Effects of nitrogen additions on mesophyll and stomatal conductance in Manchurian ash and Mongolian oak

Kai Zhu1,2, Anzhi Wang1, Jiabing Wu1, Fenghui Yuan1✉, Dexin Guan1✉, Changjie Jin1, Yushu Zhang3 & Chunjuan Gong1,2

The response of plant CO₂ diffusion conductances (mesophyll and stomatal conductances, $g_m$ and $g_{sc}$) to soil drought has been widely studied, but few studies have investigated the effects of soil nitrogen addition levels on $g_m$ and $g_{sc}$. In this study, we investigated the responses of $g_m$ and $g_{sc}$ of Manchurian ash and Mongolian oak to four soil nitrogen addition levels (control, low nitrogen, medium nitrogen and high nitrogen) and the changes in leaf anatomy and associated enzyme activities (aquaporin (AQP) and carbonic anhydrase (CA)). Both $g_m$ and $g_{sc}$ increased with the soil nitrogen addition levels for both species, but then decreased under the high nitrogen addition level, which primarily resulted from the enlargements in leaf and mesophyll cell thicknesses, mesophyll surface area exposed to intercellular space per unit leaf area and stomatal opening status with soil nitrogen addition. Additionally, the improvements in leaf N content and AQP and CA activities also significantly promoted $g_m$ and $g_{sc}$ increases. The addition of moderate levels of soil nitrogen had notably positive effects on CO₂ diffusion conductance in leaf anatomy and physiology in Manchurian ash and Mongolian oak, but these positive effects were weakened with the addition of high levels of soil nitrogen.

Nitrogen (N) is an important nutrient for plant photosynthesis because it alters N allocation between photosynthetic components1, but excessive nitrogen depositions break the soil nitrogen balance and have a strongly negative effect on photosynthesis2,3. With global climate change, nitrogen deposition has increased dramatically worldwide4,5, and its effects on CO₂ diffusion conductance (both mesophyll and stomatal conductances, $g_m$ and $g_{sc}$) in photosynthesis have attracted considerable attention in global change, physiological ecology, plant physiology and other fields6–8. In contrast to $g_{sc}$, $g_m$ has often been neglected in previous studies, with its supply being assumed to be unlimited9,10; the importance of $g_m$ is only being highlighted in recent decades with the advent of advanced instruments and measuring technologies, and studies of $g_m$ have also increased correspondingly1,11–14.

The relationship of $g_{sc}$ with soil nitrogen has been widely explored; $g_{sc}$ increased with soil nitrogen additions overall15–17, and $g_m$ also showed a positive correlation with moderate soil nitrogen supplementation in general16,7. However, excessive nitrogen application resulted in a decrease in the ability to scavenge reactive oxygen species (ROS) in wheat18. Many changes under excessive nitrogen application may also occur in photosynthesis electron transport rate ($J_f$) or actual photochemical efficiency of photosystem II ($\Phi_{PSII}$). In addition, several recent papers also reported that high N conditions reduced evapotranspiration, which resulted in constraining N uptake in almond trees19–20, and this seemed related to the slight decrease in $g_{sc}$ and leaf N content under high nitrogen condition. Hence, it has not been determined how $g_m$ and $g_{sc}$ responded to excessive nitrogen additions ($\geq 69$ kg N ha$^{-1}$ a$^{-1}$). Besides, the changes in leaf anatomy and associated physiological traits were both considered to be important mechanisms in determining $g_{sc}$ and $g_m$14,21–29.

Xiong et al.14 revealed that leaves with larger $g_m$ in high nitrogen supplement had a larger leaf thickness ($T_{leaf}$) and mesophyll surface area exposed to intercellular space per unit leaf area ($S_{mes}$) than those in low nitrogen supplementation, and Zhu et al.30 also showed that $T_{leaf}$ and mesophyll cell thickness ($T_{mes}$) both imposed a positive

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1Key Laboratory of Forest Ecology and Management, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, 110016, China. 2University of Chinese Academy of Sciences, Beijing, 100049, China. 3The Institute of Atmospheric Environment, China Meteorological Administration, Shenyang, 110166, China. ✉e-mail: fhyuan@iae.ac.cn; dxguan@iae.ac.cn
Figure 1. Changes of plant $g_{m}$, $g_{sc}$ and $A_{n}$ with soil N additions in Manchurian ash (I) and Mongolian oak (II) in July and August. Values were means ± SE (n = 5), and different lowercase letters (a–c) indicated significant difference at $P < 0.05$. CK, the control; LN, low nitrogen addition; MN, medium nitrogen addition; HN, high nitrogen addition.

Effect on $g_{m}$ recovery in Manchurian ash and Mongolian oak, while $S_{sc}$ had a negative effect on this parameter. In addition, Xiong et al.29 and Zhu et al.30 both revealed the determination of stomatal features (size and density) and opening status (SS) to $g_{m}$, and Zhu et al.30 suggested a positive correlation between SS and $g_{m}$ in Manchurian ash and Mongolian oak, which was also supported by the study of Xu and Zhou (2008)31. Besides, the changes of $g_{m}$ and $g_{sc}$ would also be significantly affected by aquaporin (AQP) and carbonic anhydrase (CA) activities14,21. AQP mediates the genes in the plasma membrane intrinsic protein (PIP) aquaporin family, and its increase in activity strongly promotes the expression of PIPs and improves CO2 membrane permeability32–34. CA regulates $g_{m}$ mainly by changing the dynamics of CO2 to $HCO_{3}$-28. However, it has not been determined how leaf anatomies and AQP and CA activities would change in the Changbai Mountains with the addition of various levels of soil nitrogen.

