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RESEARCH PAPER

Leaf anatomical characteristics are less important than leaf biochemical properties in determining photosynthesis responses to nitrogen top-dressing

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Abstract

The photosynthetic capacity of leaves is dramatically influenced by nitrogen (N) availability in the soil, as CO_2 concentration in chloroplasts and photosynthetic biochemical capacity are related to leaf N content. The relationship between mesophyll conductance (g_m) and leaf N content was expected to be shaped by leaf anatomical traits. However, the increased g_m in mature leaves achieved by N top-dressing is unlikely to be caused by changes in leaf anatomy. Here, we assessed the impacts of N supply on leaf anatomical, biochemical, and photosynthetic features, specifically, the dynamic responses of leaf anatomy, biochemistry, and photosynthesis to N top-dressing in tobacco. Plant performance was substantially affected by soil N status. In comparison with the leaves of plants subjected to low N treatment, leaves of plants with high N treatment photosynthesized significantly more, due to higher CO_2 diffusion conductance and photosynthetic biochemical capacity. The high g_m in high N-treated leaves apparently related to modifications in the leaf anatomy; however, the rapid response of g_m to N top-dressing cannot be fully explained by leaf anatomical modifications.

Keywords: Leaf anatomy, mesophyll conductance, nitrogen, photosynthesis, Rubisco, top dressing.

Introduction

The efficient use of fertilizer nitrogen (N) is important in determining plant distribution, survival, growth, and production. Recent decades have brought many advances in our understanding of nitrogen use efficiency (NUE), especially in crops (Li *et al.*, 2017; Hawkesford and Griffiths, 2019; Swarbreck *et al.*, 2019). In practice, NUE is defined in multiple ways; in plant ecophysiology, it is generally defined as the net photosynthetic rate achieved per unit of leaf N content, that is, the photosynthetic N use efficiency (PNUE) (Onoda *et al.*, 2017; Evans and Clarke, 2019). High PNUE at a given

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leaf N content, therefore, is achieved by a high photosynthetic rate. The photosynthesis in higher plants is determined by the stomatal conductance (g_s), mesophyll conductance (g_m), and biochemical capacity to fix carbon (Grassi and Magnani, 2005; Buckley and Diaz-Espejo, 2015), and biochemical capacity is usually represented by the maximum carboxylation rate (V_{cmax}) and maximum electron transport rate (J_{max}) in practice. Previous studies have shown that all of these traits are sensitive to N availability (Yamori *et al.*, 2011; Xiong *et al.*, 2015*b*).

By performing a synthesis analysis, we demonstrated a curvilinear relationship between the light-saturated photosynthetic rate (A) and leaf N content per unit area in rice (Xiong and Flexas, 2018). That is, A increases with leaf N content, but the rate of increase declines. Similar patterns were observed for g_s , g_m , V_{cmax} , and J_{max} . The increases in V_{cmax} and J_{max} are simply because the contents of Rubisco and the components of the thylakoid electron transport chain increase along with leaf N content (see Evans and Clarke, 2019, and references therein). The decrease of the Rubisco activation state corresponds to the decline of the rate of increase of V_{cmax} in high-N-concentration leaves (Cheng and Fuchigami, 2000). Among these traits, g_m is the most sensitive in responding to changes in leaf N (summarized by Xiong and Flexas, 2018, and see references therein).

The g_m , that is, the efficiency of CO₂ diffusion from the substomatal cavity to the chloroplast where CO₂ fixation occurs, has been estimated for hundreds of species (Flexas et al., 2008). Anatomical traits including the mesophyll cell wall thickness (T_{cw}) , mesophyll cell surface facing the intercellular airspace (S_m), and chloroplast surface facing the intercellular airspace (S_c) are strong determinants of interspecific differences in g_m (Evans et al., 2009; Tomás et al., 2013). By inputting several biochemical constants (e.g. membrane permeability and enzyme activities), the estimation of anatomical parameters allowed the establishment of a simplified anatomical model of diffusion (a steady-state model) that gave estimations of g_m very close to those estimated from gas exchange (Tomás et al., 2013; Tosens et al., 2016). Beyond leaf anatomical features, membrane permeability, facilitated by aquaporins and carbonic anhydrase, is also involved in regulating g_m (Flexas *et al.*, 2006; Sade *et al.*, 2014; Momayyezi et al., 2020). Previous studies have shown that leaf anatomical traits are remarkably modified by N availability, and the increased S_c due to the enlargement of chloroplasts under high N caused the increase of g_m (Xiong et al., 2015b). However, these results were based on long-term N treatments in which the anatomical and biochemical features may already be optimized to adapt to the experimental N conditions.

In agricultural systems, multi-split N fertilizer top-dressing is widely adopted as part of nutrient management for crop production; for instance, delaying N application has been suggested (reviewed by Peng *et al.*, 2010). Delaying N application has many advantages, such as developing a healthy canopy structure and reducing N losses by leaching to the environment. At the very late growth stage, leaf anatomical adjustments for maximizing top-dressed N use are unlikely to happen, as no new leaves appear in the canopies of most cereal crops at this stage; in this case, biochemical adjustment might be the major factor in determining the responses of photosynthesis to N availability. As one example, Xiong *et al.* (2015*b*) found that the rapid responses of g_m to environmental factors are likely independent of leaf anatomy, indicating that the response of g_m to N availability may be also regulated by carbonic anhydrase and/or aquaporins. However, to the best of our knowledge, no study has investigated the responses of physiological and structural traits to N top-dressing in mature leaves.

