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**Anatomical variation of mesophyll conductance due to salt stress
in *Populus cathayana* females and males growing
under different inorganic nitrogen sources**

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Running title: Sexually different responses to salt and N forms

Abstract Synergistic regulation in leaf architecture and photosynthesis is essential for salt tolerance. However, how plant sex and inorganic nitrogen sources alter salt stress-dependent photosynthesis remains unknown. Leaf anatomical characteristics and photosynthesis of *Populus cathayana* Rehder females and males were investigated under salt stress conditions combined with NO_3^- and NH_4^+ supplies to clarify the underlying mechanisms. In salt-stressed females, we observed an increased mesophyll spongy cell density, a reduced chloroplast density, a decreased surface area of chloroplasts adjacent to the intercellular air space (S_c/S) and an increased mesophyll cell area per transverse section width (S/W), consequently causing mesophyll conductance (g_m) and photosynthesis inhibition, especially under NH_4^+ supply. Conversely, males with a greater mesophyll palisade tissue thickness and chloroplast density, but a lower spongy cell density had lower S/W and higher S_c/S , and higher g_m and photosynthesis. NH_4^+ -fed females had a lower CO_2 conductance through cell wall and stromal conductance perpendicular to the cell wall, but a higher chloroplast conductance from the cell wall (g_{cytl}) than females supplied with NO_3^- , while males had a higher chloroplast conductance and lower CO_2 conductance through cell wall when supplied with NO_3^- instead of NH_4^+ under salt stress. These findings indicate sex-specific strategies in coping with salt stress related to leaf anatomy and g_m under both types of N supplies, which may contribute to sex-specific CO_2 capture and niche segregation.

Keywords: Leaf anatomy, mesophyll conductance, N form, salt stress, sexual dimorphism

Introduction

Salt stress reduces plant biomass and crop production, and these changes are typically strongly correlated with a decreasing rate of photosynthesis (Praxedes et al. 2010, Wankhade et al. 2013). Photosynthesis is a critical factor that determines energy conversion efficiency, thereby regulating plant productivity and performance (Pinheiro and Chaves 2011, Ort et al. 2015). It has been suggested that CO₂ stomatal limitations, including stomatal (g_s) and mesophyll (g_m) conductance limitation, are primary factors restricting leaf photosynthesis (A) in salt-stressed leaves (Flexas et al. 2016, Zait et al. 2019, Veromann-Jürgenson et al. 2020). Leaf dehydration induced by drought and/or salt stress reduces g_s to control the transpirational water flow from leaves (Golldack et al. 2014). However, lower g_s reduces CO₂ flux into leaves, and then results in the limitation of A and carbon starvation in plants. The g_m value indicates CO₂ diffusion from the sub-stomatal cavities to the sites of carboxylation. According to a previous simulation performed by Zhu et al. (2010), doubling of g_m could improve photosynthetic rates by nearly 20% under normal growth conditions. In stressed plants, the photosynthetic limitation due to g_m can be much greater than in non-stressed plants (Martins et al. 2014, Niinemets and Keenan 2014). Therefore, it is important to clarify, whether and how salt stress affects g_m and then leaf A in dioecious plants.

67 The g_m values of given genotypes or species are associated with changes in leaf
68 anatomical traits, especially under environmental stresses (Niinemets et al. 2009a, Lu
69 et al. 2016). Generally, leaves with a robust structure and higher *LMA* show lower g_m
70 and photosynthesis (Niinemets et al. 2009b, Tomás et al. 2013). Plants with higher
71 *LMA* usually have a greater biomass investment in supporting tissues, thicker cell
72 walls, a lower cytoplasm and higher stroma conductance to CO₂ diffusion inside
73 leaves (Niinemets 2007, Tomás et al. 2013). Inside a leaf, CO₂ from substomatal
74 cavities diffuses through the intercellular air space and liquid phase consisting of the
75 cell wall, cytoplasm, plasma membrane, chloroplast stroma and envelope (Tosens et al.
76 2012b, Cano et al. 2013). The CO₂ liquid-phase resistance accounts for 80-90% of
77 mesophyll resistance (Lu et al. 2016, Tosens et al. 2016).

78

79 Among CO₂ liquid-phase diffusion components, the cell wall thickness of the leaf
80 mesophyll and the chloroplast surface area exposed to intercellular air space (S_c/S)
81 have been considered as the strongest limiting factors of g_m , but these traits are highly
82 different among species (Tomás et al. 2013, Berghuijs et al. 2016). An increased
83 mesophyll cell wall thickness and decreased S_c/S can probably increase the CO₂
84 diffusion path length and area, which will limit g_m in plants (Terashima et al. 2011,
85 Tomás et al. 2013). In addition, the increased mesophyll surface area exposed to the
86 intercellular air space (S_m/S) facilitates CO₂ diffusion from the cytosol into
87 chloroplasts, which promotes photosynthesis via greater g_m (Ren et al. 2019, Hu et al.

2020). Other cellular components, such as chloroplasts, plasmalemma and cytoplasm also affect CO₂ diffusion into leaves (Tomás et al. 2013, Lu et al. 2016). A higher mesophyll cell density typically decreases the air space size, which probably decreases the CO₂ gas-phase conductance (Tomás et al. 2013, Lu et al. 2016). Also, a recent study has indicated that a large total mesophyll cell area per transverse section width (S/W) is advantageous as balancing the leaf structure and CO₂ assimilation (Hu et al. 2020). Therefore, knowledge of leaf structural and anatomical traits affecting g_m is crucial for understanding photosynthetic efficiency, especially under abiotic stresses.

Generally, salt stress induces leaf dehydration and leads to the reduction of g_m and g_s in plants (Wang et al. 2018, Zait et al. 2019). Leaf dehydration reduces the internal air space volume and increases the cell wall thickness (Henry et al. 2000, Tosens et al. 2012a). The size and shape of chloroplasts and mesophyll cells are also strongly affected by leaf dehydration (Scoffoni et al. 2017). Such changes probably have major implications for g_m (Tosens et al. 2012a) and suggest that salt stress may lead to analogous changes. How g_m is affected by salinity is still an open question, especially in trees. Moreover, little is known about the effects of salt stress on leaf anatomy and photosynthesis under different inorganic N sources, e.g. NH₄⁺ vs. NO₃⁻.

Poplars are dioecious species, in which females and males have significant differences in biomass partitioning, photosynthesis, carbon-nutrient balance and leaf anatomical

traits when exposed to abiotic stresses, including salt stress (Li et al. 2016, Chen et al. 2016, Liu et al. 2020a, Liu et al. 2021), which is further affected by N forms (Chen et al. 2011, Liu et al. 2020b). Poplars preferentially take up NO_3^- when both NH_4^+ and NO_3^- are given at the same concentration, and growth may be accelerated despite high energy requirements (Bi et al. 2020). Energy costs do not differ between NO_3^- and NH_4^+ when the assimilation of NO_3^- occurs in the shoots, especially under abiotic stress (Guo et al. 2007). Interestingly, poplar females have a higher specific root length (SRL), which indicates a lower carbon cost but a weaker root exploration capacity compared to males, while lower SRL in males may facilitate the utilization of less-mobile NH_4^+ (Xia et al. 2020). Sex- and N form-dependent root system architecture investments largely reflect energy requirement for foliage construction and, thereby, may affect g_m and photosynthesis. Niinemets (1999) has proposed that the fraction of the annual biomass invested in foliage decreases in older trees, which results from biomass being largely invested in roots and stems. As discussed above, we hypothesize that N forms affect root biomass allocation with further modifications of leaf anatomy and g_m .

