1 Reduced mesophyll conductance by cell wall thickening and
2 chloroplasts decreasing driven the decline of photosynthesis under
3 sole NH₄⁺ supply

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17 The date of submission: 5 January 2022

18 The number of tables and figures: 3 and 6, respectively

19 Word count: 3554

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22 Highlight: Cell wall and chloroplast variability determining the mesophyll
23 conductance under different nitrogen forms

24 **Abstract:** The relationship between nitrogen (N) sources and photosynthetic capacity
 25 of leaf differs between species. However, the leaf anatomical variabilities related to
 26 photosynthesis (A) of shrubs under different forms of N remain imperfectly known.
 27 Here, *Lonicera Japonica* (a shrub) was grown hydroponically in the presence of three
 28 forms of N (sole NH_4^+ , 50%/50% $\text{NH}_4^+/\text{NO}_3^-$ and sole NO_3^-). A and photosynthetic N
 29 use efficiency significantly decreased under sole NH_4^+ supply, in parallel with
 30 down-regulated stomatal conductance (g_s), mesophyll conductance (g_m), and electron
 31 transfer rate (J). Up to the total A decline of 41.28% in sole NH_4^+ supply (compare
 32 with sole NO_3^-), the g_m attributed to 60.3% of the total limitations. Besides, the
 33 decreased internal air space explained the increase of gas-phase resistance, and the
 34 increased liquid-phase resistance in sole NH_4^+ supply was ascribed to the thicker cell
 35 wall thickness (T_{cw}) and decreased chloroplasts exposed surface area per unit leaf area
 36 (S_c/S). The discrepancy of S_c/S could be interpreted by the altered chloroplasts
 37 numbers and the distance between adjacent chloroplasts ($D_{chl-chl}$). These results
 38 indicate the alteration of T_{cw} and chloroplast numbers were the main causes of the
 39 difference in g_m in coping with varied N sources.

40 **Key words:** Ammonium; leaf anatomy; *Lonicera Japonica*; mesophyll conductance;
 41 N form; nitrate; photosynthetic limitations; shrub

42 Introduction

43 *Lonicera Japonica* is a widespread semi-deciduous shrub species of the
44 Caprifoliaceae family utilized in traditional medical practices (Schierenbeck, 2004;
45 Shang *et al.*, 2011). The stomatal and photosynthetic function are suggested the key
46 traits for enhancing yield and quality of medicinal plants, as the photosynthesis
47 improvement could increase carbon fixation and the production of biomaterials.
48 Mesophyll conductance (g_m) represents the diffusion conductance to CO₂ in the
49 mesophyll tissues from the sub-stomatal cavities to the carboxylation sites inside
50 chloroplast (Peguero-Pina *et al.*, 2012), which was proposed to be the first limiting
51 factor in photosynthesis (Lu *et al.*, 2016; Ren *et al.*, 2019).

52 g_m is determined by the CO₂ diffusion characteristic. CO₂ diffusion, according to
53 Fick's law, depends on CO₂ diffusivity, leaf temperature, diffusion distance, and the
54 nature of the media in which diffusion occurs (e.g., mesophyll tissues) (Flexas *et al.*,
55 2012), thus it is not strange that even tiny leaf anatomical traits could drive g_m
56 variability. Tholen *et al.* (2012a) had reported that altered leaf anatomy significantly
57 influences the CO₂ diffusion in leaves. Leaf mass per area (M_A) is an important
58 integrated leaf morphological characteristic that regulates photosynthesis by
59 influencing g_m (Niinemets, 2001; Poorter *et al.*, 2009). Early studies reported a
60 negative correlation between M_A and g_m (Flexas *et al.*, 2008; Niinemets *et al.*, 2009b),
61 and further in species with high M_A , photosynthesis is more limited by g_m , on average
62 by 57% (Tomás *et al.*, 2013); analyses on the relationship between g_m and leaf
63 density/leaf thickness, two components of M_A also corroborated this idea, despite the
64 influences are opposite. The increase of M_A due to increased leaf density might result
65 in a densely packed mesophyll cell, which will ultimately reduce the g_m by decreased
66 mesophyll surface area exposed to intercellular airspace per unit leaf area (S_{mes}/S)
67 (Flexas *et al.*, 2008; M. Weraduwaage *et al.*, 2016).

68 In the journey of CO₂ diffusion, the mesophyll resistance is comprised of
69 gas-phase resistance (i.e., from the intercellular air space) and liquid-phase resistance

(i.e., from the cell wall, lipid membrane, cytoplasm, stroma, and chloroplast envelope). In particular, liquid-phase limitation accounts for more than 80% of the total limitation, in which the cell wall thickness (T_{cw}) and chloroplast surface area exposed to intercellular airspace per unit leaf area (S_c/S) are the two dominating components that affect g_m (Flexas *et al.*, 2021). There are ample studies showing a significant positive correlation between S_c/S and g_m , and the S_c/S was considered the uppermost parameter in determining g_m (Hu *et al.*, 2020; Tomás *et al.*, 2013). While the cell wall is often negligible, because it accounts for a tiny fraction of the CO₂ diffusion length (Carriquí *et al.*, 2020), has a comparative larger pore size and variability (Carpita *et al.*, 1979), and quite a static influence on photosynthesis (Evans *et al.*, 2009). Interestingly, in *Lycopodiales* (a species of fern), the accountability of T_{cw} in robust leaves of the total g_m was up to 70% (Tosens *et al.*, 2016). Other components, such as the mesophyll thickness, plasma membrane, and chloroplast density, despite not that important, also play an important role in regulating g_m , as observed in different plant species (Lu *et al.*, 2016; Niinemets *et al.*, 2009b; Veromann-Jurgenson *et al.*, 2020a; Veromann-Jurgenson *et al.*, 2020b). Overall, the leaf anatomies might vary independently and compensate for each other to achieve substantial g_m in some cases (Peguero-Pina *et al.*, 2017).

