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 (Pn) 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 Pn/Ci response curve, indicating that N supplement improved photosynthesis by enhancing carboxylation and CO2 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 CO2/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 (Pn) should increase with leaf N (Makino *et al. 1997*), but Rubisco activity is limited by CO2 concentration at the chloroplast carboxylation sites (Ci) (Adachi *et al. 2013*), and a reduction in Rubisco lowers the Pn (Marcus *et al. 2008*). Although early studies suggested that Ci is limited mainly by stomatal conductance (gs), it was recently reported that mesophyll conductance (gmm) has as strong effect on Ci as gs (Evans *et al. 2009*, Xiong *et al. 2015*). Additionally, effects of N on gmm have been demonstrated (Buckley and Warren 2014, Xiong *et al. 2015*, Barbour and Kaiser 2016). Nutritional stress influences gmm as well as gs; consequently, it also affects Pn (Yamori *et al. 2011*). The differential

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*Received 24 June 2018, accepted 21 November 2018.*

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*Abbreviations: Cc – atmospheric CO2 concentration; Cci – ambient CO2 concentration in the cuvette; Ci – CO2 concentration at the carboxylation sites inside the chloroplast; CE – carboxylation efficiency; CEi – initial slope of the Ps/Ci response curve; CEmax – initial slope of the Ps/Ci response curve; Chl – chlorophyll; Cc – intercellular CO2 concentration; Ci – apparent CO2 light-compensation point; Fm’ – steady-state fluorescence; Fm’ – maximum fluorescence; gmm – mesophyll conductance; gs – stomatal conductance; J – rate of electron transport; Jmax – RuBP regeneration supported by electron transport; Pn – photosynthetic rate; Rb – day respiration; RuBP – ribulose-1,5-bisphosphate; TPU – triose phosphate use; Vc – carboxylation rate; Vcmax – maximum carboxylation rate limited by Rubisco; Vo – oxygenation rate; a – apparent quantum yield; Φ – ratio of Vc to Vc; ΦPSII – actual photosynthetic efficiency of PSII; I* – CO2-compensation point in the absence of respiration.

**Acknowledgements:** This work was supported by the Foundation of the Science and Technology Department of Sichuan Province, China (Grant No. 2011NZ0034), the Foundation of Education Department of Sichuan Province, China (Grant No. 2013SZX0054), and the Social Practice and Technology Service Foundation for the graduate students of Sichuan Agricultural University (Grant No. ACT201304).

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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 $P_{\text{N}}$, 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% silty, 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 ($N_i$-$N_{-i}$) and five replicates, each consisting of one tree, for a total of 35 plants which were all fertilized with 60 g of phosphorus [CaP$_2$H$_5$O$_7$; P$_{2}O_5$ ≥ 12%] and 120 g of potassium [K$_2$SO$_4$; K$_{2}O ≥ 50.0$%]. Nitrogen [CO(NH$_2$)$_2$; N ≥ 46.67%] was applied at 0 (N$_0$), 30 (N$_{30}$), 60 (N$_{60}$), 90 (N$_{90}$), 120 (N$_{120}$), 150 (N$_{150}$), and 180 (N$_{180}$) 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$_2$H$_5$O$_7$ application model, and 20:30:40:10% for the K$_2$SO$_4$ 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 ($C_{\text{ave}}$) 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 $C_{\text{ave}}$ 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 ($\varphi$) 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 $P_{\text{N}}/C_i$ 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 ($F_{\text{m}}$) were recorded after reaching the steady state. $F_{\text{m}}$ 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 $F_{\text{m}}$ at 1,600 μmol(photon) m$^{-2}$ s$^{-1}$ and 400 μmol(CO$_2$) mol$^{-1}$ were used to calculate ($F_{\text{m}} - F_s$)/$F_{\text{m}}$. Carboxylation efficiency (CE) for each N treatment was calculated as the initial slope of the $Ps/C_i$ 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 $P_s$ and $C_i$ as previously described (Flexas and Ribas-Carbó 2007, Li et al. 2009).

**Diurnal respiration ($R_o$) and apparent CO$_2$ light-compensation point ($C_i^\circ$):** $R_o$ and $C_i^\circ$ were determined between 00:00 and 04:00 h by the Laish method (Laish 1977), using the same leaves used for measurements of $Ps/C_i$ and light-response curves (Guo et al. 2007). Briefly, $Ps/C_i$ 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,
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$_2$SO$_4$-H$_2$O$_2$ at 260–270°C. An automatic Kjeldahl nitrogen determination apparatus (ATN-300, Shanghai Dra-well 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).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Period</th>
<th>CO(NH$_2$)$_2$ (N ≥ 46.67%) [g per plant]</th>
<th>CaP$_2$H$_4$O$_8$ (P$_2$O$_5$ ≥ 12%) [g per plant]</th>
<th>K$_2$SO$_4$ (K$_2$O ≥ 50.0%) [g per plant]</th>
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Models: The actual photochemical efficiency of PSII (ΦPSII) was calculated as follows:

\[ \Phi_{PSII} = \frac{F_m - F_i}{F_m} \]  

