of Rubisco was present in its inactive form. Thus, relatively more of the Rubisco

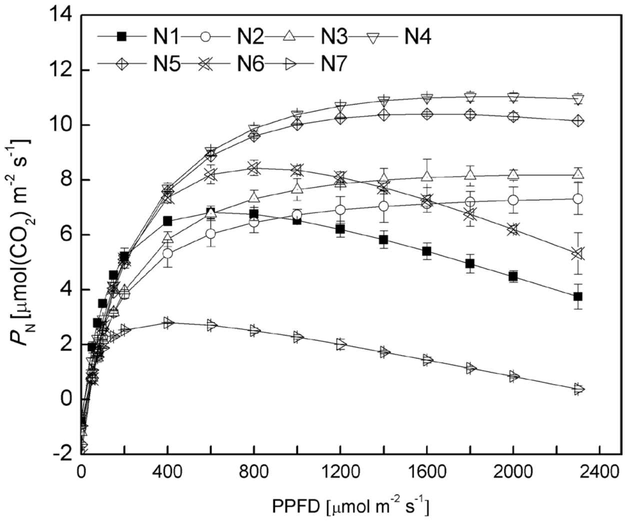

Fig. 5. Effects of N supplement on photosynthesis (P_N) response to PPFD. Values are means ± SE of three replicates. N_1, N_2, N_3, N_4, N_5, N_6, and N_7 stand for 0, 30, 60, 90, 120, 150, and 180 g(N) year^{-1}, respectively.

Table 2. Effects of different N levels on stomatal density, stomatal major axis, and stomatal minor axis of 'Huangguogan' citrus plants. Data are means ± SD of ten replicates. Significant differences (P<5%) among treatments are indicated by *different letters*. N_1, N_2, N_3, N_4, N_5, N_6, and N_7 stand for 0, 30, 60, 90, 120, 150, and 180 g(N) year^{-1}, respectively.

Treatment	Stomatal density [No. mm^{-2}]	Stomatal major axis [µm]	Stomatal minor axis [µm]
N_1	937.00 ± 14.00^{a}	20.99 ± 0.42^{b}	15.87 ± 3.23^{a}
N_2	864.33 ± 11.68^{b}	23.95 ± 1.43^{ab}	16.42 ± 2.23^{a}
N_3	718.00 ± 6.56^{c}	23.99 ± 5.09^{a}	15.89 ± 3.17^{a}
N_4	567.33 ± 10.69^{d}	26.97 ± 2.49^{a}	16.08 ± 7.00^{a}
N_5	533.00 ± 8.72^{e}	28.27 ± 1.05^{a}	21.45 ± 3.77^{a}
N_6	506.33 ± 8.33^{f}	24.87 ± 2.57^{a}	16.05 ± 2.33^{a}
N_7	488.67 ± 3.51^{g}	26.76 ± 0.54^{a}	14.37 ± 2.88^{ab}

enzyme present was inactivated under high N application rates**.** This transformation may influence N storage (Manter and Kerrigan 2004, Li *et al.* 2009). According to the FvCB model, when C_i is low, the photosynthetic rate is determined by C_i, and the photosynthetic rate is positively correlated with V_{cmax}. This association reflects the control of Rubisco over photosynthetic rate. As C_i increases, the photosynthetic rate increase begins to slow down and is gradually limited by RuBP regeneration. When photosynthetic rate no longer increases with increasing CO_2, photosynthetic limitation has entered the TPU-restriction stage (Farquhar *et al.* 1980, Sun *et al.* 2014, Niinemets *et al.* 2015). In this study, J_{cmax} and V_{cmax} were higher than P_N at atmospheric CO_2 concentration. Therefore, C_c did not saturate the carboxylation reaction. Elevated P_N in the leaves of cultivar 'Huangguogan' under high N supply was associated with high mesophyll conductance and chloroplast CO_2 concentration. However, these results also indicate that excessive N supply lowered Rubisco activity and content, CE, g_m, and C_c, all of which together eventually led to a decrease in P_N.

Conclusions: The aim of this study was to elucidate the effects of N supply on photosynthesis. We confirmed that photosynthetic rate is positively correlated with leaf N and Rubisco content. The photosynthetic rate of leaves of cultivar 'Huangguogan' increased with increasing N up to an optimal supply level of 120 g per year, but decreased thereafter with increasing N. Our results suggest that limitation of the photosynthetic capacity of cultivar 'Huangguogan' was correlated with the decrease of g_m, C_c, and Rubisco content and activity caused by excess N supply. The ultimate purpose and advantage of assessing the comparative effects of various N contents on photosynthetic performance are related to optimizing the yield and quality of cultivar 'Huangguogan.' Our study can thus contribute to a better understanding of the photosynthetic capacity response to nitrogen supply and can provide a reference for the rational application of nitrogen fertilizer in orchards of the hybrid citrus cultivar 'Huangguogan.'

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