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Stomatal, mesophyll and biochemical limitations to soil drought and rewatering in relation to intrinsic water-use efficiency in Manchurian ash and Mongolian oak

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Abstract

The balance between stomatal and mesophyll conductance has been reported to directly influence intrinsic water-use efficiency (WUE$_i$), but it is unclear whether variations in stomatal and mesophyll limitations (l_{sc} and l_m, respectively) affect WUE$_i$ during soil drought and rewatering. Limitations (l_{sc}, l_m and biochemical limitation, l_b) and WUE$_i$ were measured in Manchurian ash (*Fraxinus mandshurica* Rupr.) and Mongolian oak (*Quercus mongolica* Fish. ex Ledeb) saplings exposed to two levels of water stress (moderate and severe) and rewatering in this study. The primary limiting factors for photosynthesis among l_{sc}, l_m, and l_b and their correlations with WUE$_i$ were assessed. Both l_{sc} and l_m increased with water stress, while l_b decreased, and during recovery, l_{sc} and l_m decreased, while l_b increased. Photosynthesis limitations directly influenced WUE$_i$, with WUE$_i$ being larger after rewatering than during water stress, increasing as biochemical capacity weakened at the expense of diffusional limitations (l_{sc} and l_m). Stomatal closure was more limiting to net photosynthesis in Mongolian oak than in Manchurian ash during the drought and recovery treatments; consistently higher WUE$_i$ was measured in the former species, possibly due to a more developed root system and larger leaf hydraulic conductance and stem vessel diameter in the former species.

Keywords: biochemical capacity; diffusional limitations; intrinsic water-use efficiency; rewatering; soil drought.

Highlights

- Both stomatal limitation (l_{sc}) and mesophyll limitation (l_m) increased with water stress, while biochemical limitation (l_b) decreased
- During recovery, l_{sc} and l_m decreased, while l_b increased
- Photosynthesis limitations directly influenced WUE$_i$

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Abbreviations: C_i – intercellular CO$_2$ concentration; C_c – chloroplast CO$_2$ concentration; D_{stem} – stem vessel diameter; ETR – electron transport rate; F$_m$' – maximum fluorescence; F$_s$ – steady-state fluorescence; g_m – mesophyll conductance; g_{sc} – stomatal conductance to CO$_2$; g_{sw} – stomatal conductance to water; K_{leaf} – hydraulic conductance; l_b – biochemical limitation; l_m – mesophyll limitation; l_{sc} – stomatal limitation; P_N – net photosynthetic rate; RWC – relative soil water content; SWC – soil water content; S_L, MC_L, and B_L – the contributions of g_{sc}, g_m, and biochemical capacity to dP_N/P_N; R_d – the mitochondrial respiration in the light; V_{cmax} – maximum carboxylation rate; WUE$_i$ – intrinsic water-use efficiency; α – the total leaf absorptance; β – the partitioning of absorbed quantum between PSI and PSII; Γ^* – CO$_2$-compensation point in the absence of respiration; Φ_{CO2} – the quantum efficiency of CO$_2$ fixation; Φ_{PSII} – the actual photochemical efficiency of photosystem II; Ψ_{pd} – predawn leaf water potential.

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Introduction

With the rapid climate change during recent decades, global precipitation distributions have become seriously unbalanced (Dore 2005, IPCC 2013). Consequently, soil drought events have become more frequent, longer, and more intense in some areas around the world, with profound consequences for ecosystems (Tezara *et al.* 1999). Soil water availability is the principal environmental factor affecting plant photosynthesis, growth, and primary production globally (Nemani *et al.* 2003, Zhou *et al.* 2014). In C_3 photosynthesis, CO_2 diffuses from the air surrounding the leaf to the sites of fixation within the chloroplasts (Flexas *et al.* 2018). This process is greatly affected by changes in stomatal (g_{sc}) and mesophyll conductances (g_m) and biochemical capacity, as reflected by parameters such as leaf maximum carboxylation and enzyme activity (Luterbacher *et al.* 2004, Flexas *et al.* 2006, Joos and Spahni 2008, Perez-Martin *et al.* 2011, Xiong *et al.* 2015a). The contributions of diffusional conductance to CO_2 (*i.e.*, g_{sc} and g_m) and biochemistry to net photosynthetic rate (P_N) may be described in terms of relative limitations to P_N, namely, stomatal limitation (l_{sc}), mesophyll limitation (l_m), and biochemical limitation (l_b) (Chaves 1991, Lawlor 1995, Cornic and Massacci 1996, Grassi and Magnani 2005, Flexas *et al.* 2009), which are severely influenced by soil drought.