Manchurian ash (Fraxinus mandshurica Rupe.) and Mongolian oak (Quercus mongolica Fish. ex Ledeb.), two species in Oleaceae and Fagaceae, respectively, are widely distributed in the Changbai Mountains, China. Their responses of CO2 diffusion conductance to soil nitrogen additions have rarely been reported; and the mechanisms governing $g_{m}$ and $g_{sc}$ in leaf anatomy and physiology have not been determined. Hence, we measured both $g_{m}$ and $g_{sc}$ and their related anatomical and physiological traits, including leaf N content and AQP and CA activities, to explore the responses of CO2 diffusion conductance to the addition of various levels of soil nitrogen. The present study will advance our mechanistic understanding of global nitrogen deposition impacts on carbon cycling in tree species.

Results

Effects of soil nitrogen additions on $g_{m}$. Considerable changes in $g_{m}$ were observed in both species after soil nitrogen addition compared with the control values (CK) (Fig. 1a,b). The $g_{m}$ gradually increased with the addition of various levels of soil nitrogen, reaching the maximum with the medium level of 46 kg N ha$^{-1}$ a$^{-1}$ (MN) and then decreasing with the addition of 69 kg N ha$^{-1}$ a$^{-1}$ (HN) while remaining greater than the $g_{m}$ observed for the CK group. Overall, $g_{m}$ showed significant differences among the four nitrogen-addition treatments in both species, in which it was significantly larger in the MN treatment than in the other treatments ($P < 0.05$). In addition, the $g_{m}$ in August was slightly lower than that in July in both species overall.

Effects of soil nitrogen additions on $g_{sc}$. Concurrently, soil nitrogen addition also resulted in a significant increase in $g_{sc}$, with a different trend being observed between Manchurian ash and Mongolian oak (Fig. 1c,d). In Manchurian ash, $g_{sc}$ continued to increase with nitrogen addition in July and August, but it increased from low nitrogen (LN, 23 kg N ha$^{-1}$ a$^{-1}$) to MN and then decreased with the addition of HN in Mongolian oak. The $g_{sc}$ with the nitrogen addition treatments was significantly larger than that of the control values in July and August in both species ($P < 0.05$). Overall, the $g_{sc}$ in Manchurian ash was considerably larger than that in Mongolian oak. In addition, influenced by the changes in $g_{m}$ and $g_{sc}$, leaf $A_{n}$ also increased first and then decreased with the addition of progressively higher levels of soil nitrogen (Fig. 1e,f), indicating that plant photosynthetic capacities could also be considerably strengthened by soil nitrogen additions.

Effects of soil nitrogen addition on leaf anatomical characteristics. Effects on mesophyll anatomical traits. The addition of soil nitrogen caused some changes in mesophyll anatomical traits (Table 1), including leaf ($T_{leaf}$) and mesophyll cells ($T_{mes}$) thicknesses, the surface area of mesophyll cells exposed to intercellular space ($S_{mes}$) and the ratio of mesophyll surface ($A_{mes}$) to total leaf surface area ($A_{T}$) corrects for the actual area available for CO2 diffusion ($A_{T}/A_{mes}$). $T_{leaf}$, $T_{mes}$, $S_{mes}$ and $A_{T}/A_{mes}$ all increased after soil nitrogen addition in overall without $T_{leaf}$ in HN and $A_{T}/A_{mes}$ in LN treatment and were lower than the CK values. When nitrogen supplementation was no more than 69 kg N ha$^{-1}$ a$^{-1}$, $T_{leaf}$ and $S_{mes}$ increased with nitrogen addition in both species, and $T_{mes}$ also showed increases with the addition of soil nitrogen in Manchurian ash and reached a maximum in the MN treatment, while it reached a maximum in the LN treatment in Mongolian oak. Significant differences between
Changes of leaf g_{\text{liq}} and g_{\text{m}}. Changes of T_{\text{mes}} and A_{\text{r}}/A_{\text{mes}} to soil nitrogen additions also directly caused changes in leaf gas-phase (g_{\text{liq}}) and liquid-phase CO₂ diffusion conductance (g_{\text{m}}) inside mesophyll cells (Table 1). Both g_{\text{liq}} and g_{\text{m}} gradually increased with MN and then decreased under HN treatment. A significant difference between nitrogen addition treatments was observed in g_{\text{liq}}, while g_{\text{m}} did not change significantly overall (P < 0.05).

Effects on stomatal parameters. Correspondingly, soil nitrogen addition also caused considerable changes in stomatal parameters, mainly including stomatal pore length and width at the centre of the stoma (PL and PW), stomatal opening status (SS) and density (Dₜ) (Table 2). As two important components to SS, PL and PW, both increased with the MN treatment and were similar in both species, but in the HN treatment, they both fell below the CK values. Consequently, leaf SS gradually increased with soil nitrogen addition and then significantly decreased to a minimum under HN treatment. Besides, significant increases also happened to leaf Dₜ after soil nitrogen additions (P < 0.05) (Table 2), in which Manchurian ash showed a gradually increased Dₜ with soil nitrogen addition, reaching the maximum under HN treatment, while Dₜ increased first then decreased in Mongolian oak, reaching its maximum under LN treatment.