In fact, g_m is a rapidly adapting trait, and thus its value presents a large variability across species or environments. Up to now, mesophyll conductance to CO₂ had been studied on various plant species in a scale of interspecific variation and diverse environmental conditions. The review of Flexas *et al.* (2008) summarized the g_m in different pooled groups of plants, which showed an apparent increasing tendency of g_m from conifers (slightly above 0.1 mol m⁻² s⁻¹) to grasses (0.2-0.4 mol m⁻² s⁻¹), and the semi-deciduous shrubs generally had a g_m value of 0.2-0.3 mol m⁻² s⁻¹. The varying environmental conditions, such as light, water availability, salinity, and temperature had also been studied in regulating g_m (Flexas *et al.*, 2008; Niinemets *et al.*, 2009b; Pons *et al.*, 2009; Tosens *et al.*, 2012a). In contrast, the effects of nutrition

on g_m , particularly on quantitative limitations of g_m are somewhat incomplete, and only recently have the effects of nutrition (e.g., potassium, nitrogen) been reported (Gao *et al.*, 2020; Hu *et al.*, 2020; Xie *et al.*, 2020; Xiong *et al.*, 2015b). Nitrogen (N) is an important nutrition element for plant growth and photosynthesis. There is sufficient evidence of the strong interplays between the photosynthetic process and plant endogenous N status (Perchlik and Tegeder, 2018; Xiong *et al.*, 2015). N in soil is available mainly in two inorganic N sources, i.e., nitrate (NO_3^- -N) and ammonium (NH_4^+ -N). Due to the different assimilation processes and energy consumption, or sometimes the toxic effect of NH_4^+ , photosynthesis of species showed quite different responses/preferences to NH_4^+ or NO_3^- . In some studies, the plant supplied with NH_4^+ was observed increased chloroplast numbers and chloroplast volume in comparison with NO_3^- (Golvano *et al.*, 1982), yet the other study observed an elevated S_c /Rubisco in NO_3^- supply (Gao *et al.*, 2020); this effect on photosynthetic capacity and g_m , according to some studies, could be ascribed to N allocation trade-off and source-sink balance within leaves (Evans and Clarke, 2019; Hikosaka, 2004). The studies on the relative importance of N source to the response of g_m variation in a scale of leaf structural traits had been reported in herbs (Gao *et al.*, 2020) and trees (Liu *et al.*, 2021), while the study on shrubs is largely lost. Moreover, major limiting factors that restrict the leaf photosynthetic capacity of *L. Japonica* under different inorganic source is largely unknown.

In the present study, *L. Japonica* was grown in a hydroponic experiment with three forms of N (sole NH_4^+ , 50%/50% $\text{NH}_4^+/\text{NO}_3^-$ and sole NO_3^-) to investigate: (1) the variations of leaf photosynthesis and leaf anatomy under different inorganic nitrogen sources; (2) the crucial role of g_m on leaf photosynthesis of *L. Japonica*; (3) the impact of mesophyll conductance on leaf photosynthesis through leaf structure variations.

Material and Methods

Plant material and experimental condition

The hydroponic experiment was conducted in a greenhouse with a light intensity of 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the leaf level using 14-h photoperiod and day/night temperature of 28/18°C. The experimental site is located in Nanjing (118°51'E, 32°1'N), China. One-year-old seedlings of *Lonicera japonica* were transferred to a 12 L rectangular plastic box (64 cm×23 cm×18 cm) and a one-half-strength mixture of NH_4^+ and NO_3^- nutrient solution was supplied (for composition, see below). After two weeks, the seedlings were supplied with full-strength nutrition solution for two weeks, after which the uniform seedlings were transplanted to a 12 L rectangular plastic box (64 cm×23 cm×18 cm). The nutrition solution contained 2.0 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.8 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.03 mM K_2SO_4 , 0.32 mM KH_2PO_4 , 9.10×10^{-3} mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.52×10^{-3} mM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 37×10^{-3} mM H_3BO_3 , 0.15×10^{-3} mM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.16×10^{-3} mM $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 35.8×10^{-3} mM Fe-EDTA. In our preliminary experiment, plant growth was highest at a ratio of 50%/50% $\text{NH}_4^+/\text{NO}_3^-$ compared with 75%/25% $\text{NH}_4^+/\text{NO}_3^-$ and 25%/75% $\text{NH}_4^+/\text{NO}_3^-$ at a N level of 2.8 mM. Thus, the N was supplied at 2.8 mM with three different treatments: sole $(\text{NH}_4)_2\text{SO}_4$ (A), sole $\text{Ca}(\text{NO}_3)_2$ (N) or mixed N (combination of 50% $(\text{NH}_4)_2\text{SO}_4$ and 50% $\text{Ca}(\text{NO}_3)_2$, AN). CaCl_2 was added to the solutions of A and AN treatment to adjust the Ca level to N treatment (2.8 mM). Dicyandiamide was added to each nutrition solution as nitrification inhibitor. The nutrition solution was aerated for 1-h/1-h day/night and renewed every 4 days, while the pH was adjusted to 6.0 ± 0.1 each day. The containers were placed randomly to prevent the position effect.

Gas exchange and chlorophyll fluorescence measurement

Leaf gas exchange and chlorophyll fluorescence were measured simultaneously using an open gas exchange system equipped with multiphase flash (LI-6800XT; LI-COR Inc., Lincoln, NE, USA) from 8:30 to 15:00. For each treatment, new fully-expanded leaves were randomly selected for the measurements with five replications. Measurements were obtained at a leaf temperature of $28 \pm 0.5^\circ\text{C}$, CO_2 concentration inside the chamber of $400 \pm 6 \mu\text{mol mol}^{-1}$, and a photosynthetic photon

154 flux density (PPFD) of 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. For fluorescence parameters,
 155 steady-state fluorescence yield (F_s) and maximum fluorescence (F_m') were recorded
 156 using a fluorometer chamber during a light-saturating pulse of approximately 8000
 157 $\mu\text{mol m}^{-2} \text{s}^{-1}$. During the photosynthetic CO_2 responses (A/C_i curve) measurements,
 158 the ambient CO_2 concentration (C_a) was set in a series from 400 to 300, 200, 150, 100,
 159 and 50 $\mu\text{mol CO}_2 \text{mol}^{-1}$ and then increased from 50 to 400, 600, 800, and 1000 μmol
 160 $\text{CO}_2 \text{mol}^{-1}$ at a constant PPFD of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with four replicates. The
 161 maximum carboxylation rate (V_{cmax}) was calculated according to Long and Bernacchi
 162 (2003), and the carboxylation efficiency (CE) was estimated as the slope of the A/C_i
 163 curve fitting line over a C_i range of 50-200 $\mu\text{mol mol}^{-1}$. The effective quantum
 164 efficiency of photosystem II (ΦPSII) was quantified as follows: $\Phi\text{PSII}=(F_m'-F_s)/F_m'$.
 165 The potential electron transport rate (J) was calculated as $J=\Phi\text{PSII}\times\text{PPFD}\times\alpha\times\beta$,
 166 where α is the leaf absorption and β is the proportion of quanta absorbed by PSII,
 167 assumed as 0.85 and 0.5, respectively. There were no differences in chlorophyll
 168 contents between A, N, and AN leaf, thus eliminating out the confounding effect of
 169 different leaf optical properties as a result of N forms among the treatments.

170 Then we estimated the mesophyll conductance (g_m) and chloroplast CO_2
 171 concentration (C_c) by variable J method proposed by Harley *et al.* (1992).

$$g_m = \frac{A}{C_i - \frac{\Gamma^*(J+8(A+R_d))}{J-4(A+R_d)}} \quad (1)$$

$$C_c = C_i - \frac{A}{g_m} \quad (2)$$

172 where A , C_i , C_c , and J were calculated as described previously, Γ^* is the CO_2
 173 compensation point in the absence of mitochondrial respiration and R_d is the
 174 mitochondrial respiration rate in the light. In this study, Γ^* was assumed to be 40.0
 175 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and R_d , according to the previous data, was assumed to be 1.0 $\mu\text{mol m}^{-2}$
 176 s^{-1} .