In this study, we employ *Populus cathayana* to examine sex-specific adaptive strategies to salt stress under NO_3^- and NH_4^+ supplies with the following hypotheses: (1) sex-specific trade-offs between leaf anatomical properties and g_m determine sex-specific differences in salt adaptation; (2) N forms affect root energy allocation, with further modifications in leaf anatomy and g_m under salt stress in a sex-dependent

manner. This study will not only help us to improve understanding of the response of foliar architecture to photosynthesis under salt stress and different N forms, but it will aid to predict photosynthetic behaviour in tree species with different N source preferences and spatiotemporal changes in N assimilation at rising atmospheric CO₂.

Materials and methods

Plant material and experimental design

Cuttings of *P. cathayana* were obtained from different trees of each sex from populations in riparian and valley flat habitats of the Qinghai Province, China (30° 67' N, 104° 06' E), as explained in detail by Liu et al. (2020a). The cuttings were planted in March 2019 at the Hangzhou Normal University, China, (30° 01' N, 104° 07' E). The seedlings were cultivated in a semi-controlled greenhouse with a daytime temperature of 21-25 °C, a night-time temperature of 15-18 °C, a photoperiod of 12-14 h, and a relative humidity of 76-81% throughout the growth period. After growing for 5 weeks, 32 uniform and healthy seedlings (16 females and 16 males) with heights of 30-40 cm were transplanted into 10-l plastic pots with a 10-kg mixture of sand, vermiculite and perlite (1:1:1).

The experiment was completely randomized with two sexes (males, females), two N regimes (NO₃⁻, NH₄⁺), and two NaCl levels (-NaCl, +NaCl). Each treatment was

replicated four times. The plants were fertilized every 2 days with 200 ml nutrient solution (pH = 6.0) containing either 3.75 mM NH_4^+ or 3.75 mM NO_3^- as the N source (see Table S1 for the full composition of nutrient solutions). After the seedlings had grown for a further 30 days, half of the cuttings were treated with 200 ml NaCl (200 mM) administered every day until the final NaCl concentration reached 50 mM kg^{-1} growth substrate. Each day, the control cuttings received the same amount of distilled water.

Gas exchange and fluorescence measurements

Chlorophyll fluorescence and gas exchange characteristics of mature leaves were measured with a portable photosynthesis measuring system (LI-6400; Li-Cor Inc., Lincoln, NE, USA) equipped with an integrated leaf chamber fluorometer (LI-6400-40). The measurements were conducted between 09:00 h and 16:30 h. The standard environmental conditions during measurements of leaf photosynthetic characteristics were as follows: leaf temperature of 25 °C, saturating photon quantum flux density (PPFD) of 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (with 90% red light and 10% blue light), relative humidity of 75%, air flow rate of 500 $\mu\text{mol s}^{-1}$ and CO_2 concentration in the cuvette (C_a) of 400 $\mu\text{mol mol}^{-1}$. After leaf gas exchange rates stabilized, typically in 20-30 min, the steady-state fluorescence yield (F_s) was recorded, and a saturating light flash of 8000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 0.8 s was given to measure the maximum fluorescence yield at the light-adapted state (F_m'). For measurements of chlorophyll

fluorescence, leaves were adapted to the dark for 30 min using an aluminium foil cover. The minimum fluorescence (F_o) and maximum fluorescence (F_m) were measured. After these measurements, CO₂ response curves were measured with C_a lowered stepwise from 400 to 300, 200, 100 and 50 $\mu\text{mol mol}^{-1}$, and then raised from 50 to 400, 600, 800 and 1000 $\mu\text{mol mol}^{-1}$, while all other environmental conditions were maintained. The chlorophyll fluorescence characteristics, F_s and F_m' , were also measured at each C_a .

Estimation of g_m by chlorophyll fluorescence and gas exchange

The photochemical efficiency of photosystem II (Φ_{PSII}) was calculated according to Genty et al. (1989):

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

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

$$J = \Phi_{\text{PSII}} \cdot Q \cdot \alpha \cdot \beta \quad (2)$$

where the product $\alpha\beta$ (β is the fraction of light absorbed by PSII, and α is leaf absorptance) is obtained from the correlation between photosynthetic rate (A) and Q

• $\Phi_{PSII}/4$ using the light response curve under non-photorespiratory conditions ($[O_2] < 1.0\%$) (Yin et al. 2009). The light response curve under low $[O_2]$ was measured based on the method of Lu et al. (2016). Q is PPFD.

The CO_2 response curves were used to estimate g_m according to the variable J method proposed by Harley et al. (1992); g_m was calculated as follows:

$$g_m = \frac{A}{C_i - \frac{I^*(J + 8(A + R_d))}{J - 4(A + R_d)}} \quad (3)$$

where R_d is the dark respiration rate continuing in light, and I^* is the hypothetical CO_2 compensation point in the absence of R_d (Bernacchi et al. 2001, Tosens et al. 2012b). Then, g_m was used to transform A/C_i response curves into A/C_c response curves by the equation $C_c = C_i - A/g_m$ and $I^* = C_i^* + R_d/g_m$. C_c was used to calculate the maximum velocity of carboxylation and the photosynthetic electron transport according to the method of Bernacchi et al. (2002). Each g_m was replicated four times for every specimen. R_d and C_i^* were measured according to Brooks and Farquhar (1985). Briefly, the A/C_i curve was generated at PPFD of 150, 300 and 600 $\mu mol\ m^{-2}\ s^{-1}$, with each having four C_a chambers (50, 75, 100 and 150 $\mu mol\ CO_2\ mol^{-1}$). The x-axis and y-axis of the intersection point of three A/C_i curves represented C_i^* and R_d , respectively.

Estimation of leaf construction costs

Leaf construction costs [CC, g glucose (g dry mass)⁻¹] were calculated from ash [g g⁻¹(d.m.)], N [g g⁻¹(d.m.)] and H_c [kJ g⁻¹(d.m.)] according to Williams et al. (1987):

$$CC = \{[(0.06968 \times H_c - 0.065) (1 - \text{ash})] + [(K \times N/14.0067)(180.15/24)]\}/0.89 \quad (4)$$

where K is the substrate of nitrogen oxidation state, +5 for nitrate and -3 for ammonium. H_c is the ash-free heat of combustion.

Microscopic observations and anatomical measurements

Leaf segments (1×1 mm) were taken from leaves avoiding major veins, and fixed in a formaldehyde, acetic acid and alcohol solution (1:1:8 v/v) under vacuum for 20 min. Thereafter, the samples were thoroughly washed three times with deionized water and dehydrated in a graded ethanol series. Sections were embedded in glycol methacrylate under vacuum. Semi-thin cross-sections were stained with safranin-fast green and viewed under a light microscope (Nikon Corporation, Kyoto, Japan) at 20× magnification. The following characteristics were measured from light micrographs: the thickness, size and density of leaf palisade and spongy tissues, and the sum of spongy and palisade tissue cell area per transverse section.

In addition, leaf samples were cut and fixed in 4% (v/v) glutaraldehyde in sodium

phosphate buffer (0.2 M, pH = 7.2) overnight. The samples were thoroughly washed three times with a sodium phosphate buffer and then post-fixed in 1% (w/v) osmium tetroxide for 1 h. After dehydration in a graded ethanol series, the samples were immersed in 1:3 (v/v) Spurr's resin and acetone. The dehydrated samples were embedded in Spurr's resin and dried on glass slides. Ultrathin (90 nm) cross sections were stained with 2.0% uranyl acetate (w/v) and lead citrate, and viewed with a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan). Cell wall thickness (T_{cw}), the distance between two neighbouring chloroplasts (ΔL_{chl}), cytoplasm thickness (T_{cyt}), chloroplast distance from the cell wall ($\Delta L_{cyt,l}$), and chloroplast thickness (T_{chl}) and length (L_{chl}) were measured at 20,000-40,000 $\times$ magnification. Each anatomical characteristic per specimen was determined from four to six different fields of view for each section and calculated based on the method of Tomás et al. (2013). All photographs were analysed with Image J (Wayne Rasband/NIH, Bethesda, MD, USA).