Effects of soil nitrogen additions on leaf N content. Changes in leaf N content in both species during soil nitrogen additions are shown in Table 3. The leaf N content was enlarged by soil nitrogen addition and increased with medium nitrogen addition to MN and then decreased under HN treatment. Overall, no significant differences in leaf N content between soil nitrogen addition treatments were observed in both species.

| Manchurian ash | Mongolian oak |
|----------------|---------------|
| T_{\text{mes}} (μm) | T_{\text{mes}} (μm) | S_{\text{mes}} (μm²) | A_{\text{r}}/A_{\text{mes}} | Dₜ (10⁻³ N cm⁻²) | PL (μm) | PW (μm) | SS (μm²) | PL (μm) | PW (μm) | SS (μm²) | Dₜ (10⁻³ N cm⁻²) |
| CK | 130.1 ± 11.0² | 116.3 ± 5.1² | 12.1 ± 2.0³ | 1.91 ± 0.08³ | 4.47 ± 0.37³ | 1.08 ± 0.06³ | 206.8 ± 3.2³ | 158.1 ± 6.7³ | 12.9 ± 1.3³ | 1.81 ± 0.11³ | 5.41 ± 0.33³ | 1.14 ± 0.06³ |
| LN | 135.9 ± 5.1³ | 122.9 ± 4.2³ | 14.2 ± 3.8³ | 1.74 ± 0.11³ | 11.93 ± 0.79³ | 1.19 ± 0.14³ | 213.7 ± 3.7³ | 185.9 ± 3.5³ | 13.0 ± 1.5³ | 1.67 ± 0.40³ | 6.21 ± 0.62³ | 1.24 ± 0.12³ |
| MN | 148.2 ± 7.3³ | 150.2 ± 2.5³ | 15.2 ± 2.6³ | 2.35 ± 0.53³ | 10.13 ± 0.74³ | 1.28 ± 0.09³ | 227.4 ± 6.0³ | 171.2 ± 8.0³ | 16.0 ± 2.0³ | 1.85 ± 0.34³ | 7.22 ± 0.22³ | 1.32 ± 0.07³ |
| HN | 122.9 ± 3.6³ | 112.9 ± 3.3³ | 18.0 ± 5.0³ | 2.43 ± 0.16³ | 8.11 ± 1.0³ | 1.00 ± 0.07³ | 202.6 ± 2.4³ | 167.1 ± 4.1³ | 14.4 ± 1.7³ | 1.78 ± 0.22³ | 5.76 ± 0.59³ | 1.17 ± 0.04³ |

Table 1. Values of leaf anatomical characteristics for different nitrogen addition treatments in Manchurian ash and Mongolian oak. All data were means ± SE (n = 5). Different lowercase letters (a, b, c) indicated significant differences between nitrogen addition treatments at P < 0.05. CK, the control; LN, low nitrogen addition; MN, medium nitrogen addition; HN, high nitrogen addition.

| PL (μm) | PW (μm) | SS (μm²) | Dₜ (10⁻³ N cm⁻²) |
|---------|---------|----------|-----------------|
| CK | 7.01 ± 0.70³ | 3.04 ± 0.97³ | 17.57 ± 1.72³ | 6.32 ± 0.45³ | 6.36 ± 0.52³ | 2.47 ± 0.58³ | 12.78 ± 1.15³ | 9.48 ± 0.45³ |
| LN | 7.26 ± 0.14³ | 3.18 ± 0.27³ | 18.10 ± 1.43³ | 8.28 ± 0.94³ | 6.53 ± 0.19³ | 2.54 ± 0.35³ | 13.06 ± 2.20³ | 11.89 ± 0.69³ |
| MN | 7.66 ± 0.19³ | 3.28 ± 0.27³ | 19.74 ± 2.11³ | 8.43 ± 1.14³ | 6.71 ± 0.30³ | 3.13 ± 0.12³ | 16.52 ± 1.42³ | 11.14 ± 0.26³ |
| HN | 7.35 ± 0.05³ | 2.73 ± 0.13³ | 15.74 ± 0.68³ | 9.93 ± 0.90³ | 6.25 ± 0.29³ | 2.40 ± 0.19³ | 11.81 ± 1.51³ | 9.63 ± 0.69³ |

Table 2. Values of stomatal parameters for different nitrogen addition treatments in Manchurian ash and Mongolian oak. All data were means ± SE (n = 64). Different lowercase letters (a, b, c) indicated significant differences between nitrogen addition treatments at P < 0.05. SS, stomatal opening status; PL and PW, stomatal pore length and width at the centre of the stoma; Dₜ, stomatal density. CK, the control; LN, low nitrogen addition; MN, medium nitrogen addition; HN, high nitrogen addition.