177 Leaf physiology

178 Four new fully expanded leaves from each treatment were randomly selected and

179 pictured. The leaf area (A_L) obtained from the picture was calculated by Image-Pro
 180 Plus (Media Cybernetics, Silver Spring, MD, USA), after which the leaves were
 181 oven-dried at 105°C for 15 min and then dried to constant weight at 65°C and
 182 weighted. Dried leaf samples were weighed and digested with $H_2SO_4-H_2O_2$ at 270 °C,
 183 and the leaf N concentration was determined using the digital colorimeter
 184 (AutoAnalyzer 3; Bran+Luebbe). The total chlorophyll concentration was determined
 185 according to the method proposed by Sartory and Grobbelaar (1984). Approximately
 186 0.5 g fresh leaf discs (avoiding the main vein) were extracted with 50 ml 95%(v/v)
 187 ethanol (analytically pure, Sinopharm Chemical Reagent Co., Ltd) in the dark until
 188 they were blanched (usually no more than two days). The extract solutions were used
 189 for the determination of chlorophyll *a* and *b* using a spectrophotometer (HBS-1096A,
 190 Shanghai, China) at 665 and 649 nm, respectively. The total chlorophyll was
 191 calculated as the sum of chlorophyll *a* and *b*. There were four replications for
 192 chlorophyll concentration determination.

193 **Quantitative limitation analysis of A**

194 To separate the relative controls on A resulting from the difference in stomatal
 195 conductance, mesophyll diffusion, and biochemical capacity, quantitative analysis was
 196 conducted. The limitation of A under different types of N forms, according to Grass
 197 and Magnani (2005), was then composed of stomatal limitation (S_L), mesophyll
 198 conductance (MC_L), and biochemical limitation (B_L), which could be expressed as
 199 follows:

$$\frac{dA}{A} = l_s * \frac{dg_s}{g_s} + l_m * \frac{dg_m}{g_m} + l_b * \frac{dJ}{J} \quad (3)$$

200 where the l_s , l_m , and l_b ($l_s+l_m+l_b=1$) were the relative limitation of stomatal
 201 conductance, mesophyll diffusion and biochemical capacity, which were calculated as
 202 follows:

$$l_s = \frac{g_{tot}/g_s * \partial A / \partial C_C}{g_{tot} + \partial A / \partial C_C} \quad (4)$$

$$I_m = \frac{g_{\text{tot}}/g_m * \partial A/\partial C_c}{g_{\text{tot}} + \partial A/\partial C_c} \quad (5)$$

$$I_b = \frac{g_{\text{tot}}}{g_{\text{tot}} + \partial A/\partial C_c} \quad (6)$$

203 where the g_{total} is the total conductance to diffuse CO_2 from the leaf surface (i.e.,
204 inside the leaf boundary layer) to the carboxylation site of chloroplast, which can be
205 calculated as $\frac{1}{g_{\text{tot}}} = \frac{1}{g_s} + \frac{1}{g_m}$. $\partial A/\partial C_c$ was estimated as the slope of A/C_i curves over a
206 C_i range of 50-100 $\mu\text{mol mol}^{-1}$.

207 Then, the limitations of A can be approximately calculated as follows:

$$S_L = I_s * \frac{g_{\text{sref}} - g_s}{g_{\text{sref}}} * 100 \quad (7)$$

$$MC_L = I_m * \frac{g_{\text{mref}} - g_m}{g_{\text{mref}}} * 100 \quad (8)$$

$$B_L = I_b * \frac{J_{\text{ref}} - J}{J_{\text{ref}}} * 100 \quad (9)$$

208 where g_{sref} , g_{mref} , and J_{ref} are the reference values of stomatal conductance (g_s),
209 mesophyll conductance (g_m), and the potential electron transport rate (J), respectively.
210 The reference values were obtained from the parameters from the optimal treatment
211 from A, AN, and N. Here, we adopted the N treatment as the optimal treatment.

212 **Anatomical analysis**

213 After the photosynthetic parameter measurements, leaf pieces (approximately
214 5mm×5 mm) were cut between the main veins from each of the four different plants
215 for anatomical measurements and transmission electron microscope (TEM) analysis.
216 For anatomical measurement, leaf segments were quickly fixed with FAA (95%
217 ethanol: glacial acetic acid: formalin: distilled water = 10:1:2:7, v/v) and dehydrated in
218 dimethylbenzene-ethanol series. The pieces were then staining with safranin-fast
219 green and embedded in paraffin. After cutting into 6 μm transverse sections, the
220 pieces were photographed at a magnification of 400× with Nikon Eclipse E100
221 microscope equipped with a Nikon microscope camera (Nikon DS-U3). For TEM
222 analysis, leaf materials were quickly fixed with glutaraldehyde (2.5%, v/v) in 0.1 M
223 phosphate buffer (pH 7.4) under vacuum. Afterward, the segments were postfixed

with 2% osmium tetroxide and dehydrated in a graded ethanol series, followed by washing in propylene oxide. The dehydrated segments were then embedded in Epon 812 resin. Ultrathin cross-sections were cut with a Power Tome-XL ultramicrotome, stained with 2% uranyl acetate, and then viewed under an H7650 transmission electron microscope (H-7650, Hitachi, Japan). Photos were taken at 2000-8000× direct magnification to measure the cell wall thickness and chloroplast characteristics. Leaf thickness (T_L), leaf density (D_L), leaf volume per area ($V_A=A_L \times T_L$), mesophyll thickness between two epidermises (T_{mes}), and the volume fraction of intercellular air space (f_{ias}) were calculated according to the light and TEM micrographs. The D_L and f_{ias} were determined as follows:

$$D_L = \frac{M_A}{T_L} \quad (10)$$

$$f_{ias} = 1 - \frac{\Sigma S_s}{T_{mes} W} \quad (11)$$

where M_A is the specific leaf weight (mg cm^{-2}), ΣS_s is the total cross-section area of the mesophyll cells and W is the width of the measured framed range.