Here, we investigated the dynamic responses of leaf biochemical, structural, and photosynthetic traits to N addition in mature tobacco leaves. We aimed to uncover (i) whether photosynthetic traits of mature leaves can be quickly improved by N top-dressing and (ii) how quickly the anatomical, biochemical, and photosynthetic traits of mature leaves respond to N top-dressing.

Materials and methods

Plant materials and N treatments

Tobacco (Nicotiana tabacum L.) var. Samsun seeds were sown in seed trays filled with horticultural substrate. Two-week-old seedlings were transplanted to 4.0 litre pots containing mixed low-N organic soil and perlite (mean \pm SD N concentration 0.89 \pm 0.05 mg g⁻¹; *n*=4). Plants were kept in a growth chamber with a 12 h light/12 h dark cycle and an air temperature of 25 °C (light)/20 °C (dark); the mean ±SD light intensity (expressed as photosynthetic photon flux density) on the soil surface of pots as measured by a LI-COR LI-190R Quantum Sensor (LI-COR, Lincoln, NE, USA) was $456\pm57 \mu mol m^{-2} s^{-1}$. Plants were watered daily to avoid drought stress. Every week, 20 ml of N-free Hoagland's solution and full-elements Hoagland's solution was applied to low N treatment (LN) and high H treatment (HN) pots, respectively. To minimize the effects of leaf age, the newest fully expanded leaf of each plant was labeled at 44 days after transplanting, and 1.5 g N (as NH₄NO₃ solution) was added to half of the LN pots for the top-dressed high N treatment (THN) early in the morning of day 45 after transplanting. In this study, 30 plants were used for each N treatment. The pots of LN and THN plants were randomly mixed but separated from HN pots to avoid shading effects, as the HN plants were much taller and larger than the LN and THN plants (Table 1). All measurements were performed on the labeled leaves, to investigate the rapid response of leaf functional traits to N addition. For this purpose, day 45 after transplanting was treated as day 0 after the addition of N to the THN pots.

Gas exchange and chlorophyll fluorescence measurements

Three carefully calibrated open-flow gas exchange systems (LI-6400XT, LI-COR, Lincoln, NE, USA) with integrated fluorescence leaf chambers (LI-6400–40, LI-COR) were used to simultaneously measure leaf gas exchange and chlorophyll fluorescence. To minimize the influences of circadian rhythm, the gas exchange measurements were performed between 2 h and 6 h after the light source was switched on each day. For each treatment, CO_2 response curves were measured in three individual plants. It should be noted that the HN plants were not measured on the first and third day after the addition of N to the THN plants, as the light-response curves under low O_2 conditions were measured on those days (see details below). During the measurement,

Table 1. Influences of N supplementation on plant morphological,
biochemical, and photosynthetic traits measured 44 days afterN to the T
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	HN	LN	
Leaf number	12.0±1.2 ª	5.2±0.4 ^b	
Plant height (cm)	106.6±20.4 ^a	24.5±3.7 ^b	
Leaf area (cm ²)	102.4±18.5 ^a	51.7±11.3 ^b	
Biomass (g plant ⁻¹)	28.5±5.5 ^a	9.6±1.4 ^b	
LMA (g m ⁻²)	26.8±4.7 ^b	52.3±3.9 ^a	
Leaf thickness (mm)	1.01±0.09 ^b	1.67±0.20 ^a	
f _{IAS} (%)	38.5±3.2	41.2±1.4	
$T_{\rm cw}$ (nm)	217.2±13.8 ^b	265.4±46 ^a	
S _m (m ² m ⁻²)	17.4±2.3 ^b	21.4±2.9 ^a	
S _c (m ² m ⁻²)	16.3±3.1 ^a	13.3±2.1 ^b	
$g_{\rm m}$ -anatomy (mol m ⁻² s ⁻¹)	0.109±0.010 ^a	0.084±0.012 ^b	
Leaf N (g m ⁻²)	1.70±0.10 ^a	0.37±0.01 ^b	
SPAD	41.3±1.0 ^a	15.5±1.4 ^b	
Rubisco (g m ⁻²)	2.50±0.36 ª	0.55±0.23 ^b	
$A_{\rm sat}$ (µmol m ⁻² s ⁻¹)	24.9±1.6 ª	8.6±0.4 ^b	
$g_{\rm s} ({\rm mol}\;{\rm m}^{-2}\;{\rm s}^{-1})$	0.486±0.014 ª	0.243±0.013 ^b	
$g_{\rm m}$ -Harley (mol m ⁻² s ⁻¹)	0.467±0.139 ^a	0.088±0.015 ^b	
$A_{\rm max}$ (µmol m ⁻² s ⁻¹)	37.9±2.6 ª	15.9±1.6 ^b	
$V_{\rm cmax}$ (µmol m ⁻² s ⁻¹)	92.4±6.7 ^a	29.7±2.1 ^b	
J_{max} (umol m ⁻² s ⁻¹)	173.6±5.7 ª	73.9±4.4 ^b	

transplanting

Data are means ±SE. Different letters indicate statistically significant differences (*P*<0.05) between low N (LN) and high N (HN) treatments. LMA, leaf mass per area; *f*_{IAS}, intercellular airspace fraction; *T*_{cw}, mesophyll cell surface area facing the intercellular airspace; SPAD; a measure of chlorophyll content using SPAD 502 plus chlorophyll meter; *S*_c, chloroplast surface area facing the intercellular airspace; *A*_{sat}, light-saturated photosynthetic rate at ambient CO₂; *g*_s, stomatal conductance; *g*_m, mesophyll conductance; *A*_{max}, maximum photosynthetic rate; *V*_{cmax}, maximum carboxylation rate; *J*_{max}, maximum electron transport rate. *A*_{max}, *V*_{cmax}, and *J*_{max} were derived from CO₂ response curves (for details see the Materials and methods).