S_c/S and mesophyll surface area (S_m/S) exposed to intercellular air space per leaf area were calculated as follows:

$$S_c/S = \frac{L_{mes}}{W} F \quad (5)$$

$$S_m/S = \frac{L_c}{L_{mes}} S_m/S \quad (6)$$

where L_c and L_m are the distances of the chloroplast surface area and the mesophyll cells facing the intercellular air space, respectively; W is the width of the measured section; and F is the cell curvature correction factor, which is calculated according to the method of Thain et al. (1983). The values of F ranged from 1.4-1.5 for palisade cells and 1.16–1.4 for spongy cells.

The fraction of intercellular air space (f_{ias}) in leaf mesophyll, f_{ias} was estimated as:

$$f_{ias} = 1 - \frac{\sum S_s}{t_{mes} W} \quad (7)$$

where t_{mes} is mesophyll thickness.

All parameters were analysed at three different fields and at three different sections. The weighted average was calculated based on tissue volume fractions according to Tomás et al. (2013).

Determination of leaf area, leaf dry mass per unit area, and leaf sodium content

Leaf samples were washed three times with deionized water and dried at 75 °C to a constant mass. Dry samples were ground and digested with 3:1 (v/v) HNO₃ and HClO₄. Na concentration was determined using inductively coupled plasma mass spectrometry (Agilent 7500a, Agilent Technologies, CA, USA).

Quantitative photosynthetic limitation analysis

A quantitative limitation analysis was used to distribute the relative controls on A among stomatal limitation (S_L), mesophyll conductance limitation (MC_L) and biochemical limitation (B_L) (Grassi and Magnani 2005), as modified by Tomás et al. (2013). The relative changes in photosynthesis are expressed as:

$$\frac{dA}{A} = S_L + MC_L + B_L = l_s \frac{dg_{sc}}{g_{sc}} + l_{mc} \frac{dg_m}{g_m} + l_b \frac{dV_{cmax}}{V_{cmax}} \quad (8)$$

$$l_s = \frac{g_{tot}/g_s \cdot \partial A / \partial C_c}{g_{tot} + \partial A / \partial C_c} \quad (9)$$

$$l_m = \frac{g_{tot}/g_m \cdot \partial A / \partial C_c}{g_{tot} + \partial A / \partial C_c} \quad (10)$$

$$l_b = \frac{g_{tot}}{g_{tot} + \partial A / \partial C_c} \quad (11)$$

where l_m , l_s and l_b are the corresponding relative limitations due to g_m , g_s and biochemical capacity (V_{cmax}), respectively; ($l_s + l_m + l_b = 1$). The total CO_2 diffusion conductance, g_{tot} , was given by:

$$g_{tot} = \frac{1}{1/g_{sc} + 1/g_m} \quad (12)$$

$\partial A/\partial C_c$ was estimated as the slope of A/C_c curves with the range of C_c at 50-100 $\mu\text{mol mol}^{-1}$ (Tomás et al. 2013). At least three A/C_c curves of each treatment were used, and average values were calculated.

Relative changes in A , g_m , V_{cmax} and stomatal conductance to CO_2 (g_{sc}) due to the salt treatment were estimated as follows (Chen et al. 2015):

$$\frac{dA}{A} \approx \frac{A_{\text{max}}^{\text{ref}} - A}{A_{\text{max}}^{\text{ref}}} \quad (13)$$

$$\frac{dg_m}{g_m} \approx \frac{g_m^{\text{ref}} - g_m}{g_m^{\text{ref}}} \quad (14)$$

$$\frac{dV_{\text{cmax}}}{V_{\text{cmax}}} \approx \frac{V_{\text{cmax}}^{\text{ref}} - V_{\text{cmax}}}{V_{\text{cmax}}^{\text{ref}}} \quad (15)$$

$$\frac{dg_{\text{sc}}}{g_{\text{sc}}} \approx \frac{g_{\text{sc}}^{\text{ref}} - g_{\text{sc}}}{g_{\text{sc}}^{\text{ref}}} \quad (16)$$

where g_m^{ref} , $g_{\text{sc}}^{\text{ref}}$, $A_{\text{max}}^{\text{ref}}$ and $V_{\text{cmax}}^{\text{ref}}$ are the reference (maximum) values of g_m , g_{sc} , A and V_{cmax} , respectively (Grassi and Magnani 2005). The reference values for A , g_{sc} , g_m and V_{cmax} correspond to NO_3^- and NH_4^+ treatments without salt stress.

The CO_2 liquid-phase diffusion conductance in cytosol, cell wall and stromal

conductance were calculated according to a general formula:

$$g_i = \frac{1}{r_i} = \frac{r_{f,1} \cdot D_w \cdot p_i}{\Delta L_i} \quad (17)$$

where g_i denotes the individual component conductance (r_i is the CO₂ liquid-phase diffusion resistance), and D_w , p_i (m³ m⁻³) and ΔL_i (m) are the CO₂ aqueous-phase diffusion coefficient (1.79×10^{-9} m² s⁻¹ at 25 °C), effective porosity for the given diffusion pathway component and the length of the diffusion path in the corresponding diffusion component, respectively. The dimensionless factor $r_{f,1}$ considers the reduction of diffusion in different cellular components due to the presence of solutes and macromolecules; $r_{f,1}$ was taken as 0.3 for cytosol and stroma, and 1.0 for cell walls (Niinemets and Reichstein 2003, Rondeau-Mouro et al. 2008). The effective porosity was 1.0 for stroma and cytosol. The p_i value of the cell wall was estimated by a least-squares iterative analysis to obtain the best fit between the modelled and measured g_m values (Tosens et al. 2012b, Tomás et al. 2013). The p_i value of the cell wall was set at 0.3 for the thinnest cell walls and 0.028 for the thickest cell walls (Nobel 1991, Tosens et al. 2012b). In this study, the conductance of the chloroplast envelope (g_{env}) and plasma membrane (g_{pl}) was set to 0.0035 m s⁻¹ (Tosens et al. 2012a).

The total liquid-phase conductance consisted of two parallel pathways and was calculated as follows (Tomás et al. 2013):

$$g_{\text{liq}} = \frac{S_c}{(r_{\text{cw}} + r_{\text{pl}} + r_{\text{cel},1})} + \frac{S_m - S_c}{(r_{\text{cw}} + r_{\text{pl}} + r_{\text{cel},2})} \quad (18)$$

where r_{cw} , r_{pl} , $r_{\text{cel},1}$, and $r_{\text{cel},2}$ are the resistances of cell wall, plasma membrane, cell wall parts with chloroplasts and the interchloroplastial areas, respectively (Tomás et al. 2013). The $r_{\text{cel},1}$ and $r_{\text{cel},2}$ were calculated as follows:

$$g_{\text{cel},1} = \frac{1}{r_{\text{cyt},1} + r_{\text{env}} + r_{\text{st},1}} \quad (19)$$

$$g_{\text{cel},2} = \frac{1}{r_{\text{cyt},2} + r_{\text{env}} + r_{\text{st},2}} \quad (20)$$

where $r_{\text{st},1}$ and $r_{\text{st},2}$ are the stromal resistance perpendicular and parallel to the cell walls, respectively. The $r_{\text{cyt},1}$ value is defined as the CO_2 resistance through the plasmalemma inner surface to the outer surface of chloroplasts, and $r_{\text{cyt},2}$ as the resistance from interchloroplastic cell wall portions to the outer surface of chloroplasts (Tomás et al. 2013). These equations give g_m in m s^{-1} . The conversion to molar units is given as follows:

$g_m [\text{mol m}^{-2} \text{s}^{-1}] = g_m [\text{m s}^{-1}] 44.6 [273.16/(273.16 + T)(P/101325)]$, where T is leaf temperature in $^{\circ}\text{C}$ and P is air pressure in Pa.