Chloroplast (S_c/S) and mesophyll (S_{mes}/S) surface area exposed to intercellular airspace per unit leaf area were also calculated from light and TEM micrographs, as reported by Evans *et al.* (1994) and Syvertsen *et al.* (1995).

$$S_{mes}/S = \frac{L_{mes}}{W} * F \quad (12)$$

$$S_c/S = \frac{L_c}{W} * F = \frac{L_c}{L_{mes}} * S_{mes}/S \quad (13)$$

where the L_{mes} is the total mesophyll surface length facing intercellular air space per leaf area, and L_c is the chloroplast surface length facing intercellular air space per leaf area. F is the curvature correction factor, which depends on the shape of mesophyll cell and was calculated according to Thain (1983). Briefly, according to the differences of palisade and spongy cells (i.e., cell arrangement direction and axial/length ratio), F was calculated as a weight average of spongy (F_1) and palisade (F_2) mesophyll distributions:

$$F_1 = \frac{1+2\bar{b}_1/\bar{a}_1}{1+4\bar{b}_1/\pi\bar{a}_1} \quad (14)$$

$$F_2 = [(\bar{a}/\bar{b}) + (1/e) \sin^{-1} e]/E \quad (15)$$

where $\bar{a}$ and $\bar{b}$ are the average of the length and thickness of the mesophyll. e is the eccentricity, and E is the elliptical integral, which were calculated as follows, respectively:

$$e = \sqrt{1 - (\bar{a})^2 / (\bar{b})^2} \quad (16)$$

$$E \approx \frac{\pi}{2} \left(1 - \frac{e^2}{4} - \frac{3e^4}{64} - \frac{5e^6}{256} \right) \quad (17)$$

Besides, cell wall thickness (T_{cw}), chloroplast length (L_{chl}) and thickness (T_{chl}), the distance between two neighboring chloroplasts ($D_{chl-chl}$), chloroplast distance from the cell wall (L_{cyt}), and the chloroplast number per mesophyll cell were measured from TEM micrographs at a 2000-8000 $\times$. The images were analyzed with Image-Pro Plus software (Media Cybernetics, Sliver Spring, MD, USA).

g_m modelled from anatomical characteristics

To determine g_m , the one-dimension gas diffusion model modified by Tomas *et al.* (2013) was applied in this study. g_m , a composite conductance, is shared by different leaf anatomical characteristics and decided by within-leaf gas and liquid components:

$$g_m = \frac{1}{\frac{1}{g_{ias}} + \frac{RT_k}{H^* g_{liq}}} \quad (18)$$

where g_{ias} is the gas-phase conductance which stands for the gas-phase pathway from substomatal cavities to the outer surface of cell wall, and g_{liq} is the liquid conductance that from the outer surface of cell wall to chloroplast. R is the gas constant ($\text{Pa m}^3 \text{ K}^{-1} \text{ mol}^{-1}$), T_k is the absolute temperature (K), and H is the Henry's law constant ($\text{Pa m}^3 \text{ mol}^{-1}$). Due to the g_m in this equation, is defined as a gas-phase conductance, thus H/RT_k (dimensionless form of Henry's constant) is needed to convert to g_{liq} to the corresponding gas-phase equivalent conductance (Niinemets and Reichstein, 2003).

In this model, gas-phase conductance depends on gas-phase porosity (f_{ias}) and

269 effective diffusion path in the gas-phase (ΔL_{ias}):

$$g_{ias} = \frac{1}{r_{ias}} = \frac{D_a * f_{ias}}{\Delta L_{ias} * \varsigma} \quad (19)$$

270 where ς is the diffusion path tortuosity ($m\ m^{-1}$) and Da ($m^2\ s^{-1}$) is the diffusion
271 coefficient for CO_2 in the gas-phase (1.51×10^{-5} at $25^\circ C$). ΔL_{ias} is taken as half of the
272 mesophyll thickness (T_{mes}).

273 The total liquid-phase diffusion conductance was determined by different
274 components in mesophyll cell, including the conductance in the cell wall (g_{cw}),
275 plasma membrane (g_{st}), cytosol (g_{cyt}), chloroplast envelope (g_{en}), and stroma (g_{st}).
276 Thus, the g_{liq} was given as:

$$g_{liq} = \frac{S_c}{(r_{cw} + r_{pl} + r_{cyt} + r_{en} + r_{st})S} \quad (20)$$

277 where r_{cw} , r_{pl} , r_{cyt} , r_{en} , r_{st} are the reciprocal term of g_{cw} , g_{st} , g_{cyt} , g_{en} , g_{st} , respectively.
278 Alternatively, according to Tholen *et al.*(2012b), the CO_2 diffusion inside the cell in
279 different ways: one for cell wall parts with chloroplast ($g_{cel,1}$), and the other for
280 inter-chloroplast areas ($g_{cel,2}$), and the corresponding resistance was expressed as $r_{cel,1}$
281 and $r_{cel,2}$. Thus, the equation of g_{liq} was converted as:

$$g_{liq} = \frac{S_{mes}}{(r_{cw} + r_{pl} + r_{cel,1} + r_{cel,2})S} \quad (21)$$

282 In addition, the conductance of liquid-phase diffusion pathway, either for cell
283 wall (g_{cw}), cytosol (g_{cyt}), or stroma conductance (g_{st}) is given as follows:

$$g_i = \frac{1}{r_i} = \frac{r_{fi} * D_w * p_i}{\Delta L_i} \quad (22)$$

285 where r_{fi} (dimensionless factor) accounts for the decrease of CO_2 diffusion in the
286 aqueous phase compared with free diffusion in water and was taken as 1.0 for cell
287 wall and 0.3 for cytosol and stroma, as reported by Rondeau-Mouro *et al.*(2018) and
288 Niinemets *et al.*(2003). ΔL_i (m) is the diffusion path length in the corresponding
289 component of the diffusion pathway, and p_i ($m^3\ m^{-3}$) is the effective porosity in the
290 given part. The value of p_i was set to 1.0 for cytosol and stroma, and 0.29 for the cell
291 wall. D_w is the CO_2 diffusion coefficient in aqueous phase ($1.79 \times 10^{-9}\ m^2\ s^{-1}$ at $25^\circ C$).
292 Besides, conductance of plasma membrane (g_{pl}) and chloroplast envelope (g_{env}) was

293 assumed as constant value of 0.0035 m s^{-1} (Evans *et al.*, 1994; Tosens *et al.*, 2012b).

294 **The quantitation of the anatomical limitation of the g_m**

295 The determinants of g_m were shared by gas-phase conductance (l_{ias}) and
296 liquid-phase conductance (l_i). The proportion limited by l_{ias} was calculated as:

$$l_{ias} = \frac{g_m}{g_{ias}} \quad (23)$$

297 The share of g_m by the cellular-phase conductance (l_i), which stands for the
298 limitation of cell wall, the plasmalemma, and inside the cells was determined as:

$$l_i = \frac{g_m}{g_i * \frac{S_{mes}}{S}} \quad (24)$$

299 where g_i is the component diffusion conductance of the corresponding pathways. The
300 fraction of exposed cell wall area lined with chloroplasts and fraction free of
301 chloroplast were used to weighted limitations imposed by different cellular
302 components (cytoplasm, chloroplast envelope, and stroma).