the light intensity was set at 1500 μ mol m⁻² s⁻¹ (10%:90% blue:red light), the reference CO₂ concentration was adjusted by a CO₂ mixer, the block temperature was set at 25 °C, the leaf-to-air vapor pressure deficit was kept between 1.5 kPa and 2.0 kPa, and the flow rate was 300 μ mol s⁻¹. After the leaf reached a steady state, auto-progress of the CO₂ response curve was applied. The reference CO₂ concentrations were subsequently set at 400, 300, 200, 100, 50, 400, 600, 800, 1000, 1200, 1500, 2000, and 400 μ mol CO₂ mol⁻¹ air.

According to Genty *et al.* (1989), the actual photochemical efficiency of photosystem II (Φ_{PSII}) was calculated as follows:

$$\Phi_{\rm PSII} = \frac{F'_{\rm m} - F_{\rm s}}{F'_{\rm m}}$$

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

$$J = \Phi_{\rm PSII} \cdot {\rm PPFD} \cdot \alpha\beta$$

where α is the leaf absorptance and β is the partitioning of absorbed quanta between photosystems II and I. The product $\alpha\beta$ was determined, following Valentini *et al.* (1995), from the relationship between $1/4\Phi_{PSII}$ and Φ_{CO2} obtained by varying either light intensity or CO₂ concentration under non-photorespiration conditions (O₂ <1%). To do this, six light-response curves for each treatment were determined under low O₂ conditions on the first and the third day after the addition of N to the THN plants, following the protocol in Xiong *et al.* (2015*b*). No difference in $\alpha\beta$ values (range 0.415–0.459) between N treatments was observed and, hence, the average value of 0.438 was applied to all the treatments. The relationship between Φ_{PSII} and Φ_{CO2} is shown in Supplementary Fig. S1.

The variable *J* method (Harley *et al.*, 1992) was used to calculate the mesophyll conductance to CO_2 (g_m) and the chloroplast CO_2 concentration (C_c). C_c and g_m were calculated as follows:

$$C_{\rm c} = \frac{\Gamma^*(J + 8(A + R_d))}{J - 4(A + R_d)}$$
$$g_{\rm m} = \frac{A}{C_{\rm i} - C_{\rm c}}$$

where C_i is the intercellular CO₂ concentration and Γ^* represents the CO₂ compensation point in the absence of respiration, which is species dependent at a given temperature. In this study, a value of 40 µmol mol⁻¹ for Γ^* was used as reported in Walker *et al.* (2013). It is noteworthy that we assumed Γ^* was constant over the growing period, although Γ^* was reported to vary with leaf age, and with S_c/S_m ratios, because of the reassimilation of photorespired CO₂ (Busch *et al.*, 2013). For each data point generated, we checked whether it met the reliability criterion ($10 > dC_c/dA > 50$) as suggested by Harley *et al.* (1992). Day respiration (R_d), maximum carboxylation rate (V_{cmax}), and maximum electron transport rate (J_{max}) were estimated from $A-C_i$ curves using the *plantecowrap* R package, which provides wrapping functions to add to capabilities to the previous *plantecophys* package (Duursma, 2015). In the present study, the $A-C_c$ curve-based V_{cmax} and J_{max} were also fitted by using the g_m or g_m -anatomy (see below).

Photosynthesis limitation analysis

Limitation analysis is a helpful tool to quantify the stress effects of changes in various factors on photosynthesis (Grassi and Magnani, 2005; Buckley and Diaz-Espejo, 2015), and it has been widely used in recent years (Wang *et al.*, 2018; Xiong *et al.*, 2018). Relative photosynthetic limitations, comprising stomatal (l_s), mesophyll (l_s), and biochemical (l_b) relative limitations, were calculated according to Grassi and Magnani (2005):

$$l_{\rm s} = \frac{g_{\rm t}/g_{\rm s} \cdot \partial A/\partial C_{\rm c}}{g_{\rm t} + \partial A/\partial C_{\rm c}}$$
$$l_{\rm m} = \frac{g_{\rm t}/g_{\rm m} \cdot \partial A/\partial C_{\rm c}}{g_{\rm t} + \partial A/\partial C_{\rm c}}$$
$$l_{\rm b} = \frac{g_{\rm t}}{g_{\rm t} + \partial A/\partial C_{\rm c}}$$

where g_t represents the total CO₂ diffusion conductance of g_s and g_m ($g_t=1/(1/g_s+1/g_m)$), and $\partial A/\partial C_c$ is the slope of the *A* versus C_c response curve. For light-saturated conditions under ambient CO₂ concentrations, when Rubisco limits *A*, the $\partial A/\partial C_c$ can be modified from the Farquhar model (Farquhar *et al.*, 1980):

$$\partial A/\partial C_{\rm c} = V_{\rm cmax} \frac{\Gamma * + K_{\rm m}}{(C_{\rm c} + K_{\rm m})}$$

 $K_{\rm m} = K_{\rm c} \cdot \left(1 + \frac{O}{K_{\rm o}}\right)$

where K_c and K_o are the Michaelis–Menten constants for CO₂ and O₂, respectively, and O is the atmospheric O₂ concentration. Both K_c and K_o were taken from Bernacchi *et al.* (2001).