During the past few decades, many efforts have been made to explore the changes in l_{sc}, l_m, and l_b during soil drought and their primary roles in photosynthesis (Flexas *et al.* 2002, Grassi and Magnani 2005, Cai *et al.* 2010, Aranda *et al.* 2012a, Rho *et al.* 2012). For instance, reductions in both g_{sc} and g_m have been found to account for most of the photosynthesis decrease under moderate water stress conditions, whereas photosynthesis has been found to be affected mainly by biochemical capacity (*i.e.*, metabolic impairment) only under severe stress conditions (Nadal and Flexas 2019). Recently, a few studies have explored the responses of photosynthesis limitations. For example, in response to both water stress and rewatering, congeneric species have been found to exhibit contrasting responses linked to differences in tolerance to water stress (Cano *et al.* 2014). Furthermore, in a study of species in the Balearic Islands, Galmés *et al.* (2007) found that l_m was the strongest factor influencing photosynthesis recovery, whereas l_{sc} and l_b were of similar magnitudes throughout the entire range. Similarly, Cai *et al.* (2015) emphasized the primary role of l_m in the photosynthesis of *R. delavayi* during water stress and recovery. In addition, a dominant role of l_{sc} in photosynthesis had been suggested in studies by Flexas *et al.* (2009) and Perez-Martin *et al.* (2014). Ennahli and Earl (2005) confirmed the important contribution of photosynthesis biochemistry to relative photosynthesis limitations. Additionally, Warren *et al.* (2011) found that the complete recovery of net photosynthesis after rewatering in several tree species was associated with a lower CO_2 concentration in the chloroplasts (C_c) than the concentration observed in control plants, suggesting that water stress leads to increased amounts and/or activity of Rubisco (*i.e.*, photosynthesis capacity). Furthermore, Grassi and Magnani (2005), Cano *et al.* (2013), and Peguero-Pina *et al.* (2018a) asserted that diffusive limitations (*i.e.*, l_{sc} + l_m) strongly affect light-saturated assimilation and that l_b is quantitatively important only during leaf development and senescence. Nevertheless, the responses of l_{sc}, l_m, and l_b and their primary roles in photosynthesis during soil recovery and drought acclimation have been less well studied than those during drought, especially in the Changbai Mountains, Northeast China.

Previous studies have revealed that leaf water-use efficiency reflects the relationship between water consumption and carbon assimilation, and it has been widely used to assess plant adaption to water stress (Flexas *et al.* 2013, Xia *et al.* 2017, Pirzad and Mohammadzadeh 2018). Leaf intrinsic water-use efficiency (WUE_i) greatly influences photosynthesis and strongly correlates with CO_2 diffusion (Tomás *et al.* 2014, Barbour and Kaiser 2016). The balance between g_m and g_{sc} has been reported to influence directly leaf WUE_i and gas-exchange recovery (Duan *et al.* 2010, Flexas *et al.* 2013, Cano *et al.* 2014), but the relationship regarding photosynthesis limitation with WUE_i is unclear.