303 **Statistical analysis**

304 Statistical analysis was conducted with SPSS 25.0 (SPSS Inc., Chicago, IL,
305 USA). All data were subjected to a one-way analysis of variance (ANOVA), and the
306 significant differences between treatments were compared using the least significant
307 difference (*LSD*) at $P < 0.05$. Linear regression analyses were used to obtain the
308 relationships among photosynthetic capacity and the main limiting factors, the key
309 structural parameters and mesophyll conductance, and the values of mesophyll
310 conductance estimated from different methods. Graphics and regression analyses were
311 performed using Origin Pro 2021 software (Origin Lab Corporation, Northampton,
312 MA, USA).

313 **Results**

314 **Leaf morphology characteristics**

315 Sole NH_4^+ supply significantly decreased leaf area (A_L) and increased leaf
316 thickness (T_L) in comparison with sole NO_3^- and mixed N supply (Table 1).
317 Correspondingly, the leaf volume per area (V_A) of sole NH_4^+ supply was almost half

of the V_A of mixed N supply. The variation in leaf density (D_L) was 1.5-fold with sole NH_4^+ supply having the densest leaves (0.36 g cm^{-3}) and sole NO_3^- supply the least dense (0.27 g cm^{-3}). Notably, M_A seemed uninfluenced among the treatments. The leaf N concentration (N_L) of sole NO_3^- supply was approximately 11% lower compared with mixed N and sole NH_4^+ supply, yet the photosynthetic N use efficiency was dramatically upregulated by NO_3^- treatments.

Leaf physiological characteristics

In comparison to sole NH_4^+ supply, net photosynthetic rate (A) was increased by both sole NO_3^- and mixed N supply, by 39.2% and 26.6%, respectively. Whilst the intercellular CO_2 concentration (C_i) seemed indifferent among the treatments, chloroplast CO_2 concentration (C_c) was significantly down-regulated by sole NH_4^+ supply. For the stomatal conductance (g_s) and mesophyll conductance (g_m), sole NO_3^- and mixed N supply had higher value than sole NH_4^+ supply and coincided with elevated electron transfer rate (J) and carboxylation efficiency (CE) (Table 2).

In order to ascertain the photosynthetic component that had the largest effect on net assimilation rate, we performed a correlation analysis, and the net photosynthesis rate (A) was positively correlated with stomatal conductance (g_s), mesophyll conductance (g_m), and electron transport rate (J) (Figure 2). The CO_2 drawdown ($C_i - C_c$) from sub-stomatal cavities (C_i) to chloroplasts (C_c) ranged from 86.3 to 107.3 $\mu\text{mol mol}^{-1}$ and was higher in leaves with decreased g_m under sole NH_4^+ supply.

Leaf anatomical traits

Among the leaf ultrastructural characteristics estimated from TEM (Table 3; Figure 1), the mesophyll thickness (T_{mes}), the volume fraction of intercellular air space (f_{ias}), mesophyll surface area (S_{mes}/S), and the chloroplast surface area (S_c/S) exposed to intercellular airspace per unit leaf area were significantly down-regulated by sole NH_4^+ supply. Notably, there were marked differences in cell wall thickness (T_{cw}) among the treatments, for sole NO_3^- supply exhibited the thinnest cell wall (0.16 μm), while sole NH_4^+ supply had the thickness cell walls with the maximum value of

0.25 μm . For chloroplast characteristics, the length (L_{chl}) and thickness (T_{chl}) of the chloroplasts, and the distance of the chloroplast from the cell wall (L_{cyt}) seemed unaffected by N forms supply, whereas NH_4^+ supply significantly elevated the distance between adjacent chloroplasts ($D_{\text{chl-chl}}$). Besides, chloroplasts amount in per sponge cell and palisade cell showed more specific changes among the treatments. In the comparison with the other treatments, chloroplasts of both sponge and palisade cell were dramatically up-regulated in sole NO_3^- supply (Supplementary Figure S1).

Besides, g_m was not correlated with T_{mes} , reflecting the circumstance that T_{mes} seemed more invariable. The positive and significant correlation between g_m and S_i/S_a , f_{ias} were observed, combined with the negative relationship observed between g_m and T_{cw} suggested the importance of anatomical components to the intercellular CO_2 diffusion.

Estimation of g_m with different methods

In the present study, the value of g_m was measured according to the gas exchange and chlorophyll fluorescence (Harley *et al.*, 1992), and compared with g_m modelled by A/C_i response curves (Bernacchi *et al.*, 2002) and anatomical characteristics (Tomás *et al.*, 2013). The correlation analysis indicated that the estimated g_m from different methods was mainly positively correlated (Supplementary Figure S3). However, the slope was different from unity, and the Harley *et al.*-based and Tomás *et al.*-based estimates of g_m were strongly correlated with a R^2 of 0.76 ($P < 0.01$).

Limiting components analyses of A

The relative limitations of stomatal conductance (l_s), mesophyll diffusion (l_m), and biochemistry capacity (l_b) on photosynthesis are shown in Figure 3(a). In the NO_3^- -treatment and mixed N treatment, the percentages of the three limiting components are relatively close, and l_b (36.4% and 39.5%, respectively) accounts for the most important relative limitation of photosynthesis. While for sole NH_4^+ supply, the relative limitation of l_s , l_m , and l_b significantly varied, and the l_m (44.2%) was considered more important of photosynthesis, followed by l_s (30.8%) and l_b (25.0%).

374 Meanwhile, according to quantitative limitations analysis of A , mesophyll
375 conductance limitation (MC_L) was the highest constrain of A under sole NH_4^+ supply,
376 accounting for the 24.39 % of A decline compared with the NO_3^- treatment (Figure 3).

377 **Key structural factors regulating A through g_m**

378 For the different components of the whole CO_2 diffusion pathway, restrictions in
379 liquid-phase CO_2 conductance were the dominant limiting factors on g_m , accounting
380 for approximately 90% of the total limitation (Figure 4b). Among the different
381 components of the liquid-phase, the stroma limitation was the main limiting factor in
382 the liquid-phase, whereas the stroma limitation seemed unaffected by the treatments.
383 The liquid-phase limiting component of g_m is also associated with the cytoplasm, with
384 significantly upregulated by sole NH_4^+ supply. In comparison with the sole NH_4^+
385 supply, plasma membrane and chloroplast envelope limitation proposed a slightly
386 higher percentage in sole NO_3^- supply, with 24.3% and 24.3%, respectively. For the
387 absolute value of the limitations, the cell walls appeared to have a more specific
388 change among the treatments that limited the internal diffusion of CO_2 varied from
389 303.69 to 480.37 $s\ m^{-1}$ (Figure 4a). Besides, the variation in cytoplasm resistance was
390 1.7-fold, with sole NO_3^- treatment having the lowest resistance (450.13 $s\ m^{-1}$) and
391 sole NH_4^+ supply having the highest (664.23 $s\ m^{-1}$).