| PL (μm) | PW (μm) | SS (μm²) | Dₜ (10⁻³ N cm⁻²) |
|---------|---------|----------|-----------------|
| July | August | July | August |
| CK | 13.1 ± 0.88³ | 10.4 ± 0.31³ | 13.0 ± 1.22³ | 10.2 ± 0.93³ |
| LN | 14.4 ± 0.83³ | 11.8 ± 0.55³ | 16.4 ± 3.05³ | 15.7 ± 2.73³ |
| MN | 15.2 ± 0.46³ | 11.9 ± 0.32³ | 19.1 ± 1.12³ | 15.5 ± 0.47³ |
| HN | 15.0 ± 1.85³ | 11.1 ± 1.10³ | 16.0 ± 1.48³ | 12.8 ± 0.93³ |

Table 3. Values of leaf N content (g kg⁻¹) during soil nitrogen additions in July and August in both species. All values were means ± SE (n = 3). Different lowercase letters (a, b, c) indicated significant differences between nitrogen addition treatments at P < 0.05. CK, the control; LN, low nitrogen addition; MN, medium nitrogen addition; HN, high nitrogen addition.
P < 0.05), and Mongolian oak saplings maintained a higher leaf N content in nitrogen addition treatments than did Manchurian ash saplings.

Effects of soil nitrogen addition on AQP and CA activities. Soil nitrogen addition also had great effects on leaf AQP and CA activities in both species (Table 4). In this study, the activities of AQP and CA were both enlarged after soil nitrogen addition, and they also gradually strengthened with soil nitrogen added to MN and then weakened with the HN treatment. Similarly, the activities of these two enzymes also did not show significant differences between the four nitrogen addition treatments overall (P < 0.05).

Discussion

Increases in $g_m$ and $g_sc$ mostly resulted from the improvement of leaf N content. Our results showed that both $g_m$ and $g_sc$ were enlarged by soil nitrogen addition; specifically, these values increased with nitrogen addition from LN to MN and then decreased with the addition of HN. Concurrently, leaf N content also presented a similar change with soil nitrogen addition (See Table 3). We believed that the increases of $g_m$ and $g_sc$ were largely related to the enlargement of leaf N content in this study because the relationships between leaf N content and $g_m$ and $g_sc$ strongly supported the promotion of leaf N content to $g_m$ and $g_sc$ increases, in which $g_m$ and $g_sc$ both showed a positive correlation with leaf N content, even though they were highly significant in Mongolian oak (P < 0.01) (Fig. 2). This finding was supported by the positive links between leaf N content and CO2 diffusion conductance (both $g_m$ and $g_sc$) in studies of Li et al. and Yamori et al.

| Manchurian ash | Mongolian oak |
|----------------|---------------|
| AQP (U g⁻¹)   | CA (U g⁻¹)    | AQP (U g⁻¹) | CA (U g⁻¹) |
| CK 5.57 ± 0.29p | 1.54 ± 0.09p  | 5.57 ± 0.15e | 1.42 ± 0.10e |
| LN 5.78 ± 0.09p | 1.57 ± 0.05p  | 6.00 ± 0.13e | 1.40 ± 0.03e |
| MN 5.92 ± 0.04p | 1.64 ± 0.08p  | 6.80 ± 0.06e | 1.51 ± 0.02e |
| HN 5.91 ± 0.13p | 1.81 ± 0.09p  | 6.17 ± 0.31e | 1.38 ± 0.03e |

Table 4. Values of leaf AQP and CA activities during soil nitrogen additions in both species. All values were means ± SE (n = 3). Different lowercase letters (a, b, c) indicated significant differences between nitrogen addition treatments at P < 0.05. CK, the control; LN, low nitrogen addition; MN, medium nitrogen addition; HN, high nitrogen addition.

Figure 2. The correlations between $g_m$, $g_sc$ and leaf N content in Manchurian ash (a,b) and Mongolian oak (c,d). The coefficient of correlation (r) and significant correlation were at P < 0.05 and **P < 0.01.
The positive correlation between leaf N content and \( g_{\text{m}} \) found in the current study supported earlier observations that if leaves were treated individually, leaf N content explained 32% of measured variability in \( A_i \), but only 11% of that in \( g_{\text{m}} \), while variation in \( g_{\text{m}} \) explained 33% of variation in \( A_i \). With three-quarters of leaf N associated with photosynthesis (mostly Rubisco and chlorophiphy), the relationship between N and \( g_{\text{m}} \) might simply reflect the relationship between \( A_i \) and \( g_{\text{m}} \). In this study, leaf N content increased under soil nitrogen addition conditions (Table 3), this made great improvements to leaf photosynthetic capacity and \( g_{\text{m}} \) (from CK to MN, see Fig. 1), but the excessive N supply (high N addition of 69 kg N ha\(^{-1}\) a\(^{-1}\), HN) would lower Rubisco activity and content, \( g_{\text{m}} \) and \( C_{\text{w}} \).