Manchurian ash (*Fraxinus mandshurica* Rupr.) and Mongolian oak (*Quercus mongolica* Fish. ex Ledeb) are two dominant tree species in the primitive broad-leaved Korean pine forests of northeast China. The two species exhibit contrasting drought tolerances; Mongolian oak has much stronger drought-tolerance capacity due to its more developed root system than that of Manchurian ash (Aranda *et al.* 2012b). We previously showed (Zhu *et al.* 2019) that upon rewatering Manchurian ash and Mongolian oak after different degrees of water stress, g_{sc} and g_m recovered only partially in a species- and stress intensity-dependent manner. Here, we aimed to assess whether variation in l_{sc} and l_m during soil drought and rewatering in the two species result in variation in WUE_i. To address this question, the values of l_{sc}, l_m, and l_b and leaf WUE_i during soil drought and rewatering were measured in Manchurian ash and Mongolian oak saplings. This study enhances our understanding of the drought-tolerance mechanisms of trees and the relationships of g_m and g_{sc} with leaf water-use efficiency.

Materials and methods

Plant material: Two dominant broad-leaved tree species of Manchurian ash (*Fraxinus mandshurica* Rupr.) and Mongolian oak (*Quercus mongolica* Fish. ex Ledeb) in a primitive broad-leaved Korean pine forest in the Changbai Mountains, Northeast China (42°24'N, 128°06'E, 738 m a.s.l.) were selected. The region has a typical temperate continental monsoon climate, a mean annual air temperature of 3.6°C and an average annual precipitation of 695 mm. In April 2015, five-year-old saplings of both species with similar growth status collected from a primitive broad-leaved Korean pine forest in the Changbai Mountains of Northeast China were transplanted into individual pots, each with a volume of 29.28 L (30.0 cm height, 34.3 cm diameter) and containing 27 L of surface soil collected from the same forest from which the saplings were collected, with a field moisture capacity (FC) of 0.426 g cm^{-3}.

Experimental design: Potted saplings were proportionally divided into three groups subjected to three treatments, with five replicates per treatment: well irrigated (control, CK, 90 ± 5% FC), moderate water stress (MW, 40 ± 5% FC), and severe water stress (SW, 20 ± 5% FC). Before starting the water control experiment, the potted saplings were well irrigated daily from 20 May to 20 June to FC. Then, the water-stressed saplings were allowed to dry naturally without water input while the controls were well irrigated for the next 10 d. Soil water content (SWC, volumetric water content) at a 15-cm depth was monitored every 30 min using a soil humidity real-time observation system (*93640 Hydra*, Stevens, USA). SWC was converted to relative SWC (relative soil water content – RWC; RWC = SWC/FC × 100%) to describe the variation in SWC in the pots. When RWC declined to the designated stress levels, the water control experiment was started. During the experiment, all saplings were watered (not every day) according to the real-time RWC obtained from the *93640 Hydra* system to maintain their designated water stress or non-stress status. In addition, five replicate plants per treatment were used for gas-exchange measurements in this study, and all measurements for the control, water-stressed, and rewatered saplings were collected within 3 d. Specifically, the measurements were conducted after 2 d of rewatering, and the plants were well watered during the recovery period.

The saplings were blocked from outside water by placing pedestals under the pots. In addition, all potted saplings were placed under a 10-m long, 5-m wide, and 3.5-m high rain shelter covered with transparent plastic film (95% light transmittance) that was well ventilated with open sidewalls. The variations in daily soil RWC during soil drought and rewatering are shown in Fig. 1.

Predawn leaf water potential: To reflect soil moisture stress, we measured predawn leaf water potential (Ψ_{pd} [MPa]) in three different leaves per treatment using a pressure chamber (*1505D*, *PMS Instrument Company*, USA). The detailed measurement processes are described in Zhu *et al*. (2019), and the Ψ_{pd} values are shown in Fig. 2.