392 **Discussion**

393 **g_m dominated the decrease of A in sole NH_4^+ supply**

394 In the present study, inorganic N sources significantly decreased the
395 photosynthetic rate (A) of *Lonicera Japonica*. Leaf in sole NH_4^+ treatment was
396 observed a dramatically downregulated A , in parallel with a larger dropdown of CO_2
397 from the sub-stomatal cavities (C_i) to the sites of carboxylation inside the chloroplasts
398 (C_c). Among the limiting factors, mesophyll conductance limitation (MC_L) controlled
399 60.3 % of A decline, followed by stomatal conductance (S_L , 26.4%) and biochemistry
400 limitation (B_L , 13.3%). These results suggested the mesophyll diffusion resistance to
401 CO_2 is a key limiting factor to A . Early studies suggested that g_m was driven by

integrated leaf characteristics, notably and negatively correlated with leaf mass per area (M_A) (Flexas *et al.*, 2008; Han, 2011; Niinemets *et al.*, 2009a; Onoda *et al.*, 2017). Nevertheless, the M_A did not show any significant difference among treatments in this study, despite some declining tendency of sole NO_3^- treatment. Whilst the unchanged M_A , the present data showed that the NH_4^+ -fed leaves were manifested by higher leaf thickness (T_L) and density (D_L), and a smaller leaf area (A_L) and leaf volume (V_A) (Table 1). The leaf traits are generally observed in NH_4^+ -fed plants compared to those supplied with NO_3^- (Guo *et al.*, 2007), suggesting the high leaf structure plasticity and varied leaf carbon-expensive structure in relation to environmental conditions.

As there is ample agreement that the use of integrated traits such as M_A as proxies of g_m might not be valid in all cases, the intercellular anatomical traits that limit CO_2 effective diffusion length and area, in particularly the cell wall thickness (T_{cw}) are especially crucial (Evans *et al.*, 2009; Momayyezi and Guy, 2017; Tosens *et al.*, 2012b). In the present study, a one-dimensional within-leaf gas diffusion model considering all of the leaf anatomical limitations was applied as previously modified by Tomas *et al.* (2013). The g_m modelled from anatomical traits was tightly correlated with measured g_m (Supplementary Figure S3), which, to some extent, verified the view that the variation of g_m is related to the intercellular structures involved in CO_2 diffusion. Nevertheless, the partial conductance components are generally assumed to be composed of a single medium in the estimation of g_m , which might be unrealistic (Gago *et al.*, 2020). In addition, the carbonic anhydrases, as well as aquaporins were not considered in the g_m -modelled (Flexas *et al.*, 2012). Consequently, the correlation between g_m -gas exchanges and g_m -modelled frequently deviates from the 1:1 relationship.

Mesophyll conductance is a composite conductance of an intercellular gas-phase (g_{ias}) and a liquid-phase (g_{liq}). The g_{ias} mainly depends on the intercellular air space and mesophyll thickness, while the g_{liq} was affected by apoplast and cellular components of the CO_2 pathway, which can be scaled by the chloroplast surface area

exposed to intercellular air space per unite leaf area (S_c/S) (Flexas *et al.*, 2012). It was observed that the limitations were mainly represented by liquid-phase, and the gas-phase accounts for only approximately 10% (Figure 4b), analogous to the value obtained in rape (Lu *et al.*, 2016). Despite the low proportion, a significant discrepancy of g_{ias} among the treatments appeared, in that gas-phase limitation was upregulated by sole NH_4^+ supply. This result was somewhat inconsistent with Liu *et al* (2021) and Gao *et al* (2020), as NO_3^- caused higher limitation on g_{ias} . This could be explained by the variation of the environment light intensity, concentrations of N addition, experiment period as well as the plant species. Integrated all the determinants into account, there was a positive correlation between g_m and the volume fraction of intercellular air space (f_{ias}) ($R^2 = 0.71$, $P < 0.01$), while a weak relationship with the mesophyll thickness (Figure 5), suggesting that f_{ias} was more variable in regulating g_m . Additionally, f_{ias} under sole NO_3^- supply was approximately 2-folds higher than that of sole NH_4^+ supply, similar to the result of Liu *et al* (2021). The flexible f_{ias} were general in plants, which may be ascribed to acclimation-related changes, e.g., a larger f_{ias} was usually observed in more favorable growth conditions (Binks *et al.*, 2016; Muller *et al.*, 2009).

Structural trade-off in driving the share of A limitations

As suggested in various studies, S_c/S is more influential in determining g_m across species (Loucos *et al.*, 2017; Ren *et al.*, 2019; Terashima *et al.*, 2011; Veromann-Jürgenson *et al.*, 2020; Veromann-Jurgenson *et al.*, 2017). Here, the down-regulation of S_c/S by sole NH_4^+ supply was speculated to be attributed to the fewer chloroplast numbers and the lower mesophyll surface area exposed to intercellular airspace per leaf area (S_{mes}/S) (Table 3), similar to the observation of (Carriqui *et al.*, 2015); while the length and thickness of chloroplasts were unchanged among the treatments. Despite the dropdown of r_{st} , a fact that cannot be ignored is the relative control of chloroplast stroma on g_{liq} was less affected by N forms, with no significant change among the treatments.

There rises a crucial concern that whether g_m was necessarily controlled by the component owning the largest proportion, because the contribution of components that limit CO_2 diffusion may vary among species. Here, the discrepancy in liquid-phase resistance was suggested to be related to the cytoplasm and cell wall. For cytoplasm, it accounts for approximately 15% of liquid resistance in the present study, corresponds well with previous reports (Lu *et al.*, 2016; Tosens *et al.*, 2012a; Tosens *et al.*, 2012b). Commonly, the chloroplasts are arranged against the cell periphery to absorb light and CO_2 (Sage *et al.*, 2009), thus the distance of chloroplast from cell wall (L_{cyt}) could be responsible for most of the cytoplasm resistance, as reported by (Sharkey *et al.*, 1991). However, L_{cyt} was the same among the treatments (Table 3) in this study, and therefore, the upregulated r_{cyt} in NH_4^+ treatment may be explained by the increase of distance between adjacent chloroplasts ($D_{\text{chl-chl}}$), by up to 50% compared with mixed N treatment.

Previous studies have highlighted the importance of cell wall in determining g_m among species, as the cell wall could account for 50% of g_m by restricting CO_2 diffusion (Evans *et al.*, 2009; Terashima *et al.*, 2006). Although the limitation is often neglected, cell wall limitation sometimes is greater than S_c/S , and together these constitute the primary anatomical factors for setting the maximum g_m (Carriquí *et al.*, 2019; Carriqui *et al.*, 2019). Here, the cell wall thickness (T_{cw}) varied over a range of 0.16 to 0.25 μm , and T_{cw} of sole NO_3^- treatment decreased by 27% to 36% in the comparison with mixed N and sole NH_4^+ supply, resulting in a dramatic difference among the treatments. Głazowska *et al.* (2019) had reported that the distinct cell wall remodeling was mediated by inorganic N supply. NH_4^+ -mediated cell wall thicken, as observed in early studies may be ascribed to changes in the contents of polysaccharide, lignin, or cellulose of cell walls (Ellsworth *et al.*, 2018; Podgórska *et al.*, 2017). Meanwhile, as previously mentioned, a more rigid cell wall structure could explain the reduction in A_L in ammonium supply, by limiting cell expansion. Besides, the T_{cw} and g_m were observed strongly and positively correlation in this study (Figure 5 (b),

486 $R^2 = 0.53$, $P < 0.01$), the reduced g_m and thicker cell wall were reported to be an
487 adaption of plant species to dry or nutrition poor environment (Niinemets *et al.*, 2009a;
488 Niinemets *et al.*, 2009b).