On the other hand, the release of photosynthetic CO\(_2\) occurred in the bundle-sheath mitochondria might make an enriched CO\(_2\) partial pressure in the bundle-sheath chloroplast in C\(_3\) species and result in an increased amount of CO\(_2\) to be refixed by Rubisco. The amount of (photo)respiratory CO\(_2\) that was refixed by Rubisco can be calculated from the diffusion resistances. The \( g_{\text{m}} \) as an apparent conductance, could also be strongly affected by respiratory and photosynthetic CO\(_2\) diffusing towards the chloroplasts from the mitochondria. In this study, both CO\(_2\) compensation point in the absence of respiration \( (I^* \text{ } \Gamma) \) and the mitochondrial respiration in the light \( (R_D) \) differed among nitrogen addition levels in Manchurian ash and Mongolian oak (Table S2). This difference might enrich CO\(_2\) partial pressure in the bundle-sheath chloroplast in Manchurian ash and Mongolian oak, and increase the amount of CO\(_2\) refixed by Rubisco, finally making large influences on \( g_{\text{m}} \) responding to soil nitrogen additions. However, \( I^* \) and \( R_D \) did not show significant declines under high nitrogen addition condition as other gas exchange parameters (Table S2). This might be due to the bias of the Laisk method in the estimation \( I^* \). The Laisk method was pointed out that what it estimated is the intercellular CO\(_2\) partial pressure \( (C_i^* \text{ } \Gamma) \), rather than the true CO\(_2\) photocompensation point. In other words, the \( I^* \) measured in the current study might be the \( C_i^* \) actually, and this would lead to an enlarged CO\(_2\) high nitrogen addition condition, but this only held under the assumption of combined resistance of chloroplast envelope and stroma \( (r_s) \) being negligible. If \( r_s \) made up a significant portion of mesophyll resistance \( (r_m) \), the \( C_i^* \) would no longer necessarily smaller than the true \( I^* \); it might be equal to \( I^* \), or even greater. Hence, the \( I^* \) value estimated using the classical Laisk method and its response to soil N supplement might exist uncertainties under high or excessive nitrogen addition condition.

Since a positive relationship between leaf N content and CO\(_2\) assimilation was reported in numerous studies in which N increased Rubisco content and activity, the positive effect of leaf N content on \( g_{\text{m}} \) and \( g_{\text{m}} \) might also be achieved, as it improved the activities of related enzymes, such as AQP and CA. Furthermore, the diffusion of CO\(_2\) in mesophyll cells was not only a complexly affected by respiratory and photorespiratory CO\(_2\) diffusing towards the chloroplasts from the mitochondria. The Laisk method was pointed out that what it estimated is the intercellular CO\(_2\) partial pressure \( (C_i^* \text{ } \Gamma) \), rather than the true CO\(_2\) photocompensation point. In other words, the \( I^* \) measured in the current study might be the \( C_i^* \) actually, and this would lead to an enlarged CO\(_2\) high nitrogen addition condition, but this only held under the assumption of combined resistance of chloroplast envelope and stroma \( (r_s) \) being negligible. If \( r_s \) made up a significant portion of mesophyll resistance \( (r_m) \), the \( C_i^* \) would no longer necessarily smaller than the true \( I^* \); it might be equal to \( I^* \), or even greater. Hence, the \( I^* \) value estimated using the classical Laisk method and its response to soil N supplement might exist uncertainties under high or excessive nitrogen addition condition.

Strong promotion of soil nitrogen addition on \( g_{\text{m}} \) and \( g_{\text{m}} \) as explained in leaf anatomy. In this study, leaf anatomical structures were adjusted by soil nitrogen additions from the changes in anatomical characteristics (See Tables 1 and 2), which exhibited notably positive effects on leaf anatomy.

In this study, both CO\(_2\) compensation point in the absence of respiration \( (I^* \text{ } \Gamma) \) and the mitochondrial respiration in the light \( (R_D) \) differed among nitrogen addition levels in Manchurian ash and Mongolian oak (Table S2). This difference might enrich CO\(_2\) partial pressure in the bundle-sheath chloroplast in Manchurian ash and Mongolian oak, and increase the amount of CO\(_2\) refixed by Rubisco, finally making large influences on \( g_{\text{m}} \) responding to soil nitrogen additions. However, \( I^* \) and \( R_D \) did not show significant declines under high nitrogen addition condition as other gas exchange parameters (Table S2). This might be due to the bias of the Laisk method in the estimation \( I^* \). The Laisk method was pointed out that what it estimated is the intercellular CO\(_2\) partial pressure \( (C_i^* \text{ } \Gamma) \), rather than the true CO\(_2\) photocompensation point. In other words, the \( I^* \) measured in the current study might be the \( C_i^* \) actually, and this would lead to an enlarged CO\(_2\) high nitrogen addition condition, but this only held under the assumption of combined resistance of chloroplast envelope and stroma \( (r_s) \) being negligible. If \( r_s \) made up a significant portion of mesophyll resistance \( (r_m) \), the \( C_i^* \) would no longer necessarily smaller than the true \( I^* \); it might be equal to \( I^* \), or even greater. Hence, the \( I^* \) value estimated using the classical Laisk method and its response to soil N supplement might exist uncertainties under high or excessive nitrogen addition condition.