Quantitative analyses of anatomical limitations of g_m

The quantitative contributions of different anatomical traits to g_m were separated according to Tosens et al. (2012b) and Tomás et al. (2013). The share of g_m in the gas phase was calculated as follows:

$$l_{ias} = \frac{g_m}{g_{ias}} \quad (21)$$

The limitations of g_m by different liquid-phase components (l_i , where i stands for a diffusion pathway component, including cell wall, cytosol, chloroplast stroma, chloroplast envelope and plasmalemma) were calculated as follows:

$$l_i = \frac{g_m}{g_i \frac{S_m}{S}} \quad (22)$$

where g_i is the corresponding CO_2 conductance of the diffusion pathway.

Estimation of g_m by a curve-fitting method

Estimation of g_m was performed by the method of Ethier and Livingston (2004). The g_m estimation was performed with the A/C_i curves with a non-rectangular hyperbola version of the Farquhar's biochemical model for leaf photosynthesis (Farquhar et al. 1980). The g_m value from the fitting of A/C_i and measured from combined gas exchange (g_m) and chlorophyll fluorescence measurements (g_m -gas exchange) were

significantly correlated ($r^2 = 0.79$, $P < 0.05$; Figure S1).

Statistical analyses

Statistical analyses were conducted using the SPSS software (version 22.0). Before analyses of variance (ANOVAs), the data were checked for normality. The differences between mean values were compared by Duncan's tests at a significance level of $P < 0.05$. Three-way analyses of variance were performed to examine the effects of sex, N forms and salt levels. Structural equation modeling was used to evaluate the indirect and direct relationships between leaf structure, biomass allocation, chloroplast exposed surface to leaf area ratio (S_c/S), mesophyll cell area per transverse section width (S/W), CO₂ mesophyll conductance (g_m) and photosynthesis (A). Before the construction of the structure equation modeling, we conducted a principal component analysis (PCA) to create the multivariate functional index due to the correlations among the factors within each group. The structural equation modeling analyses were performed using the R package "lavaan" v. 0.6–1 (Rosseel 2012).

Results

Morphological and anatomical traits of leaves

Salt stress significantly decreased leaf A , g_m , g_s , leaf area, leaf biomass, F_v/F_m ,

chlorophyll concentration, the rate of photosynthetic electron transport (J , Eq. 2) and V_{cmax} in females and males grown under both N forms (except for F_v/F_m under NH_4^+ supply and leaf biomass under both N forms in males). The salt effects were greater in females than in males (Figure 1; Table 1; Table S2). Salt stress increased leaf dry mass per unit area (LMA) and leaf thickness (Figure 1; Table 1). Compared with control conditions, salt stress increased LMA by 37% and 59% in females, and by 21% and 16% in males under NO_3^- and NH_4^+ supply, respectively. Leaf thickness increased by 59% and 56% in females, and by 26% and 19% in males by salt stress under NO_3^- and NH_4^+ supply, respectively (Table 2). The leaf construction cost with salt stress exposure was slightly higher in males than in females under NH_4^+ supply, but it was similar in both sexes under NO_3^- supply (Table 1). The leaf dark respiration rate (R_d) increased by salt stress, and the effect of salt stress on R_d was greater in females than in males under both N forms. Salt stress induced R_d more in females under NO_3^- supply than under NH_4^+ supply; there was no significant difference in R_d in males between the two N forms (Table S2). The SRL value in females was greater than that in males, and the increase in SRL was more significant under NO_3^- supply in males (Table 1). The contrary was true for root length density (RLD) under salt stress. The root biomass and the ratio of root to shoot were higher in females than in males with salt stress under both N forms (Table 1; Table S2).

The density of both mesophyll palisade and spongy tissue cells was higher in females than in males exposed to salt stress under both N forms (Table S2). The contrary was

true for the thickness of palisade and spongy tissues. Palisade and spongy cells were denser in NH_4^+ -fed females than in those supplied by NO_3^- under salt stress, while a higher density of palisade and spongy cells was found in males with NO_3^- supply under salt stress (Table S2). Leaf thickness, f_{ias} , S_{m}/S , S_{c}/S , and chloroplast density and length were higher in males than in females with salt stress under both N forms (Table 2). NH_4^+ -fed males with salt stress had lower S/W and chloroplast density but higher f_{ias} under NO_3^- supply (Table 2). In contrast, there was no significant difference in leaf thickness, S_{m}/S , S/W , or chloroplast density and length in females with salt stress under either N form, but S_{c}/S and f_{ias} were lower under NH_4^+ supply (Table 2). T_{cw} , T_{chl} , $l_{\text{cyt},1}$ and $l_{\text{cyt},2}$ in salt-stressed plants were higher under NO_3^- and NH_4^+ supply in both sexes compared with control plants (Figure 1; Table 2). In males, salt stress increased T_{cw} , T_{chl} , $l_{\text{cyt},1}$ and $l_{\text{cyt},2}$ by 23%, 15%, 43% and 20%, respectively, under NO_3^- supply, and by 15%, 24%, 66% and 28%, respectively, under NH_4^+ supply (Table 2). In contrast, in females, T_{cw} , T_{chl} , $l_{\text{cyt},1}$, and $l_{\text{cyt},2}$ increased by 19%, 34%, 62% and 37%, respectively, under NO_3^- supply, and by 29%, 40%, 32% and 39%, respectively, under NH_4^+ supply in salt-stressed plants (Table 2).

Correlations of g_{m} with physiological characteristics of leaves

The net photosynthesis rate (A) exhibited a significant positive correlation with g_{m} and g_{s} in both sexes under both NO_3^- and NH_4^+ supply (Figure 2a, b). Under both N forms, A at a given g_{s} and g_{m} was higher in males than in females irrespective of salt stress

(Figure 2, $P < 0.05$, Duncan's test), reflecting a greater photosynthetic capacity in males. With an increasing Na^+ concentration in leaves, g_m decreased significantly in both sexes, especially in males under NH_4^+ supply (Figure 2c). Both g_m and the CO_2 drawdown from intercellular air space to chloroplasts ($C_i - C_c$) decreased under salt stress, but the reductions were greater in females than in males, especially under NH_4^+ supply (Figure 2d).

Correlations of g_m with anatomical traits of leaves

Strong positive correlations between g_m and f_{ias} were observed in females and males under all treatments ($P < 0.001$, Duncan's test) (Figure 3a). In males, f_{ias} decreased by salt stress more under NO_3^- than under NH_4^+ supply, whereas lower f_{ias} was observed under NH_4^+ than under NO_3^- supply (Figure 3a). According to multiple regression analyses, g_m was positively associated with S_m/S and S_c/S in all treatments in both sexes ($P < 0.001$; Duncan's test, Figure 3b, c). At given S_m/S , g_m was greater in males than in females, but a similar relationship between g_m and S_c/S was observed in both sexes (Figure 3b, c). A strong negative relationship was observed between g_m and T_{cw} in both sexes under both N forms ($P < 0.001$; Duncan's test, Figure 3d). The slopes of g_m vs. T_{cw} were similar in males and females, but because salt stress led to higher T_{cw} in females than in males (see above), the g_m vs. T_{cw} line was shifted towards higher T_{cw} values along the common slope (Figure 3d). S_c/S and T_{cw} better explained the sexually differential g_m relative to f_{ias} and S_m/S . In contrast, f_{ias} and S_m/S explained the

differences among treatments for each sex.