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Light and transmission electron microscopy

After the daily gas exchange measurement (~6 h after the light source was switched on in the growth chamber), the leaves on which the gas exchange measurements had been performed were sampled for anatomical and biochemical analyses. Small sections of ~4.0 mm×1.2 mm were cut from the top, middle, bottom of each leaf for anatomical analysis, and another part of the same leaf was further sampled using a leaf tissue punch (2 cm^2) for measurement of leaf biochemical traits including leaf N and Rubisco (see below). The leaf sections for anatomical analysis were infiltrated with the fixative 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.6) at 4 °C in a vacuum chamber, and post-fixed in 2% buffered osmium tetroxide at 20 °C for 2 h. The samples were then embedded in Spurr's epoxy resin. For light microscopy, semi-thin leaf cross sections were cut using a fully automated rotary microtome (Leica RM2265, Leica Microsystems, Milton Keynes, UK). The leaf sections were stained with 1% (w/v) toluidine blue in 1% (w/v) Na₂B₄O₇, and they were examined at ×40 and ×100 magnification with an Olympus IX71 light microscope (Olympus Optical, Tokyo, Japan). For transmission electron microscopy, an H-7650 (Hitachi, Tokyo, Japan) was used for observation and photography. Three leaves per treatment were sampled every day. The total cross-sectional area of mesophyll tissues (S_{mes}) and intercellular airspace area (S_{IAS}) , the total length of the mesophyll cell wall exposed to intercellular airspace (l_{mes}) , the total length of chloroplasts touching the plasma membrane appressed to the intercellular airspace (l_{chl}) , and the width of the analyzed leaf cross section (L) were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The volume fraction of intercellular airspace (f_{ias}) was calculated as:

$$f_{\rm IAS} = \frac{S_{\rm IAS}}{S_{\rm m}}$$

 $S_{\rm m}$ and $S_{\rm c}$ were then calculated as follows:

$$S = \frac{l}{L} \cdot F$$

where S is S_m or S_c , l is l_{mes} or l_{chl} and the F is the curvature correction factor, which was measured and calculated for each species according to Tomás *et al.* (2013) for palisade and spongy cells by measuring their width and height and calculating an average width/height ratio. Curvature factors of 1.31 for spongy cells and 1.44 for palisade cells were used in this study.

Modeling of mesophyll conductance from anatomical characteristics

The anatomical-based $g_{\rm m}$ -anatomy was estimated by dividing the diffusivity of each individual component along the diffusion path (Tomás *et al.*, 2013; Carriquí *et al.*, 2019). First, $g_{\rm m}$ -anatomy is divided into a gas-phase conductance between the substomatal cavities and the outer surface of the cell walls ($g_{\rm ias}$), and a liquid-phase conductance between the outer surface of the cell walls and the site of carboxylation in the chloroplast stroma ($g_{\rm iag}$):

$$g_{\rm m}\text{-}anatomy = \frac{1}{\frac{1}{g_{\rm iss}} + \frac{RT}{H \cdot g_{\rm liq}}}$$

where R is the gas constant, T is the absolute temperature, and H is the Henry constant.

The g_{ias} is calculated based on the f_{ias} and the length of the diffusion path in the gas phase, which is assumed to be half of the mesophyll thickness (T_{leat}):

$$g_{\rm ias} = \frac{D_{\rm a} \cdot f_{\rm ias}}{\frac{1}{2} T_{\rm leaf} \cdot q}$$

where D_a (m² s⁻¹) is the diffusion coefficient for CO₂ in the gas phase (1.51×10⁻⁵ at 25 °C), and ς is the diffusion path tortuosity (m m⁻¹), which was fixed at 1.57 as in previous studies.

The g_{liq} is calculated as:

$$g_{\rm liq} = \frac{1}{\sum \frac{1}{g_{\rm i}}} \cdot S_{\rm c}$$

where g_i is the conductance of the cell wall, plasma membrane, cytosol, chloroplast envelope, or chloroplast stroma. The conductance of a given component of the diffusion pathway can be calculated as:

$$g_{\rm i} = \frac{D_{\rm w} \cdot p_{\rm i} \cdot \gamma_{\rm i}}{\Delta L_{\rm i}}$$

where D_w is the aqueous-phase diffusion coefficient for CO₂, p_i is the effective porosity, which is variable with cell wall thickness, ΔL_i is the diffusion path length, which is usually represented by the thickness of a component, and γ_i is a dimensionless factor accounting for a decrease of diffusion conductance in the cytosol and in the stroma compared with free diffusion in water. Because the structural parameters of the plasma membrane and chloroplast envelope are impossible to estimate from light or electron microscopic images, an estimate of 0.0035 m s⁻¹ for both plasma membrane conductance (g_{pl}) and chloroplast envelope conductance (g_{en}) was used, as in previous studies (Tomás et al., 2013; Carriquí et al., 2019).