Both \( T_{\text{leaf}} \) and \( T_{\text{mes}} \) increased with soil nitrogen added to MN level and then decreased with the addition of HN (See Table 1); these treatments enlarged total leaf area \( (A_l) \) and mesophyll surface areas \( (A_{\text{mes}}) \) for the actual areas available for CO\(_2\) diffusion (data not shown), widened CO\(_2\) flow pathways, and correspondingly enlarged \( g_{\text{m}} \). However, \( g_{\text{m}} \) was often suggested to be the minor component to \( g_{\text{m}} \) while \( g_{\text{m}} \) was the major one. Hence, suggested by our previous conclusion that the recovery of \( g_{\text{m}} \) after soil rewatering was mainly resulted from the increase of \( g_{\text{m}} \) in Zhu et al., we believed the important promotion of soil nitrogen addition on \( g_{\text{m}} \) increase in this study would also be mainly resulted from the enlargement in \( g_{\text{m}} \). The \( g_{\text{m}} \) is tightly related to \( S_{\text{mes}} \), cell wall thickness \( (T_{\text{cw}}) \) and the chloroplast surface facing the intercellular space per unit leaf area \( (S_i) \). In this study, the \( S_{\text{mes}} \) in this study (See Table 1) enlarged the touching area between CO\(_2\) and mesophyll cells and then improved the efficiency of CO\(_2\) transmembrane diffusion. Although we did not measure \( T_{\text{cw}} \) and \( S_i \), the \( S_{\text{mes}} \) should be enlarged, while \( T_{\text{cw}} \) decreased, by soil nitrogen addition in this study, as \( S_i \) had been revealed to increase with nitrogen supply in rice, and \( T_{\text{cw}} \) was negative with both \( S_{\text{mes}} \) and \( g_{\text{m}} \). The changes in \( S_{\text{mes}} \) and \( T_{\text{cw}} \) would also strongly promote increased \( g_{\text{m}} \). Finally, the increase of \( A_l/A_{\text{mes}} \) caused by mesophyll anatomical changes directly resulted in the enlargement of \( g_{\text{m}} \). In addition, leaf PL and PW were enlarged by soil nitrogen additions in both species (See Table 2), which resulted in the improvement of stomatal opening status \( (S) \) and broadened CO\(_2\) diffusion pathway from leaf surface to substomatal cavities. Since Xiong et al. suggested that the change of \( S \) would affect \( g_{\text{m}} \) in leaf anatomy, the enlargement of \( S \) in this study would largely promote the increase of \( g_{\text{m}} \) in both species.

In summary, our data showed a different effect of soil nitrogen addition levels on leaf anatomical characteristics in this study, which reached the maximum under MN treatment and decreased under the addition of HN, indicating a decreased positive effect from high soil nitrogen addition on plant metabolism. In addition, soil...
nitrogen addition could decrease the distance between the intercellular space and catalytic site of Rubisco ($D_{r_b}$) and markedly increase chloroplast size, finally facilitating CO$_2$ diffusion in the liquid phase of mesophyll cells$^{26,28}$. This phenomenon would be another important aspect to explain the effect of leaf anatomy on $g_m$ during soil nitrogen addition.

**Conclusion**

Soil nitrogen additions could enlarge CO$_2$ diffusion conductance (both $g_m$ and $g_c$) markedly in Manchurian ash and Mongolian oak in Changbai Mountains, but its promotion was dependent on the addition levels. Moderate soil nitrogen additions ($\leq 46$ kg N ha$^{-1}$ a$^{-1}$) increased $g_m$ and $g_c$, and they reached their maximum under the addition of 46 kg N ha$^{-1}$ a$^{-1}$, but this positive effect was weakened with the addition of high level of 69 kg N ha$^{-1}$ a$^{-1}$. The effects of soil nitrogen addition on $g_m$ and $g_c$ mostly resulted from improvements in physiological traits, such as leaf N content and the activities of AQP and CA, and adjustments in anatomical characteristics, including $T_{leaf}$, $T_{mes}$, $S_{mes}$ and stomatal opening status.

**Material and methods**

**Plant material and experimental design.** Five-year-old potted saplings of similar size in Manchurian ash and Mongolian oak were selected as the materials at the National Research Station of Changbai Mountain Forest Ecosystems of the Chinese Academy of Sciences located in Jilin province of northeast China (128°06’E, 42°24’N), and they were transplanted into individual pots in 2015, filled with 271 soils collected from a broad-leaved Korean pine forest with a mean annual nitrogen forest deposition of 23 kg N ha$^{-1}$ a$^{-1}$. The volume of the pots was 29.28 L with a height of 30.0 cm and a diameter of 34.3 cm.

Four nitrogen addition levels were used to simulate nitrogen deposition intensities with the addition of no nitrogen (CK), low nitrogen (LN, 23 kg N ha$^{-1}$ a$^{-1}$), medium nitrogen (MN, 46 kg N ha$^{-1}$ a$^{-1}$) and high nitrogen (HN, 69 kg N ha$^{-1}$ a$^{-1}$). Urea solutions with different concentrations of nitrogen were sprayed into the pots once every other month from May to October in 2017. Five replicates were designed for each treatment, and all potted saplings were thoroughly watered daily to avoid water deficit. All measurements were carried out during summer growth season (July and August) to explore the changes in effects of soil nitrogen addition on $g_m$ and $g_c$.