Limitation of photosynthesis under salt stress

According to the quantitative limitation analysis, salt stress changed the share of mesophyll, stomatal and biochemical limitations in both sexes under NH_4^+ and NO_3^- supply (Figure 4a). In particular, biochemical and mesophyll diffusion limitations (Eqs 10-11) restricted photosynthetic capacity more than stomatal limitations (Eq. 9) under salt stress (Figure 4a). Mesophyll diffusion limitations due to salt stress were higher in females than in males under both N forms, and the extent of inhibition due to salt stress was greater under NH_4^+ than under NO_3^- supply (Figure 4a). Salt stress strongly increased biochemical limitations in females under both N forms, but the extent of such limitations was more significant in males under NO_3^- than under NH_4^+ supply. Stomatal limitations were higher under NO_3^- than under NH_4^+ supply, especially in females (Figure 4a, $P < 0.05$, Duncan's test). Positive relationships were observed between leaf Na and S_L , and Na and MC_L in both sexes (Figure 4b).

Limitations of g_m resulting from diffusion pathway components

In all cases, CO_2 liquid phase diffusion represented a higher proportion of the total diffusion pathway limitation than did gas phase diffusion (Figure 5a-d). For the liquid phase, decreases in the absolute values of CO_2 diffusion conductance due to salt stress

were greater in females than in males under both N forms (Figure 5d). Moreover, the salt stress-dependent increase in the absolute liquid phase conductance was lower under NH_4^+ than under NO_3^- supply in females but not in males (Figure 5d).

The analysis of different liquid phase components indicated that the chloroplast stroma and cytoplasm were the most limiting components in females and males (Figure 6a, b). In salt-stressed females, the share of overall limitation due to chloroplast stroma was reduced under NH_4^+ but unaffected under NO_3^- supply. In contrast, salt stress increased the CO_2 diffusion limitation due to cytoplasm in both sexes. Salt stress did not affect chloroplast stromal limitation under either N form in males (Figure 6a, b). The limitation of liquid phase conductance due to cell walls was not affected by salt stress under either N supply in either sex. However, salt stress led to 43% and 109% increases in the relative chloroplast envelope limitation under NO_3^- and NH_4^+ supply, respectively, in females, while the limitation of the chloroplast envelope did not change by salt stress in males under NH_4^+ supply (Figure 6a, b).

The absolute values of diffusion conductance differed among sexes and treatments (Figure 6c, d). Salt stress decreased g_{cyt1} , g_{cyt2} and g_{st1} in both sexes under NO_3^- and NH_4^+ supply, and the effects of salt stress on cytoplasm conductance were greater in males than in females (Figure 6c, d; Figure S2). The g_{st2} value of females also decreased by salt stress under NH_4^+ supply, but there was no significant difference in males under either N form (Figure 6c, d; Figure S2). Compared with control

conditions, salt stress had higher g_{st1} and g_{cw} , and lower g_{cyt1} in females under NO_3^- than under NH_4^+ supply, whereas no significant differences were found in g_{st2} and g_{cyt2} values between the two N forms (Figure S2). In males, greater g_{cw} and g_{st2} , and lower g_{cyt1} and g_{cyt2} induced by salt stress were found under NH_4^+ supply than under NO_3^- supply (Figure 6c, d; Figure S2). There was no significant difference in g_{st1} between the two N forms in males.

Structural equation modelling

As shown in Figure 7, structural equation modelling suggested that leaf S/W and g_m had direct effects on leaf photosynthesis ($r^2 = -0.49$ and 0.51 , respectively). The leaf photosynthesis was positively correlated with leaf g_m but negatively correlated with leaf S/W . Leaf S_c/S had a direct positive effect on g_m ($r^2 = 0.41$), while leaf S/W had a direct negative effect on g_m ($r^2 = -0.60$). Leaf structure had a direct positive effect on biomass allocation ($r^2 = 0.47$) and S/W ($r^2 = 0.34$), but a direct negative effect on S_c/S ($r^2 = -0.30$). By contrast, biomass allocation had a direct positive effect on S_c/S ($r^2 = 0.36$).

Schematic modes showing the sexual difference in g_m

As shown in Figure 8, under salt stress, females had less chloroplast and more mesophyll spongy cells; the contrary was true for males under both N forms. The CO_2

stroma (g_{st}) and cytoplasm conductance (g_{cyt}) were the lowest among the CO₂ liquid conductance levels. The CO₂ cell wall conductance of females was lower with NH₄⁺ supply, but lower with NO₃⁻ supply when compared to males under salt stress. The g_{st1} and g_{cyt1} values of males were higher than those of females under both N forms and salt stress. Under salt stress, g_{st2} was higher in females than in males with NH₄⁺ supply, but it showed no significant difference between the sexes under NO₃⁻ supply. NH₄⁺-fed females had greater g_{cyt1} but lower g_{st1} and g_{cw} , and similar g_{st2} and g_{cyt2} than those under NO₃⁻ supply, while males had higher g_{cyt1} and g_{cyt2} , lower g_{cw} and g_{st2} , and similar g_{st1} under NO₃⁻ supply than under NH₄⁺ supply under salt stress (Figure 8).

Discussion

The present study clearly demonstrated that salt stress imposes sex-specific restraints on CO₂ assimilation, mainly deriving from the restraint of g_m relative to g_s under NO₃⁻ and NH₄⁺ supply. Generally, stomatal closure is the first response to salt stress in plants (Wang et al. 2018). Salt stress could disturb stomatal function, leading to a decreased photosynthetic capacity (Tavakkoli et al. 2011, Pérez-López et al. 2012, Chen et al. 2015). Improving stomatal regulation has been regarded as the most effective approach to alleviate salt stress in plants (Chen et al. 2015, Qiu and Katul 2020). In this study, both g_m and g_s were positively correlated with A in females and males under both N forms (Figure 2). The relationship of g_m and g_s was stronger in

females than in males (Figure S3). However, we found that the g_m , g_s and J significantly decreased by salt stress under both N forms, especially in females (Table 1; Table S2).

The variable g_m was the main limiting factor for the sexual difference in A under salt stress. This result was a little inconsistent with a previous study on *Ziziphus spina-christi* trees suggesting that g_m plays a critical role in controlling A only under severe salinity stress (Zait et al. 2019). This could be explained by the different way of calculating l_s and l_m , as well as by the plant species. In the study by Zait et al. (2019), the authors investigated drought-tolerant *Ziziphus spina-christi* trees, which exhibited strong tolerance to salt stress. The concentration of NaCl used in our study was moderate for males but not for females. In this study, the net photosynthetic rate A and stomatal conductance g_s decreased by salt stress more significantly in *Populus cathayana* females than in males. Additionally, g_s was dynamic and changed in response to environmental conditions rapidly or even faster than g_m (Flexas et al. 2008). These results suggested that the photosynthetic limitation from g_m might be higher than that from g_s under our experimental conditions.