It is noteworthy that the inputs of the model are mainly based on parameters extracted from microscopic images, and the model is a pure physics-law-based model in which no biological regulations were considered. As the g_m -anatomy cannot catch the potential N responses of CO₂ diffusion conductance through the plasma membrane, cytosol, chloroplast envelope, and chloroplast stroma, we calculated the g_m -anatomy just to evaluate the influences of anatomical modifications to the mesophyll on g_m . The g_m -anatomy failed to explain the N-modified CO₂-response curves (Supplementary Fig. S2) and a higher g_m was expected to achieve the high *A* after N was added. As a consequence, the g_m estimated using the variable *J* method was used for subsequent analyses.

Leaf N and Rubisco contents

The punched leaf samples were stored at -80 °C until the Rubisco concentrations were measured. Each frozen leaf sample was ground in liquid nitrogen and homogenized in ice in an extraction buffer containing 50 mM Tris-HCl buffer (pH 8.0), 5 mM β-mercaptoethanol, and 12.5% glycerol (v/v). After centrifuging at 15 000 g for 15 min at 4 °C, the supernatant fluid was extracted for analysis of the Rubisco content. The samples were loaded on to a 12.5% (w/v) polyacrylamide gel and separated by SDS-PAGE. After electrophoresis, the gels were washed with deionized water several times and then dyed with 0.25% (w/v) Coomassie blue staining solution [Coomassie R250 dissolved in water:ethanol:acetic acid (5:4:1)] for 3 h, and decolorized using water:ethanol:acetic acid (5:4:1) until the background was colorless (see Supplementary Fig. S3 for an image of a typical gel). The Rubisco content was analyzed through densitometry using a gel documentation system (Bio-Rad Laboratories, Hercules, CA), and a Rubisco band of known concentration on each gel was referred to as the control.

For measurement of leaf N contents, samples of leaf with known area (sampled using a leaf punch as described above) were dried at 70 °C to a constant weight. The dry samples were digested by the micro-Kjeldahl method and the N concentrations were measured with a discrete wet chemistry analyzer (SmartChem 200, AMS-Westco, Rome, Italy).

Statistical analysis

One-way ANOVA was used to test the differences in measured traits between N treatments on the given day after N top-dressing, and two-way ANOVA was used on the time-series data to test the trait response on each day. Regression analyses were performed with mean values to test the correlations between parameters. All analyses were performed in R version 3.6.3 (R Core Team, 2020).

Results

Plant growth performance under N deficit

As expected, plant growth parameters, including leaf number per plant, plant height, leaf area of the newest fully expanded leaves, and aboveground biomass, were significantly smaller in plants subjected to the LN treatment (Table 1). Leaf number and leaf area were about 2-fold higher in HN than in LN, and biomass was 3-fold higher in HN than in LN. The greatest morphological difference between the HN and LN treatments was in the plant height, with HN plants being 4.4-fold taller than LN plants.

Responses of leaf biochemical traits to N addition

The values of leaf biochemical traits including N content per unit leaf area, chlorophyll content per unit leaf area (expressed as a SPAD value), and Rubisco content per unit leaf area for leaves developed by plants grown under HN were higher than those for plants grown under LN (Table 1, Fig. 1). However, leaf biochemical traits were extremely sensitive to the addition of N (Fig. 1, Supplementary Fig. S4). The leaf N content of THN plants increased 3-fold (from 0.35 g m^{-2} to 1.04 g m^{-2}) 1 day after N was added and achieved a maximum value of 2.3 g m⁻² 4 days after N was added (Fig. 1A). Similar response patterns were observed for SPAD values and Rubisco content (Fig. 1B, C). Leaf N (P<0.001) and SPAD (P<0.001) values of THN plants were significantly higher than the values of HN plants at 4 and 5 days, respectively, after the addition of N. We also estimated the relative rate of increase of the leaf biochemical traits by normalizing their values to the values at day 0 after the addition of N. Leaf N content showed the highest rate of increase after N addition, followed by Rubisco and SPAD values (Supplementary Fig. S4).

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Plasticity of photosynthesis in response to N availability

Compared with HN plants, the photosynthetic traits of LN plants were dramatically restricted by N availability (Table 1, Fig. 2). The values of A, g_s , and g_m of HN plants were 2.8-, 2.0-, and 5.3-fold higher than the respective values of LN plants. Similarly, the HN plants exhibited greater photosynthetic capacities, that is, higher V_{cmax} , J_{max} , and A_{max} values (Table 1, Supplementary Fig. S4). All estimated photosynthetic parameters showed a rapid response to the addition of N fertilizer in the THN plants (Figs 2, 3, Supplementary Fig. S5).

The A, g_s , and g_m of THN plants achieved similar values to those of HN plants on day 4, 2, and 5 after N top-dressing, respectively (Fig. 2). CO2 response curve analysis revealed that both $V_{\rm cmax}$ and $J_{\rm max}$ of THN plants fully recovered from LN to HN-like values within 3 days after N addition (Fig. 3). Furthermore, although the intercellular CO_2 concentration at which the transition from ribulose 1,5-bisphosphate (RuBP)-carboxylation-limited to RuBP-regeneration-limited photosynthesis occurs decreased significantly after the addition of N fertilizer, photosynthesis at ambient CO₂ concentration $(400 \ \mu mol \ mol^{-1})$ was limited by RuBP carboxylation. To compare the sensitivity of the traits to the addition of N fertilizer, the dose-response approach was applied to all the photosynthetic parameters. Overall, g_m was the most sensitive trait and $_{gS}$ was the least sensitive trait (Fig. 4A). To separate the stomatal, mesophyll, and biochemical limitations to photosynthesis, a quantitative limitation analysis was applied. The results of this analysis showed that the limitation factors on photosynthesis changed remarkably, with the major limitation factor transferring from $g_{\rm m}$ to biochemical capacity after N was added (Fig. 4B).