**Simultaneous gas exchange and chlorophyll fluorescence measurements.** We simultaneously measured gas exchange and chlorophyll fluorescence on newly and fully expanded, sun-exposed leaves using an open-flow gas exchange system (LI-6400XT; Li-Cor Inc., Lincoln, NE, USA) equipped with an integrated fluorescence leaf chamber (LI-6400-40; Li-Cor). Leaves were fully light adapted under a saturated photosynthetic active photon flux density (PPFD) of 1200 µmol m$^{-2}$ s$^{-1}$ provided by LI-6400 with a 10:90 blue: red light for 30 minutes, while leaf temperature and relative humidity and CO$_2$ concentration in the leaf chamber were maintained at 25 °C and 60 ± 5% and 400 µmol CO$_2$ mol$^{-1}$ with a CO$_2$ mixture, respectively. In addition, the gas flow rate was controlled at 300 µmol s$^{-1}$ to ensure the adequate gas exchange. After stabilization to a steady state, gas exchange parameters, steady-state fluorescence ($F_s$) and maximum fluorescence ($F_{m}’$) were recorded.

The actual photochemical efficiency of photosystem II ($\Phi_{PSII}$) was calculated according to Genty et al.$^{35}$:

$$\Phi_{PSII} = \frac{(F_m’-F_i)}{F_m’}$$

(1)

The photosynthesis electron transport rate ($J_i$) was calculated as follows:

$$J_i = \Phi_{PSII} \cdot \text{PPFD} \cdot \alpha \beta$$

(2)

where $\alpha$ is the total leaf absorptance and $\beta$ is the partitioning of absorbed quantum between PS II and PS I.

In this study, light response curves ($A_i$-PPFD curve) for controlled and nitrogen-addition saplings were also measured under a low O$_2$ concentration ($<1\%$) condition to correct the $\alpha \beta$, as it was equal to the slope of the relationship between $\Phi_{PSII}$ and $\Phi_{CO2}$ (the quantum efficiency of CO$_2$ fixation)$^{36}$. Values of $\alpha \beta$ for different nitrogen treatments were shown in Table S1.

The mesophyll conductance ($g_m$) was calculated using the ‘variable $J$ method’ described in Harley et al.$^{37}$:

$$g_m = \frac{A_i}{C_i - \frac{1}{\hbar} + \frac{\alpha \beta}{\hbar} - \frac{\alpha \beta}{\hbar} \frac{A_m + R_d}{A_m}}$$

(3)

where $A_m$ is the net photosynthetic rate, $C_i$ is the intercellular CO$_2$ concentration, and these values were directly obtained from gas exchange measurements; $I_i$ represents the CO$_2$ compensation point in the absence of respiration and $R_d$ represents the mitochondrial respiration in the light.

$I_i$ and $R_d$ were measured using the Laisk method, namely, 5 initial slopes of $A_m$-$C_i$ curves under low light and low CO$_2$ concentrations were measured for $I_i$ and $R_d$ estimations in this study.$^{38,39}$ In theory, three CO$_2$ response curves obtained by varying CO$_2$ concentrations from 150 to 40 µmol CO$_2$ mol$^{-1}$ under three PPFDs (150, 100 and 50 µmol m$^{-2}$ s$^{-1}$) would intersect with each other at a point, and the intersection point at x-axis and y-axis were considered to be $I_i$ and $R_d$, respectively. But in practice, these three linear regressions of the intersected $A_m$-$C_i$ curves formed a triangle range rather than a single point$^{40}$ (Fig. S1), and the $I_i$ and $R_d$ were calculated as the barycenter of the triangle formed by the intersection of the three lines at x-axis and y-axis, respectively, according to our previous published method$^{40}$. The values of $I_i$ and $R_d$ during soil nitrogen additions were shown in Table S2.

Stomatal conductance to CO$_2$ ($g_{sc}$, mol CO$_2$ m$^{-2}$ s$^{-1}$) was calculated from the ratio of stomatal conductance to water ($g_{sw}$, mol CO$_2$ m$^{-2}$ s$^{-1}$) to 1.6 (i.e., $g_{sc} = g_{sw}/1.6$), as $g_{sw}$ was 1.6 times larger than $g_{sc}$.$^{41}$
Measurement of leaf anatomical characteristics. After gas exchange measurements, we cut fifteen small leaf samples (4.0 mm × 1.5 mm) from five replicated leaves per treatment and fixed them in FAA (alcohol: glacial acetic acid: 90: 5: 5) to measure leaf anatomical characteristics, mainly including leaf thickness (Tmes, μm), mesophyll and stomatal anatomical characteristics, such as the thickness of mesophyll cells between the two epidermal layers (Tmes, μm) and the surface area of mesophyll cells exposed to intercellular space per unit leaf area (Smes, μm² μm⁻²) calculated using formula (4), stomatal pore length (PL, μm) and width (PW, μm) at the centre of the stomata, which were detailed described in Zhu et al.60. Besides, we counted the number of stomata per unit leaf area to calculate the stomatal density (Dn, N cm⁻²).

\[
S_{\text{mes}} = \frac{L_{\text{mes}}}{W} \cdot F
\]

where the length of mesophyll cells exposes to intercellular space (Lmes, μm) and the cross-sectional width (W, μm) are measured with Image J software. The curvature correction factor (F) was measured using the method described in the study of Evans et al.26 and Thain (1983)45, which was shown in Table S3.