Salt stress affects those morphological and anatomical traits of leaves that are associated with CO₂ diffusion into the leaves and leaf photosynthesis (Wankhade et al. 2013, Wang et al. 2018). A higher LMA value is correlated with a greater dry mass investment and it has a negative effect on g_m (Tomás et al. 2013, Onoda et al. 2017). Consistently, females with higher LMA had lower g_m under salt stress (Table 1). It is

noteworthy that males with higher A and g_m had higher LMA than females, which suggested that it was not possible to precisely determine g_m and A only from leaf LMA (Figure 7), because of the effects of the chemical composition of leaves (John et al. 2017). The S/W value included the total cell area of palisade and spongy tissues per transverse section width, representing the leaf structural investment per area, which would be a more advantageous and convenient trade-off between the leaf structure and photosynthesis (Hu et al. 2020). The results of the structural equation modelling suggested that S/W plays a critical role in determining g_m and A ($r^2 = -0.60$ for g_m and -0.49 for A) (Figure 7). A lower chloroplast density and a greater mesophyll spongy density in females would reduce light energy capture and CO_2 diffusion conductance in leaves, especially under NH_4^+ supply (Table 2).

Cell wall thickness and chloroplast distribution are critical for setting g_m limitation in plants (Terashima et al. 2011). Consistently, S_c/S and T_{cw} were those anatomical variables that best explain differences in g_m between sexes (Figures 3, 7, 8). A greater increase in chloroplast numbers observed in males compared to females facilitated an increase in S_c/S (Figure 8). Higher S_c/S and a lower cell wall thickness led to an increased CO_2 diffusion conductance into male leaves, which was consistent with previous studies (Peguero-Pina et al. 2017, Wang et al. 2018). Indeed, the quantification of g_m limitation showed that g_{cyt} was higher in females than in males during salt stress under both types of N supplies (Figure 6). The proportion of g_{cyt} in the CO_2 diffusion conductance increases along with a reduced contact between

chloroplasts and cell surfaces (Lu et al. 2016). Modifications in the chloroplast shape and structure affect the distance of cytosolic CO₂ diffusion under abiotic stress (Shu et al. 2012). Changes in the shape of female chloroplasts caused by salt stress probably decreased the g_{cyt} value and, thus, g_{m} as well.

Generally, the distance has little effect on CO₂ diffusion when the cellular stromal distance between the plasma membrane and chloroplast envelope is relatively short. However, intracellular CA could convert CO₂ into HCO₃⁻, having the highest activity in the stroma and, thus, affecting g_{m} (Evans et al. 2009). The effects of N forms on g_{st} and g_{cyt} were sex-dependent under salt stress. NH₄⁺-fed females had greater g_{cyt1} but lower g_{cw} and g_{st1} than those under NO₃⁻ supply, while males had higher g_{cw} and lower g_{st2} under NH₄⁺ than under NO₃⁻ supply (Figures 6, 8; Figure S2). Additionally, nitrogen availability and forms have been found to affect CA activities under salt stress (Siddiqui et al. 2010, Nawaz et al. 2020). Therefore, CA may increase its effect on g_{m} inside chloroplasts. In addition, increasing evidence suggests that aquaporins are capable of transporting CO₂ in plants and the aquaporin activity is associated with g_{m} in leaves (Maurel et al. 2008, Heckwolf et al. 2011). Some studies have suggested that salt stress or nitrogen availability increase aquaporin activities (Qi et al. 2009), which indicates that the variation in aquaporin activities under salt stress and N availability might partially affect g_{m} .

The effects of N forms on g_{m} and A largely depend on salt stress tolerance and the

energy allocation of plants. N forms affect matter distribution and carbohydrate consumption in plants (Guo et al. 2007, Ashraf et al. 2018). A greater investment in the leaf area and cell wall thickness results in a lower allocation of resources to leaf photosynthesis in females. Net photosynthesis could be reduced not only by a greater energy investment in structures but also by higher maintenance costs, including the effects of abiotic stresses (Guo et al. 2007). Females show a higher respiration rate than males under drought stress in *Populus cathayana* (Han et al. 2013). The higher dark respiration rate in *P. cathayana* females under salt stress found in the present study was probably associated with the leaf photosynthesis (Table S2). NO_3^- assimilation in plants requires more energy than NH_4^+ assimilation, while carbon losses through leaf and root respiration are higher under NH_4^+ than under NO_3^- supply. Whether or how N assimilation in plants affects photosynthesis under salt stress was not clear. In the future, it will be important to clarify the sexually different leaf photosynthesis under salt stress with different N form supplies based on the relationship between photosynthesis and respiration consumption.

Importantly, the sex- and N form-specific investment in the root system architecture largely reflects energy requirements for foliage construction, thereby, affecting g_m and photosynthesis. In this study, the biomass allocation did not directly explain leaf A , whereas the biomass allocation had indirect effects on leaf A through leaf structural traits (Figure 7). NH_4^+ -fed males with a higher root biomass had high root construction costs, while NO_3^- -fed males with a higher leaf biomass had high energy

cost from NO_3^- assimilation without salt stress. Such energy trade-offs between biomass and A in males led to similar g_m and photosynthesis under both N forms. Females produce thinner roots than males and they intensively facilitate the exploration of the surrounding soil and Na^+ uptake (Xia et al. 2020). Certainly, the larger root system and ion uptake enhanced the energy consumption of females exposed to salt stress, especially under NH_4^+ supply.

Conclusions

This study suggested that leaf g_m relative to g_s under salt stress was the main restraint on leaf A under NO_3^- and NH_4^+ supplies. Among leaf structural components, S_c/S and S/W were critical factors affecting g_m and A (Figure 7). Salt stress increased S/W , and reduced intercellular air space and S_c/S , resulting in an increased length of cytoplasmic and stromal paths, and inhibition of g_m and A in females (Figure 8). Conversely, males had a better tradeoff between leaf structure and photosynthesis (more chloroplasts and fewer spongy cells), and they have a good ability to regulate leaf CO_2 diffusion and light capture into the leaf, thereby alleviating g_m and A decreases under salt stress and both N forms (Figure 8). The low CO_2 diffusion conductance in NH_4^+ -fed females was mainly due to g_{stl} and g_{cw} , while NO_3^- -fed males had a higher cytoplasm conductance (Figure 8). Moreover, biomass allocation in both sexes indirectly affected leaf structural traits and A under both N forms. This study will not only help us to predict potential climate change impacts on dioecious *P.*

cathayana populations, but also to predict the photosynthetic behavior of tree species with different N-source preferences and spatiotemporal changes in N assimilation at rising atmospheric CO₂.

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Conflict of interest The authors declare that they have no conflict of interest.

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Figure legends

Figure 1 Representative light microscopy images (a, b) and transmission electron microscopy images (c, d) of the leaves of *P. cathayana* females and males grown with NO_3^- or NH_4^+ supply, and with or without salt stress (50 mM NaCl addition for 14 days). Bar = 50 μm for images a and b. Leaf upper epidermis (ue), leaf mesophyll palisade tissue (pl), leaf mesophyll spongy tissue (sp), leaf lower epidermis (le), leaf vascular tissue (v), cell wall (cw), chloroplast (ch) and starch granule (s).

Figure 2 Relationships of the light-saturated rate of photosynthesis with stomatal conductance (g_s) (a) and mesophyll conductance (g_m) (b), and the relationships of g_m with leaf Na^+ concentration (c) and CO_2 drawdown ($C_i - C_c$) (d) from sub-stomatal cavities (C_i) to chloroplasts (C_c) in *P. cathayana* females (F) and males (M) grown with NO_3^- or NH_4^+ supply, and with or without salt stress. Salt stress application as in Figure 1.