Leaf anatomy

A structural analysis was performed to investigate the responses of leaf anatomical traits to the availability of N. The



Fig. 1. Timeline of mean (A) leaf N content, (B) SPAD value (a measure of chlorophyll content), and (C) Rubisco content. HN, high N; LN, low N; THN, top-dressed high N treatment. Data are means ±SE. Two-way ANOVA was performed for each trait.



Fig. 2. Influences of N fertilizer addition on (A) CO_2 assimilation rate (A), (B) stomatal conductance (g_s), and (C) mesophyll conductance (g_m). HN, high N; LN, low N; THN, top-dressed high N treatment. Data are means ±SE. Two-way ANOVA was performed for each trait.



Fig. 3. Intercellular CO₂ concentration (C_i) response of the CO₂ assimilation rate (A) on different days after the addition of N fertilizer as a top-dressing. RuBP carboxylation (grey shaded area) and RuBP regeneration (white area) limitations on A, RuBP carboxylation rate (red line), and RuBP regeneration rate (green line), the maximum carboxylation rate (V_{cmax}), and maximum electron transport rate (J_{max}) were estimated using the Farquhar–von Caemmerer–Berry leaf photosynthesis model. The A at the ambient CO₂ concentration (400 μ mol mol⁻¹) is shown in each panel as a red symbol.

mesophyll cell wall thickness (T_{cw}), mesophyll cell surface area facing the intercellular airspace (S_m), and chloroplast surface area facing the intercellular airspace (S_c) differed between LN leaves and HN leaves (Table 1); in contrast, no significant difference was observed between LN and HN leaves for intercellular airspace fraction (f_{IAS}) (Figs 5, 6). Unexpectedly, the g_m values estimated based on anatomical traits were about 5-fold lower than the values estimated using the Harley method for HN leaves (0.109 mol m⁻² s⁻¹ versus 0.467 mol m⁻² s⁻¹, *P*<0.001), but the g_m values estimated using the two methods were similar in LN leaves (0.084 mol m⁻² s⁻¹ versus 0.088 mol m⁻² s⁻¹, *P*>0.05) (Table



Fig. 4. (A) Dose-response of leaf photosynthetic parameters to N addition and (B) photosynthetic limitation in response to N addition.

1). None of the estimated mesophyll structural traits showed a response to N fertilizer top-dressing except S_c at day 6 after N application (Fig. 5), and consequently, the $g_{\rm m}$ -anatomy estimated based on anatomical parameters showed no response to N top-dressing (Supplementary Fig. S6). When the $g_{\rm m}$ -anatomy values were used to fit the CO₂ response curves, the modeled curves lost their shapes compared with the curves measured using the gas exchange system 2 days after N top-dressing (Supplementary Fig. S2). In contrast, when the variable J-based g_m values were used, the modeled curves matched the estimated curves well in all cases (Supplementary Fig. S7). Moreover, we found a large accumulation of starch grains in LN leaves but much less accumulation in HN leaves (Fig. 6). Although leaf was larger for LN leaves than for HN leaves, no difference in leaf thickness was observed thickness between LN and THN over the timeline of the investigation (Supplementary Fig. S8). However, the leaf mass per area of THN plants decreased from 53.3 g m⁻² to 29.9 g m⁻² after N fertilizer was added to the soil.

Discussion

Photosynthetic capacity in mature leaves is rapidly shaped by N application

The growth performance of the tobacco plants was restricted by N availability. Decreased plant biomass production due to N deficiency was associated with reductions in both leaf area and leaf photosynthetic capacity. In comparison with LN leaves, the higher photosynthesis of HN leaves corresponded to higher CO₂ diffusion conductance from the ambient air to chloroplasts (i.e. g_s and g_m), as well as higher photosynthetic biochemical capacity (i.e. V_{cmax} and J_{max}) (Fig. 7). These results agree with findings from previous studies (e.g. Evans, 1983; Yamori *et al.*, 2011; Xiong *et al.*, 2015*b*; Barbour and Kaiser, 2016). The new information provided by this study is the rapid shaping of leaf functionality in mature leaves in response to N top-dressing. Overall, the tight correlation between photosynthetic rate and leaf N content per unit area has been widely confirmed (see Evans, 1989; Makino, 2011; Xiong and Flexas, 2018 for reviews); however, to the best of our knowledge, no study has tracked the dynamic responses of leaf photosynthetic capacity to N fertilizer addition. In this study, we present daily changes in leaf physiological, biochemical, and anatomical traits after the addition of N fertilizer to the soil.

The leaf N content increased 7-fold in the 4 days after N was added to the soil, which indicates that the top-dressed N fertilizer was rapidly absorbed and assimilated by the plants (Supplementary Fig. S4). N uptake and assimilation kinetics were not investigated in the present study, as the focus of the work was to understand how rapid changes in leaf N content affect photosynthesis. In fact, the sensing, uptake, and assimilation of N by plants, including the fast response to N, has been widely studied (Xu *et al.*, 2012; Liu and Wirén, 2017; Vidal *et al.*, 2020).