We also calculated the gas- and liquid-phase CO₂ diffusion conductance in mesophyll cells (i.e., \(g_{\text{gas}}\) and \(g_{\text{liq}}\)) with leaf anatomical characteristics according to formulas (5) and (6) to assess their relationships with \(g_{\text{gas}}\).26

\[
g_{\text{gas}} = \frac{D_{\text{gas}}}{\Delta L_{\text{gas}}} S
\]

\[
\frac{1}{g_{\text{liq}}} = \left( \frac{1}{g_{\text{ct}}} + \frac{1}{g_{\text{pl}}} + \frac{1}{g_{\text{en}}} + \frac{1}{g_{\text{en}}} + \frac{1}{g_{\text{ct}}} \right) \cdot A_T
\]

where \(D_{\text{gas}}\) (m² s⁻¹) is the diffusion coefficient for CO₂ in the gas phase, \(\Delta L_{\text{gas}}\) (μm) is taken as half \(T_{\text{mes}}\), and \(S\) is the mesophyll surface area corrected for the actual area available for CO₂ diffusion. \(g_{\text{ct}}, g_{\text{pl}}, g_{\text{en}}, g_{\text{en}}\) and \(g_{\text{ct}}\) are the partial conductance for the cell wall, plasmalemma, cytosol, chloroplast envelope and chloroplast stroma (m⁻¹), respectively. We used an estimation of 0.0035 m s⁻¹ for the \(g_{\text{pl}}\) and \(g_{\text{en}}\) according to the previous studies42,43, and estimated \(g_{\text{ct}}, g_{\text{en}}\) and \(g_{\text{ct}}\) using the formula of \(g_{\text{ct}} = \frac{nD_{\text{gas}}}{3A}\) in the studies of Tomás et al.26 and Niinemets and Reichstein (2003)44, where \(g_{\text{ct}}\) is either \(g_{\text{ct}}\) or \(g_{\text{ct}}\), \(g_{\text{ct}}\) is the dimensionless coefficient, \(D_{\text{gas}}\) (m² s⁻¹) is the aqueous-phase volatile diffusion coefficient for CO₂ (1.79 × 10⁻⁹ m² s⁻¹ at 25°C), \(\Delta L_{\text{gas}}\) (m) is the diffusion path length, and \(p\) (m² m⁻³) is the effective porosity. According to the previous studies of Tomás et al.26 and Niinemets and Reichstein (2003)44, \(r_1\) was valued as 1 for cell wall, and an estimate of \(r_1\) of 0.294 for \(g_{\text{ct}}\) and \(g_{\text{ct}}\) in this study, \(p\) was taken as 1 for \(g_{\text{ct}}\) and \(g_{\text{ct}}\), and 0.3 for cell walls, \(\Delta L_{\text{gas}}\) was valued as 5.0 × 10⁻² (for cell wall, \(\Delta L_{\text{gas}}\), 9.7 × 10⁻⁸ (for cytosol, \(\Delta L_{\text{ct}}\)) and 1.65 × 10⁻⁹ (for chloroplast stroma, \(\Delta L_{\text{ct}}\)). \(A_{\text{mes}}\) (μm²) and \(A_T\) (μm²) are mesophyll surface area and total leaf surface area corrected for the actual area available for CO₂ diffusion, respectively, calculated from the light microscope.

Measurement of leaf N content. Leaves were picked and over-dried at 75 °C for 24 h to constant weight during gas exchange measuring periods and then ground using a mixer oscillating mill homogenizer (MM400, Retsch, Germany). Approximately 5.0 mg leaf samples were taken to measure leaf nitrogen content per area using a C N element analyser (Elementar vario MACRO, Element, Germany).

Measurements of leaf aquaporin and carbonic anhydrase activities. For analysing the physiological mechanism of \(g_{\text{gas}}\) and \(g_{\text{liq}}\) responses to soil nitrogen additions, we sampled fifteen fresh leaves per treatment to measure the activities of aquaporin (AQP) and carbonic anhydrase (CA) using enzyme-linked immunosorbent assay (ELISA)46.47. Solid-phase antibody was made using purified plant AQP (or CA) antibody. Then, combined with antibody labelled with horseradish peroxidase (HRP), AQP (or CA) was added to microtiter plate wells to become an antibody-antigen-enzyme-antibody complex. This complex became blue with 3,3’,5,5’-tetramethyl benzidine (TMB) substrate solution after complete washing. The optical density (OD) values were measured spectrophotometrically at a wavelength of 450 nm to compare with the standard curves to determine the activity of AQP (or CA) in the samples.

Statistical analysis. SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for one-way statistical analysis of normality and homogeneity of variance between nitrogen addition treatments (one-way ANOVA). Furthermore, regression analysis between \(g_{\text{gas}}\) and leaf N content was also performed. Mean values were compared using the least significant difference (LSD) multiple comparison test at the 0.05 and 0.01 probability levels (P < 0.05 and P < 0.01) with Tukey’s honest significant difference (HSD) test.

Data availability
All data analysed during this study are included in this published article and its Supplementary Information files.

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Author contributions
F.Y., D.G. and C.J. conceived the project. K.Z., A.W. and Y.Z. performed the field experiments. K.Z., C.G. and J.W. contributed to lab work. K.Z., A.W. and J.W. interpreted the results. K.Z., F.Y. and D.G. wrote the manuscript with assistance from other coauthors.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to F.Y. or D.G.

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