(1)

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

\[ J = \Phi_{PSII} \times PPFD \times \alpha \times \beta \]  

(2)

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

\[ V_{max} = 8 \times 2.5 \times 10^{6} \times 550000 (= 36.36) \] 

times the Rubisco content (g m\(^{-2}\)). \( J_{max} \) was determined from \( J_{max} \) as follows (Farquhar et al. 1980):

\[ J_{max} = J_{max}/(4 + 4\Phi) \]  

(3)

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

\[ \Phi = V_o/V_c = 2\Gamma/C_c \]  

(4)

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

\[ C_c = \frac{J}{J - 4(P_N + R_D)} \]  

(5)

\[ g_m = \frac{P_N}{(C_t - C_c)} \]  

(6)

\( \Gamma^* \) (CO\(_2\)-compensation point in the absence of respiration) was calculated as follows (Harley et al. 1992):

\[ \Gamma^* = C_t^* + R_D/g_m \]  

(7)

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

\[ P_N = \frac{V_{max}C_c}{C_c + K_c(1 + O/K_o)} \left( 1 - \frac{J^*}{C_c} \right) - R_D \]  

(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 ‘Huangguoguan’ leaf biochemistry parameters: Relative to the control (N\(_i\)), leaf N progressively increased in N\(_2\), N\(_3\), N\(_4\), and N\(_5\), but declined in N\(_6\) and N\(_7\) (Fig. 1B). 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. 1A). 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. 1G,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\(_6\), N\(_7\), and N\(_7\) (Fig. 1C–F). In all cases, differences between treatments were significant. Overall performance of the measured indices was best in plants under N\(_i\), compared to controls (N\(_i\)). The Chl content was 15.6% higher in N\(_5\) than that in N\(_i\). The leaf N content was 177% higher in N\(_7\) than that in N\(_i\), while Rubisco content in N\(_5\) was only 37.4% higher than that in N\(_i\).

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 \( \alpha \) were significant (Fig. 2A–C). Inter-cellular CO\(_2\) dose-response curves showed that the maximum light-saturated CO\(_2\) assimilation rate (\( P_{max} \)), increased with N supply up to N\(_i\), where it peaked. \( P_{max} \) were 22.61, 21.23, 26.54, 26.55, 28.29, 23.58, and 19.70 \( \mu \)mol(CO\(_2\)) m\(^{-2}\) s\(^{-1}\) for N\(_i\), N\(_2\), N\(_3\), N\(_4\), N\(_5\), N\(_6\), and N\(_7\), respectively (Fig. 3A). The initial slope of the \( P_{C}/C \) response curve, CE\(_{PSII} \), 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 \( P_{D}/C \) response curve, CE\(_{PSII} \) (Fig. 2A,B). Similarly, there were significant differences between the N treatments in terms of \( V_{max} \), \( J_{max} \), and TPU, all of which increased with increasing N up to peak values under N\(_i\). Thereafter, they all decreased with further increasing N supply (Fig. 2D–G).

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

Responses of gas exchange to rapid changes in CO\(_2\) concentration: Fast intercellular CO\(_2\) response (\( P_{D}/C \)) curves were analyzed for the seven N treatments under evaluation here (Fig. 4C). CE\(_{PSII} \) increased with N, reached a maximum at N\(_i\), and decreased thereafter (Fig. 2A). The values for \( P_{N} \) were 8.8, 13.61, and 6.54 \( \mu \)mol(CO\(_2\)) m\(^{-2}\) s\(^{-1}\) for N\(_i\), N\(_2\), N\(_3\), and N\(_7\), respectively (Fig. 1F). Both, \( g_m \) and \( g_m \) decreased with increasing \( C \) under N supplementation (Fig. 4A,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\(_6\), N\(_7\), 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
Photosynthetic capacity of ‘Huangguogan’ is decreased under excess nitrogen supply

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 PN progressively increased in plants under N2, N3, N4, and N5, but decreased in plants under N6 and N7, compared with the control plants (N1). The relative reduction in PN at high N (150 and 180 g) might be correlated with the parallel decreases in the leaf N content and photosynthetic pigment biosynthesis.

In C3 plants, PN is limited by Rubisco carboxylation capacity and/or Ci (von Caemmerer and Evans 2010). Here, we recorded that Rubisco content and activity changed in parallel with PN 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 Ci and C3 were consistent with these findings; indeed, C3 is the product of the cooperation of C3, gs, gmax, and PN. Increases in C3, gs, and gmax and decreases in PN may increase C3. On the other hand, C3 remained stable in the present study. Therefore, C3 was determined from gs and PN. Only when gmax is small enough, it significantly affects C3. We found

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 CO2 assimilation rate (PNmax) (I) of ‘Huangguogan’ citrus plants. Data are means ± SD of five replications. Significant differences (P<5%) between treatments are indicated by different letters. N1, N2, N3, N4, N5, N6, and N7 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 $C_1$ was the reverse one. Thus, relative to $N_1$, the decrease in $C_1$ caused by an increase in $P_N$ was more significant than the increase in $C_1$ caused by the increase in $g_s$. Consequently, decreases in $C_1$ were noted for $N_3$, $N_6$, and $N_9$. In contrast, the decrease in $C_1$ caused by a decrease in $g_s$ was more significant than the increase in $C_1$ caused by a decrease in $P_N$. Therefore, $C_1$ increased under $N_5$, $N_6$, and $N_9$.