Figure 3 Relationships of CO_2 mesophyll conductance (g_m) with the volume fraction of intercellular air space (f_{ias}) (a), the surface area of mesophyll cells adjacent to intercellular air space (S_m/S) (b), the chloroplast surface area exposed to intercellular air space per leaf area (S_c/S) (c) and the cell wall thickness (T_{cw}) (d) in *P. cathayana* females (F) and males (M) grown with NO_3^- or NH_4^+ supply, and with or without salt stress. Salt stress application as in Figure 1.

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916 **Figure 4** Quantitative photosynthetic limitations in leaves of *P. cathayana* females (F)
917 and males (M) grown with NO_3^- or NH_4^+ supply, and with or without salt stress. The
918 photosynthetic limitation $dA/A = S_L + MC_L + B_L$. S_L , stomatal limitation; MC_L ,
919 mesophyll conductance; B_L , biochemical limitation. Salt stress application as in
920 Figure 1.

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922 **Figure 5** The share of limitations of g_m among gas-phase (a) and liquid-phase (b)
923 pathways, and absolute values of gas- (c) and liquid-phase (d) conductance in leaves
924 of *P. cathayana* females and males grown with NO_3^- or NH_4^+ supply, and with or
925 without salt stress. Salt stress application as in Figure 1.

926

927 **Figure 6** Anatomical limitations of CO_2 mesophyll conductance (g_m) and CO_2
928 conductance in leaves of *P. cathayana* females (F) and males (M) grown with NO_3^- or
929 NH_4^+ supply with or without salt stress. The share of CO_2 liquid-phase diffusive
930 limitation (a, b) and absolute values of conductance among cytoplasm (g_{cytl}) and
931 chloroplast stroma (g_{stl}) (c), cell wall (g_{cw}), chloroplast envelope (g_{env}) and plasma
932 membrane (g_{pl}) (d). The g_{stl} variable is the stromal conductance perpendicular to cell
933 walls, and g_{cytl} is the cytosolic conductance from the plasmalemma inner surface
934 towards the outer surface of chloroplasts. A constant value of 0.0035 m s^{-1} was used
935 for both plasmalemma and chloroplast envelope conductance. Additional components
936 of the diffusion pathway are provided in Figure S2. Salt stress application as in Figure

1.

Figure 7 Direct and indirect effects of leaf structure, biomass allocation and the surface area of chloroplast exposed to intercellular air spaces per unit leaf area (S_c/S), mesophyll cell area per transverse section width (S/W), CO₂ mesophyll conductance (g_m) and photosynthesis (A) in *P. cathayana* females and males grown with NO₃⁻ or NH₄⁺ supply, and with or without salt stress. Multiple-layer rectangles represent the first component of PCA conducted for the leaf structure and biomass allocation. The arrows indicate the hypothesized direction of causation. The arrow width represents the strength of relationships. Black solid and dotted arrows represent positive and negative relationships, respectively. Grey arrows represent nonsignificant relationships. The leaf structure traits include mesophyll palisade cell area (A_{pl}), the volume fraction of intercellular air space (f_{ias}), leaf thickness (T_l), mesophyll surface area exposed to intercellular air space per unit leaf area (S_m/S), cell wall thickness (T_{cw}), mesophyll length (L_m), mesophyll palisade tissue length (L_{pl}), mesophyll spongy tissue length (L_{sp}), leaf dry mass per unit area (LMA), mesophyll spongy cell density (D_{sp}), chloroplast length (L_{cl}), mesophyll palisade cell density (D_{pl}), chlorophyll content (Chl), chloroplast width (W_{cl}), and mesophyll spongy cell area (A_{sp}). The biomass allocation includes specific root length (SLR), root length density (RLD), stem dry mass (W_s), leaf dry mass (W_l), root dry mass, shoot dry mass/root dry mass (S/R), and leaf dry mass/(stem and root dry mass) ($L/(S+R)$). The numbers adjacent to the arrows are the standardized path coefficients. The significance values

are shown as follows: ns, not significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$. $X^2 = 7.48$, $p = 0.006$, $RMSE = 0.19$.

Figure 8 A schematic model for leaf anatomical traits, CO₂ diffusion pathway and conductance inside leaves of *P. cathayana* females and males with salt stress under NO₃⁻ or NH₄⁺ supply. The width of the red line represents the strength of CO₂ diffusion conductance into leaves. Blue dots indicate CO₂. g_{cw} , CO₂ transfer conductance through cell wall; g_{env} , CO₂ transfer conductance through chloroplast envelope; g_{pl} , CO₂ transfer conductance through plasma membrane; g_{st1} , the stromal conductance perpendicular to cell wall; g_{st2} , the stromal conductance parallel with the cell wall; g_{cyt1} , CO₂ transfer conductance through cytoplasm from the plasmalemma inner surface to the outer surface of chloroplasts; g_{cyt2} , cytosolic conductance from the plasmalemma inner surface to the outer surface of chloroplasts; T_{leaf} , leaf thickness; P, mesophyll palisade tissue; S, mesophyll spongy tissue; C, chloroplast. Salt stress application as in Figure 1. The relative values of the liquid-phase components based on the Figure 6 and Figure S2. Values of g_{cyt2} and g_{cyt1} under NO₃⁻ supply, and g_{st1} , g_{cyt1} , g_{cyt2} and g_{cw} under NH₄⁺ supply were lower in females than in males. The values of g_{st2} under NO₃⁻ supply were higher in females than females.

Figure 1

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1066 **Table 1** Leaf biomass and area, leaf structural and gas exchange characteristics, leaf construction cost and root traits in *P. cathayana* females and
1067 males under NO₃⁻ or NH₄⁺ supply, and with or without salt stress.

Salt	N	Sex	Leaf biomass	Leaf area	<i>LMA</i>	Leaf CC	<i>F_v/F_m</i>	<i>J</i>	<i>V_{cmax}</i>	SRL	RLD	Root biomass
0	NO ₃ ⁻	Female	14.14±0.93a	85.14±5.90a	37.83±3.57d	1.39±0.01a	0.814±0.01a	47.45±3.86a	65.99±1.68b	6.91±0.50cd	1.70±0.15a	8.04±0.44a
0	NO ₃ ⁻	Male	8.69±0.28cd	51.65±6.57d	45.20±2.18c	1.39±0.01a	0.808±0.01a	50.95±2.72a	74.44±4.43a	7.27±1.08cd	0.671±0.06c	4.08±0.35d
0	NH ₄ ⁺	Female	12.83±1.46b	72.09±5.54b	36.62±2.78d	1.37±0.06ab	0.815±0.01a	35.18±3.85b	58.18±5.12c	8.25±0.78c	1.24±0.13b	6.87±0.44b
0	NH ₄ ⁺	Male	8.41±0.74cd	43.77±1.20d	45.54±1.10c	1.39±0.01a	0.794±0.01b	47.78±0.99a	66.17±3.44b	6.87±1.41cd	0.847±0.06d	5.38±0.25c
50	NO ₃ ⁻	Female	9.54±0.45c	68.28±4.15bc	51.67±3.09b	1.37±0.004ab	0.769±0.01cd	17.93±2.81d	51.47±4.09d	13.611.15a	1.29±0.05b	7.12±0.56b
50	NO ₃ ⁻	Male	8.06±0.60de	50.60±5.96d	54.58±2.44ab	1.39±0.01a	0.773±0.01c	35.13±3.90b	65.79±3.12b	6.51±0.67d	1.56±0.10a	4.54±0.28d
50	NH ₄ ⁺	Female	8.44±0.95cde	63.10±4.61c	58.33±4.46a	1.35±0.06c	0.757±0.01d	13.88±1.59d	42.10±2.53e	10.75±1.09b	0.784±0.06de	4.57±0.31d
50	NH ₄ ⁺	Male	6.64±1.02f	45.93±2.18d	53.06±2.40b	1.41±0.01a	0.790±0.01b	24.18±2.16c	58.68±2.64c	7.39±0.92cd	1.01±0.14c	3.25±0.40e
		<i>P_{se}</i>	***	***	**	***	ns	***	***	***	***	***
		<i>P_{sa}</i>	***	***	***	ns	***	***	***	***	ns	***
		<i>P_n</i>	***	***	ns	ns	ns	***	***	ns	***	***
		<i>P_{se×sa}</i>	***	*	***	***	***	**	**	***	***	**
		<i>P_{se×n}</i>	Ns	ns	ns	***	ns	ns	ns	ns	***	***
		<i>P_{sa×n}</i>	Ns	**	Ns	ns	ns	ns	ns	*	***	***
		<i>P_{se×sa×n}</i>	Ns	ns	*	ns	**	***	ns	***	***	*