489 Recent evidence points to an effect of cell wall composition on photosynthesis,
490 possibly due to a trade-off of N allocation between chloroplasts and the cell wall in
491 plants (Kuusk *et al.*, 2018). In angiosperms, particular in C3 plants, chloroplast N
492 distribution accounts for almost 75 %, while the cell wall for 10% (Li *et al.*, 2017;
493 Onoda *et al.*, 2017; Wang *et al.*, 2015); the leaf N that is not allocated to
494 photosynthetic apparatus is generally used structurally in cell walls (Feng *et al.*, 2009).
495 In this study, the reduced chloroplasts numbers and the thicker T_{cw} by sole NH_4^+
496 supply were speculated to be related to the down-regulated photosynthetic N
497 allocation (Figure S1). Takashima *et al* (2004) had reported that higher N allocation to
498 the cell wall could lead to decreased PNUE. The discrepancy of N partitioning among
499 N sources may indicate a trade-off in the leaf photosynthetic capacity and the
500 persistence, while the mechanism underlying this result need further research.

501

502 **Conclusion**

503 The present study showed that N sources significantly affected the leaf
504 morphology and photosynthetic rate (A) of *L. Japonica* and suggested the mesophyll
505 diffusion resistance accounts for the most limiting of A , with more than 50%. Sole
506 NH_4^+ -fed leaves were characterized by smaller leaf area, higher leaf thickness, and
507 larger leaf density. Variations of mesophyll conductance (g_m) under different N
508 sources were ascribed to the leaf anatomy changes, notably the internal air space (f_{ias}),
509 exposed surface area of chloroplasts per unit leaf area (S_c/S) and cell wall thickness
510 (T_{cw}). Ammonium treatment reduced the f_{ias} and chloroplast numbers, resulting in an
511 increased intercellular length and inter-chloroplast length, and finally inhibition of g_m
512 and A (Figure 6).

513

514

515 **Acknowledgments**

516 This work was financially supported by the National Natural Science Foundation of
517 China (32072673), the Fundamental Research Funds for the Central Universities
518 (KYGD202007), the Young Elite Scientists Sponsorship Program by CAST
519 (2018QNRC001), and the Innovative Research Team Development Plan of the
520 Ministry of Education of China (IRT_17R56).

521

522 **Author contributions**

523 Shiwei Guo and Yiwen Cao conceived the idea and designed the experiment;
524 Yiwen Cao, Yonghui Pan, and Tianheng Liu completed the experiment; Yiwen Cao
525 analyzed the data and wrote the manuscript; Shiwei Guo, Min Wang, and Yonghui
526 Pan helped in manuscript revising; Shiwei Guo and Min Wang provided funding
527 support. All the authors contributed critically to the drafts and gave final approval for
528 publication.

529

530 **Conflict of interest**

531 The authors declare that the research was conducted in the absence of any
532 commercial or financial relationships that could be construed as a potential conflict of
533 interest.

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Tables

Table 1. Effect of different N forms on integral leaf variables

Treatment	M_A (mg cm ⁻²)	V_A (cm ³)	T_L (μm)	D_L (g cm ⁻³)	A_L (cm ²)	Chl (mg g ⁻¹)	N_a (g m ⁻²)	PNUE (μmol g ⁻¹ s ⁻¹)
A	4.45±0.49a	0.10±0.02b	114.7±10.5a	0.36±0.06a	8.43±1.48b	4.01±0.76a	1.53±0.12a	9.52±1.97c
AN	4.76±1.46a	0.20±0.02a	106.5±9.6b	0.40±0.12a	13.81±1.83a	4.18±0.42a	1.43±0.21a	13.16±1.70b
N	4.01±0.71a	0.19±0.03a	108.9±9.9b	0.27±0.04b	13.32±0.93a	4.36±0.60a	1.18±0.11b	16.87±1.80a

M_A , leaf mass per area; V_A , leaf volume per area; T_L , leaf thickness; D_L , leaf density; A_L , leaf area; Chl, total chlorophyll concentration per leaf; N_a , leaf nitrogen content per area; PNUE, photosynthetic nitrogen use efficiency, $PNUE = A/N_a$. A, AN, and N represent the sole NH_4^+ supply, mixed N supply, and sole NO_3^- supply, respectively. Data are mean ± SD of 4 replications. Different letters indicate statistically significant differences ($P < 0.05$).

Table 2. CO₂ transmission characteristics of *L. Japonica* affected by different N forms

Treatment	A ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	g_s ($\text{mol m}^{-2} \text{s}^{-1}$)	C_i ($\mu\text{mol mol}^{-1}$)	C_c ($\mu\text{mol mol}^{-1}$)	C_i-C_c ($\mu\text{mol mol}^{-1}$)	g_m ($\text{mol m}^{-2} \text{s}^{-1}$)	J ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	CE
A	14.3±0.9b	0.23±0.05b	277.0±14.6a	160.8±13.6b	107.3±16.2a	0.13±0.03b	122.8±8.4b	0.070±0.005b
AN	18.1±2.5a	0.35±0.10a	275.1±11.8a	188.8±8.0a	86.3±7.9b	0.21±0.04a	138.3±13.4ab	0.085±0.006a
N	19.9±0.5a	0.35±0.01a	266.8±3.1a	177.7±2.0a	89.1±4.2b	0.22±0.01a	156.4±4.4a	0.095±0.011a

A , net photosynthetic rate; g_s , stomatal conductance; C_i , intercellular CO₂ concentration; C_c , chloroplast CO₂ concentration; C_i-C_c , CO₂ drawdown from sub-stomatal cavities (C_i) to chloroplasts (C_c); g_m , mesophyll conductance; J , electron transfer rate; CE , carboxylation efficiency. A, AN, and N represent the sole NH₄⁺ supply, mixed N supply, and sole NO₃⁻ supply, respectively. Data are mean ± SD of 5 replications for A , g_s , C_i , C_c , C_i-C_c , g_m , J , and of 4 replications for CE ; Different letters indicate statistically significant differences ($P < 0.05$).