Stomatal conductance affects photosynthesis by changing $C_1$ (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 $g_s$.

**N supplement improved photosynthesis by enhancing carboxylation and CO₂ 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 $CE_{PNCC}$ and $a$; further, Rubisco activity was positively correlated with $CE_{PNCC}$. Indeed, there were significant differences between $N$ application rates in terms of $CE_{PNCC}$ and $CE_{PNCG}$ (Fig. 2); in general, as $N$ increased, $CE_{PNCC}$ and $CE_{PNCG}$ 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 ($C_i < C_1$) (Evans and von Caemmerer Fig. 3. Effects of different $N$ levels on light-saturated $CO_2$ assimilation ($P_{nax}$) (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 $C_i$ ($C'_C$) (G), and $CO_2$ compensation point in the absence of respiration ($C'_R$) (H). Data are means ± SD of five replicates for $C_i$ and $g_m$, and of three replicates for $P_{nax}$, $R_D$, $g_s$, $C_i$, $C'_C$, and $C'_R$. Significant differences (P<5%) between treatments are indicated by different letters. $N_1$, $N_2$, $N_3$, $N_4$, $N_5$, $N_6$, $N_7$, and $N_8$ stand for 0, 30, 60, 90, 120, 150, and 180 g(N) year⁻¹, respectively.
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 \( V_{\text{cmax}}, J_{\text{max}}, \) and TPU. These parameters help accelerate photosynthetic 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 (\( J_{\text{max}} \)) (Fig. 2). Estimated \( V_{\text{cmax}} \) were 35.74, 45.67, and 36.42 \( \mu \text{mol} \text{CO}_2 \text{m}^{-2} \text{s}^{-1} \) under \( N_1, N_5, \) and \( N_7 \), respectively. When Rubisco was fully activated, optimal Rubisco contents (Rubisco\(_{\text{opt}}\)) (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\(_{\text{opt}}\) 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

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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stomatal density [No. mm(^{-2})]</th>
<th>Stomatal major axis [µm]</th>
<th>Stomatal minor axis [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>( N_1 )</td>
<td>937.00 ± 14.00*</td>
<td>20.99 ± 0.42*</td>
<td>15.87 ± 3.23*</td>
</tr>
<tr>
<td>( N_2 )</td>
<td>864.33 ± 11.68*</td>
<td>23.95 ± 1.43*</td>
<td>16.42 ± 2.23*</td>
</tr>
<tr>
<td>( N_3 )</td>
<td>718.00 ± 6.56*</td>
<td>23.99 ± 5.09*</td>
<td>15.89 ± 3.17*</td>
</tr>
<tr>
<td>( N_4 )</td>
<td>567.33 ± 10.69*</td>
<td>26.97 ± 2.49*</td>
<td>16.08 ± 7.00*</td>
</tr>
<tr>
<td>( N_5 )</td>
<td>533.00 ± 8.72*</td>
<td>28.27 ± 1.05*</td>
<td>21.45 ± 3.77*</td>
</tr>
<tr>
<td>( N_6 )</td>
<td>506.33 ± 8.33*</td>
<td>24.87 ± 2.57*</td>
<td>16.05 ± 2.33*</td>
</tr>
<tr>
<td>( N_7 )</td>
<td>488.67 ± 3.51*</td>
<td>26.76 ± 0.54*</td>
<td>14.37 ± 2.88*</td>
</tr>
</tbody>
</table>

Fig. 4. Effects of N supplement on stomatal conductance (\( g_m \)) (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.

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.
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 Ci is low, the photosynthetic rate is determined by C, and the photosynthetic rate is positively correlated with \( V_{\text{max}} \). 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, Ninnenets et al. 2015). In this study, \( J_{\text{max}} \) and \( V_{\text{max}} \) were higher than \( P_{\text{n}} \) at atmospheric \( CO_2 \) concentration. Therefore, \( C_i \) did not saturate the carboxylation reaction. Elevated \( P_{\text{n}} \) 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, and \( C_i \), all of which together eventually led to a decrease in \( P_{\text{n}} \).

**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 \( g_{\text{so}} \), \( C_i \), 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.’

**References**


Cheng L., Fuchigami L.H.: Rubisco activation state decreases together eventually led to a decrease in \( P_{\text{n}} \).

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**References**


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2011.


Yamori W., Nagai T., Makino A.: The rate-limiting step for CO\textsubscript{2} assimilation at different temperatures is influenced by the leaf nitrogen content in several C\textsubscript{3} crop species. – Plant Cell Environ. 34: 764-777, 2011.