The photosynthetic rate increased dramatically with leaf N content once the N top-dressing had been applied to the soil (Fig. 8). The limiting step of photosynthesis is considered as two factors: (i) the CO₂ concentration in chloroplasts, determined by CO₂ diffusion conductances; and (ii) $V_{\rm cmax}$ and/or $J_{\rm max}$. In this study, we observed that the rapid enhancement of CO₂ diffusion conductance via stomata and mesophyll tissues, as well as enhancement of biochemical efficiency (i.e. $V_{\rm cmax}$ and $J_{\rm max}$), corresponded to the increased photosynthesis in leaves with high N content (Supplementary Fig. S9). The increases of $V_{\rm cmax}$ and $J_{\rm max}$ were likely caused by enrichments of the Rubisco and chlorophyll contents in leaves. A lower



Fig. 5. Responses of leaf anatomical traits to N supplementation. (A) Intercellular airspace fraction (f_{IAS}); (B) mesophyll cell wall thickness (T_{cw}); (C) mesophyll cell surface area facing the intercellular airspace (S_m); (D) chloroplast surface area facing the intercellular airspace (S_c). LN, low N treatment; HN, high N treatment; THN, top-dressed high N treatment. Two-way ANOVA was performed for each trait.

 $J_{\rm max}/V_{\rm cmax}$ ratio was correlated with a higher leaf N content (Supplementary Fig. S10), and therefore may reflect a greater limitation on photosynthesis of RuBP regeneration at high leaf N content. In fact, the intercellular CO₂ concentration at which the transition from Rubisco to RuBP regeneration limitation occurs was significantly decreased (the transition point of C_i decreased from 812 ppm at day 0 to 437 ppm at day 5 after N top-dressing; P<0.001) when leaf N content increased (Fig. 3). However, within the range of leaf N content investigated in the present study, the photosynthetic rate under ambient CO_2 (400 ppm), with a photosynthetic photon flux density of 1500 µmol m⁻² s⁻¹ and leaf temperature of 25 °C, was limited by RuBP carboxylation, indicating that N partitioning between the components determining V_{cmax} and J_{max} was responsible for limiting the photosynthetic rate under ambient CO2. These results support earlier observations for a range of species, including tobacco (Yamori et al., 2011). It would be worth noting that the rate of carbohydrate export from the chloroplasts may play a role in regulating the N response of photosynthesis, as the size and number of starch grains in the chloroplasts obviously decreased as leaf N content increased. Indeed, a role for N in regulating the remobilization of carbohydrates among plant organs has been suggested (Chen *et al.*, 2016).

The quantitative limitation analysis shows that g_s accounts for a very small proportion of the total limitation over the estimated range of leaf N content. In contrast to g_s , the limiting roles of g_m and biochemical capacity were remarkably shaped by leaf N content. gm accounted for about half of the total limitation under the LN condition; however, the limitation of $g_{\rm m}$ on photosynthesis reduced as the leaf N content increased, and photosynthetic biochemistry increased to become the major limiting factor (Fig. 4). These findings agree with our synthesis analysis in rice (Xiong and Flexas, 2018), where we found that the major photosynthetic limitation shifted from $g_{\rm m}$ to photosynthetic biochemistry at a leaf N content of ~ 0.8 g m⁻². Our results, together with those of previous studies (Barbour and Kaiser, 2016; Xiong et al., 2018; Xiong and Flexas, 2018; Nadal and Flexas, 2019), suggest that the biochemical limitation contributes to more than half of photosynthetic limitation for annual crops including rice, tobacco, cotton, sunflower, and wheat at their typical leaf N content observed on farmland,



Fig. 6. Light (A–C) and transmission electron (D–F) microscopic images of high N (HN; A, D), low N (LN; B, E) and top-dressed high N (THN; C, F) leaves. Leaves were sampled 6 days after N was added to THN pots. CW, mesophyll cell wall; IAS, intercellular airspace; SG, starch grain. Bars=500 μm in (A–C) and 1 μm in (D–F).



Fig. 7. (A) CO₂ assimilation rate (A), (B) stomatal conductance (g_s), (C) mesophyll conductance (g_m), (D) maximum carboxylation rate (V_{cmax}), (E) maximum electron transport rate (J_{max}), and (F) Rubisco content versus leaf N content for the long-term N treatments [pooled high N (HN) and low N (LN) treatments; dashed lines] and the top-dressed high N treatment (THN; solid lines). The slopes and elevations of the regressions for the long-term and THN treatments were compared using the standardized major axis method. *P<0.05, ***P<0.001; ns, not significant based on the hypothesis test.



Fig. 8. Relationships between mesophyll conductance (g_m) and leaf anatomical traits: (A) cell wall thickness (T_{cw}); (B) mesophyll cell surface facing the intercellular airspace (S_m); (C) chloroplast surface facing the intercellular airspace (S_c). Grey triangles, grey circles, and blue circles represent low N (LN), high N (HN), and top-dressed high N treatments, respectively. The grey lines were fitted using values of HN and LN treatments. g_m =-0.009× T_{cw} +2.44 (r^2 =0.98; P<0.001); g_m =-0.083× S_m +1.92 (r^2 =0.94; P<0.001); g_m =0.16× S_c =2.13 (r^2 =0.87; P<0.001).

and modifying enzymes might be the key for improvement of photosynthesis in these crops.