Table 3. Leaf anatomical structure of *L. Japonica* affected by different N forms

Treatment	T_{mes} (μm)	T_{cw} (μm)	L_{chl} (μm)	T_{chl} (μm)	$D_{\text{chl-chl}}$ (μm)	L_{cyt} (μm)	f_{ias} (%)	S_{mes}/S ($\text{m}^2 \text{m}^{-2}$)	S_{c}/S ($\text{m}^2 \text{m}^{-2}$)
A	83.5 $\pm$ 7.5b	0.25 $\pm$ 0.02a	6.10 $\pm$ 0.96a	2.03 $\pm$ 0.20a	1.08 $\pm$ 0.04a	0.21 $\pm$ 0.03a	17.1 $\pm$ 4.3b	11.4 $\pm$ 0.4b	6.16 $\pm$ 0.21b
AN	83.5 $\pm$ 4.1b	0.22 $\pm$ 0.03b	5.54 $\pm$ 0.47a	1.98 $\pm$ 0.22a	0.72 $\pm$ 0.03b	0.20 $\pm$ 0.03a	33.4 $\pm$ 0.03a	13.8 $\pm$ 0.4a	8.96 $\pm$ 0.20a
N	86.3 $\pm$ 4.4a	0.16 $\pm$ 0.02c	5.58 $\pm$ 0.58a	1.77 $\pm$ 0.15a	0.84 $\pm$ 0.03b	0.19 $\pm$ 0.04a	37.0 $\pm$ 2.3a	12.8 $\pm$ 0.4a	9.44 $\pm$ 0.43a

T_{mes} , mesophyll thickness; T_{cw} , cell wall thickness; L_{chl} , chloroplast length; T_{chl} , chloroplast thickness; $D_{\text{chl-chl}}$, the distance between adjacent chloroplasts; L_{cyt} , the distance of chloroplast from cell wall; f_{ias} , the volume fraction of intercellular air space; S_{mes}/S , mesophyll surface area exposed to intercellular airspace per unit leaf area; S_{c}/S , chloroplast surface area exposed to intercellular air space per leaf area. A, AN, and N represent the sole NH_4^+ supply, mixed N supply, and sole NO_3^- supply, respectively. Data are mean $\pm$ SD with 20 microscopic pictures for T_{mes} , f_{ias} , S_{mes}/S , S_{c}/S , and at least 30 microscopic pictures for the other ultrastructural traits; Different letters indicate statistically significant differences ($P < 0.05$).

Figure legends

Figure 1. Light micrographs (a, e, i, scale bar = 50 μm) and transmission electron micrographs (b, f, j, c, g, k, scale bar = 2 μm ; h, scale bar = 1 μm ; d, l, scale bar = 800 nm) of *L. Japonica* leaves affected by different forms of N, sole NH_4^+ supply (Fig. a-d), mixed N supply (Fig. e-h), and sole NO_3^- supply (Fig. i-l). ias: intercellular air space; SG: starch granule; CW: cell wall; G: grana.

Figure 2. Correlation of the net photosynthetic rate (A) and mesophyll conductance (g_m) (a), stomatal conductance (g_s) (b), electron transport rate (J) (c), and the relationship between CO_2 drawdown ($C_i - C_c$) from sub-stomatal cavities (C_i) to chloroplasts (C_c) and g_m (d). A, AN, and N represent the sole NH_4^+ supply, mixed N supply, and sole NO_3^- supply, respectively, corresponding to the symbol of the open triangles, the closed circles, and the open square in the plot. The data were fitted by linear regressions ($P < 0.01$).

Figure 3. Relative limitation of stomatal conductance (l_s), mesophyll diffusion (l_m), and biochemical capacity (l_b) of *L. Japonica* under sole NH_4^+ supply (a), mixed N supply (b), and sole NO_3^- supply treatment (c), the limitation of l_s , l_m , and l_b together add up to 100% at each treatment occasions; Quantitative limitation analyses of stomatal limitation (S_L), mesophyll conductance limitation (MC_L), and biochemical limitation (B_L) of *L. Japonica* photosynthesis under sole NH_4^+ supply (d), the data outside the circles represent the absolute quantitative limitations and the data inside the circles represent the share of the total limitations by S_L , MC_L , and B_L .

Figure 4. Anatomical limitations of mesophyll conductance (g_m) (a) and the share of the overall g_m limitation (b) by cell wall (cw), plasma membrane (pl), chloroplast envelope (env), stroma (st), and cytoplasm (cyt) in leaves of *L. Japonica* under different forms of N. A, AN, and N represent the sole NH_4^+ supply, mixed N supply, and sole NO_3^- supply, respectively. The inset figures showed the anatomical limitations of g_m and the share of the overall g_m limitation by gas-phase and liquid-phase. Different letters indicate statistically significant differences ($P < 0.05$).

Figure 5. Correlations of mesophyll conductance (g_m) with the intercellular air space (f_{ias}) (a), the volume fraction of cell wall thickness (T_{cw}) (b), the chloroplast

surface area exposed to intercellular air space per leaf area (S_c/S) (**c**), and mesophyll thickness (T_{mes}) (**d**). A, AN, and N represent the sole NH_4^+ supply, mixed N supply, and sole NO_3^- supply, respectively, corresponding to the symbol of the open triangles, the closed circles, and the open square in the plot. The data of each group was fitted by linear regressions ($P < 0.01$).

Figure 6. A schematic model of *L. Japonica* leaf anatomical traits and CO_2 diffusion pathway under different forms of N supply. Leaf ultrastructure model in sole NH_4^+ supply (**a**), mixed N supply (**b**), and sole NO_3^- supply (**c**), respectively. CO_2 diffusion pathways inside the mesophyll cells in sole NH_4^+ supply, mixed N supply, and sole NO_3^- supply, respectively (**d**). The chloroplast surface area exposed to intercellular air space per leaf area (S_c/S) was outlined with yellow lines in (b), and the mesophyll surface area exposed to intercellular airspace per unit leaf area (S_{mes}/S) was marked with purple lines in (c). The loose arrangement of mesophyll cells as affected by sole NO_3^- supply increased the intercellular airspace and consequently up-regulated the S_c/S and S_{mes}/S (**b**, **c**). Increased chloroplast density resulted in reduced distance between two adjacent chloroplasts ($D_{chl-chl}$) in sole NO_3^- -fed leaves (**c**). The red folded lines and black folded lines represent the strength of CO_2 diffusion resistance into the cell from cell wall (g_{cw}), plasma (g_{pl}), cytoplasm (g_{cyt}), envelope (g_{env}) and stroma (g_{st}), while the red folded lines indicated the values of this component differed between treatments and the black folded lines means no difference between treatments or not been measured in the study (**d**). The blue dot represents the CO_2 concentration (**d**). T_L , leaf thickness; W, the width of the leaf section; cw, cell wall; pl, plasm; c, chloroplast.

Sole NH_4^+ supply

Mixed N supply

Sole NO_3^- supply

(d)