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1069 Leaf biomass (g); leaf area (cm²); *LMA* (g m⁻²), leaf dry mass per unit leaf area; leaf CC (g glucose g⁻¹), leaf construction cost; F_v/F_m , maximum
1070 quantum efficiency of PSII photochemistry; J (μmol m⁻² s⁻¹), photosynthetic electron transport rate from chlorophyll fluorescence (Eq. 2); V_{cmax}
1071 (μmol m⁻² s⁻¹), the maximum carboxylase activity of Rubisco; SRL (m g⁻¹), specific root length; RLD (cm cm⁻³), root length density; root
1072 biomass (g). P_{se} , sex effect; P_{sa} , salt effect; P_{n} , N form effect (NO₃⁻ and NH₄⁺); $P_{\text{se} \times \text{sa}}$, the interaction effect of sex and salt stress; $P_{\text{n} \times \text{sa}}$, the
1073 interaction effect of N forms and salt stress; $P_{\text{se} \times \text{n}}$, the interaction effect of sex and N forms; $P_{\text{se} \times \text{n} \times \text{sa}}$, the interaction effect of sex, N forms and
1074 salt stress. Different letters indicate significant differences between treatments ($P < 0.05$, three-way ANOVA followed by Duncan's tests). Data
1075 are mean $\pm$ SE ($n = 4$). The significance values are shown as follows: ns, not significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$.

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1082 **Table 2** Leaf thickness and anatomical traits in *P. cathayana* females and males grown under NO₃⁻ or NH₄⁺ supply, and with or without salt
1083 stress. Data are mean ± SE (*n* = 4).

Salt	N	Sex	<i>f</i> _{ias} (%)	<i>S</i> _m / <i>S</i>	<i>S</i> _c / <i>S</i>	<i>S</i> / <i>W</i>	<i>T</i> _l	<i>T</i> _{cw}	<i>D</i> _{chl}	<i>L</i> _{chl}	<i>T</i> _{chl}	Δ <i>L</i> _{cyt,1}	Δ <i>L</i> _{cyt2}
form													
0	NO ₃ ⁻	Female	28.21±0.93a	9.60±0.77ab	7.36±0.99bc	71.5±6.0b	171.3±3.2b	0.139±0.004d	12.8±0.9cd	5.9±0.6ab	1.7±0.09c	0.106±0.13e	0.864±0.040e
0	NO ₃ ⁻	Male	28.28±1.67a	7.76±0.22c	7.01±0.24c	35.6±4.7d	220.6±17.8a	0.123±0.001e	16.3±1.0a	6.1±0.6a	1.3±0.06e	0.093±0.002e	0.821±0.004e
0	NH ₄ ⁺	Female	22.35±1.78b	10.04±0.36a	7.98±0.63ab	72.9±0.91b	177.8±8.8b	0.144±0.004d	11.5±0.6d	4.7±0.6c	1.7±0.06c	0.138±0.10d	0.969±0.025d
0	NH ₄ ⁺	Male	23.90±2.58b	8.54±0.66bc	8.45±0.65bc	42.5±5.7d	216.1±44.2a	0.116±0.007e	13.8±1.0bc	6.2±0.5a	1.6±0.09cd	0.074±0.003f	0.877±0.034e
50	NO ₃ ⁻	Female	18.14±0.74c	6.93±0.46d	5.93±0.69d	82.7±1.5a	177.3±1.9b	0.171±0.004b	8.8±1.0e	5.2±0.6bc	2.6±0.2b	0.202±0.016b	1.38±0.042b
50	NO ₃ ⁻	Male	22.90±2.62b	8.40±1.09bc	6.31±0.75c	52.5±8.9c	212.3±2.7a	0.160±0.007c	16.3±1.3a	5.9±0.2ab	1.4±0.05de	0.162±0.016c	1.02±0.058d
50	NH ₄ ⁺	Female	14.49±0.67d	6.22±0.16d	4.96±0.63e	89.2±2.6a	177.6±3.9b	0.203±0.009a	9.0±0.8e	5.2±0.5bc	3.2±0.3a	0.279±0.018a	1.59±0.11a
50	NH ₄ ⁺	Male	23.29±2.55b	8.97±1.45abc	7.13±0.68c	41.0±4.4d	212.5±9.2a	0.137±0.005d	14.5±0.6b	6.3±0.5a	1.6±0.1cd	0.218±0.007b	1.22±0.078c
		<i>P</i> _{se}	***	ns	***	***	***	***	***	***	***	***	***
		<i>P</i> _{sa}	***	***	***	***	ns	***	***	ns	***	***	***
		<i>P</i> _n	***	ns	ns	ns	ns	ns	***	ns	***	***	***
		<i>P</i> _{se×sa}	***	***	ns	ns	ns	***	***	ns	***	ns	***
		<i>P</i> _{se×n}	***	ns	ns	*	ns	***	*	*	ns	***	ns
		<i>P</i> _{sa×n}	***	ns	ns	*	ns	ns	ns	ns	*	***	**
		<i>P</i> _{se×sa×n}	Ns	ns	*	***	ns	***	ns	ns	**	ns	ns

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1086 f_{ias} (%), the volume fraction of intercellular air space per unit leaf area; S_m/S ($m^2 m^{-2}$), mesophyll surface area exposed to intercellular air space
1087 per unit leaf area; S_c/S ($m^2 m^{-2}$), chloroplast surface area exposed to intercellular air space per unit leaf area; S/W ($\mu m^2 \mu m^{-1}$), mesophyll cell
1088 area per transverse section width; T_l (μm), leaf thickness; T_{cw} (μm), cell wall thickness; D_{chl} (no. no), chloroplast density; L_{chl} (μm), chloroplast
1089 length; T_{chl} (μm), chloroplast thickness; $\Delta L_{cyt,1}$ (μm), the vertical distance between the chloroplasts and cell walls; $\Delta L_{cyt,2}$ (μm), the distance
1090 between the neighboring chloroplasts. P_{se} , sex effect; P_{sa} , salt effect; P_n , N form effect (NO_3^- and NH_4^+); $P_{se \times sa}$, the interaction effect of sex and
1091 salt stress; $P_{n \times sa}$, the interaction effect of N forms and salt stress; $P_{se \times n}$, the interaction effect of sex and N forms; $P_{se \times n \times sa}$, the interaction effect of
1092 sex, N forms and salt stress. Different letters indicate significant differences between treatments ($P < 0.05$, three-way ANOVA followed by
1093 Duncan's tests). Data are mean $\pm$ SE ($n = 4$). The significance values are shown as follows: ns, not significant; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq$
1094 0.01 ; *** $P \leq 0.001$.