N enhancement of g_m cannot be fully explained by leaf anatomical modifications in mature leaves

Among the traits examined, gm was the most sensitive to changes in leaf N content (Fig. 4), supporting our earlier observations for rice (Xiong and Flexas, 2018). Our simulation showed that the increase of gm was mainly caused by the increase of liquid-phase conductance (Supplementary Fig. S6), which was expected to relate to membrane permeability and mesophyll anatomy. Indeed, the changes in g_m in response to N availability have been suggested to be due to changes in leaf anatomy (Xiong et al., 2015b). That is, leaf anatomical characteristics over the growth stages can be substantially modified by N availability in the soil. Enlargement of chloroplasts, resulting in an increased chloroplast surface area facing the intercellular airspace per leaf area (S_c) , for instance, was frequently observed in rice leaves grown under HN conditions (Xiong et al., 2015a, b). In the present study, structural traits of tobacco mesophyll tissues, including T_{cw} , S_m , and S_c , were also modified by the soil N conditions over the growth period (Figs 5, 8). However, no such modifications were observed for $S_{\rm m}$ and $T_{\rm cw}$ in mature leaves of tobacco after N top-dressing, although the $g_{\rm m}$ increased dramatically. In fact, these traits have been suggested to be invariable in the short term (Evans et al., 2009; Terashima et al., 2011; Carriquí et al., 2019). Surprisingly, 6 days after N top-dressing, the S_c increased slightly but significantly (P=0.032), associated with changes in the shape of the chloroplasts (Figs 5, 6). Although small changes in S_c might potentially drive the rapid response of g_m to N top-dressing (Supplementary Fig. S6), the increase of S_c observed in the present study apparently cannot fully explain the increase of $g_{\rm m}$ after N top-dressing, yet it is still the trait among all those studied that best correlates with changes in $g_{\rm m}$ (Fig. 8).

Beyond structural barriers, the diffusion of CO₂ molecules in mesophyll tissues can be regulated by membrane permeability and the activity of carbonic anhydrase in the chloroplast and cytosol (Ainsworth and Bush, 2011; Cousins et al., 2020; Momayyezi et al., 2020). Both membrane permeability, which is mainly regulated by aquaporins, and carbonic anhydrase activity are believed to be the major factors determining the rapid responses of $g_{\rm m}$ to environmental changes, as the leaf anatomical traits cannot be modified under such a short timescale (Flexas and Diaz-Espejo, 2015; von Caemmerer and Evans, 2015; Carriquí et al., 2019). Interestingly, in our previous study in rice, we observed that the g_m of HN leaves is more sensitive to environmental changes than that of LN leaves (Xiong et al., 2015b), which once again supports the hypothesis that the N responses of g_m might be regulated by aquaporins and/or carbonic anhydrase. However, a recent study (Kromdijk et al., 2020) failed to show any difference in $g_{\rm m}$ values between aquaporin knockout lines and their wild type by estimating g_m by multiple methods, which suggests that our current understanding of the role of aquaporins in g_m regulation may need to be revised. Moreover, our simulation analysis showed that the increase of membrane permeability alone made a limited contribution to the improvement of $g_{\rm m}$, and the enhancement of CO_2 diffusion conductance in the cytosol and stroma could dramatically increase the liquidphase conductance (Supplementary Fig. S6). It is obvious that more efforts are required to help us understand how N regulates aquaporins and/or carbonic anhydrase activity for CO₂ transport.

In summary, we investigated the dynamic responses of leaf biochemical, structural, and photosynthetic traits to long-term HN treatment and to N top-dressing in tobacco. In comparison with the leaves of plants subjected to LN treatment, the leaves of HN plants had significantly higher photosynthesis due to their higher g_s , g_m , and photosynthetic biochemical capacity. The high g_m in HN leaves was tightly correlated with leaf anatomical traits including $T_{\rm cw}$ and S_m ; however, the rapid response of the photosynthetic rate and g_m to N top-dressing cannot be fully explained by leaf anatomical modifications.

Supplementary data

The following supplementary data are available at JXB online. Fig. S1. Quantum yield of photosystem II versus quantum vield of CO₂.

Fig. S2. Chloroplast CO_2 concentration response of photosynthesis.

Fig. S3. A typical SDS gel used for Rubisco quantification.

Fig. S4. Dose-response of leaf biochemical parameters to N addition.

Fig. S5. Intercellular CO_2 response of CO_2 assimilation rate on different days after N top-dressing.

Fig. S6. Timeline of mesophyll conductance (g_m) and the components estimated based on leaf anatomical parameters.

Fig. S7. Chloroplast CO_2 concentration response of photosynthesis.

Fig. S8. Responses of leaf mass per area and leaf thickness to N supplements.

Fig. S9. Correlation of assimilation rate and CO_2 diffusion conductances, and assimilation rate versus photosynthetic biochemical traits.

Fig. S10. $J_{\text{max}}/V_{\text{cmax}}$ ratio versus leaf N content for long- and short-time N treatments.

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Author contributions

DX and JF designed the research; DX performed the experiments; DX analyzed the data and wrote the paper with inputs from JF.

Data availability

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

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