Simultaneous gas exchange and chlorophyll (Chl) fluorescence measurements: Light-saturated gas exchange and Chl fluorescence were simultaneously measured on the youngest fully expanded, sun-exposed leaves of five plants per species. Treatments were done from 8:00 h to 11:30 h each day from 15–20 July (‘water stress’ period) and 23–26 August (‘recovery’ period), in the same individuals each period, 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*, *Inc*.). In the leaf chamber, a saturated PPFD of 1,200 μmol(photon) m^{-2} s^{-1} was provided by a 10:90 blue:red light source of the *LI-6400XT* instrument. The leaf temperature was maintained at 25°C, the relative humidity was approximately 60%, the leaf-to-air vapour pressure deficit (VPD) was between 1.0 and 1.4 kPa, and the flow rate was controlled at 300 μmol s^{-1}. Gas-exchange parameters, steady-state fluorescence (F_s), and maximum fluorescence (F_m') with a light-saturating pulse of 7,800 μmol(photon) m^{-2} s^{-1} were recorded after reaching a steady state in leaves fully light-adapted for 25–30 min (Loriaux *et al*. 2013). The actual photochemical efficiency of PSII

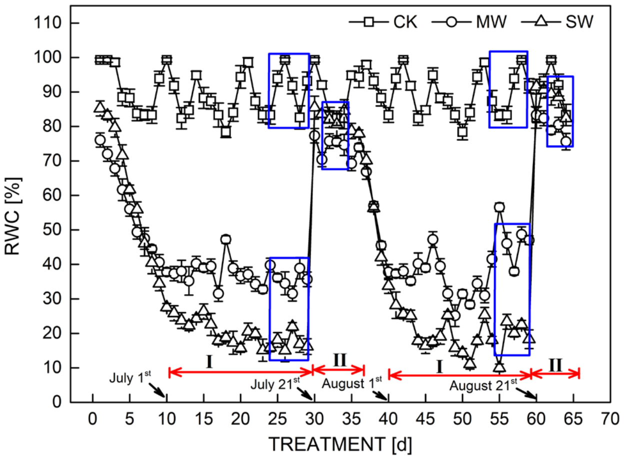

Fig. 1. Variations of daily soil relative water contents (RWC [%]) during soil drought (I) and rewatering (II) in July and August. All data were the averages of both species' pots with variations (n = 3). CK – control; MW – medium water stress; SW – severe water stress. Gas-exchange measurement and leaf sampling were performed in the days marked with *blue box* (Zhu *et al*. 2019).

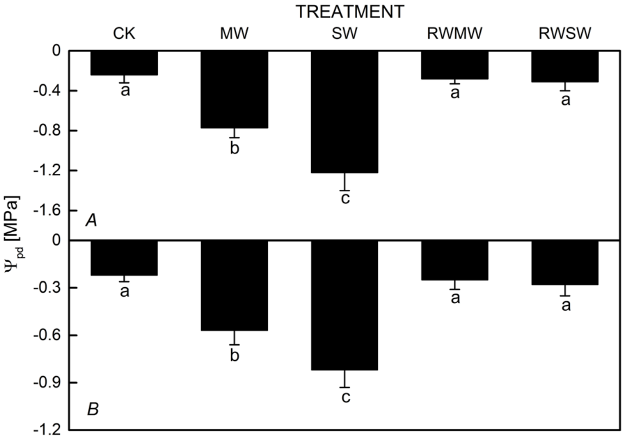

Fig. 2. Predawn leaf water potential (Ψ_{pd} [MPa]) responses to different treatments in Manchurian ash (*A*) and Mongolian oak (*B*) (n = 3). The error bar represents standard error of all measurements in each individual tree species. *Different letters* indicate significant difference between each group treatments (P<0.05). CK – control; MW – medium water stress; SW – severe water stress; RWMW – rewatering after initial medium water stress; RWSW – rewatering after initial severe water stress (Zhu *et al*. 2019).

(Φ_{PSII}) was calculated according to Genty *et al.* (1989) as follows:

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

The electron transport rate (ETR) was then calculated as:

$$\mathrm{ETR} = \Phi_{PSII} \times \mathrm{PPFD} \times \alpha\beta \quad (2)$$

where α is the total leaf absorbance and β is the partitioning of absorbed quanta between PSI and PSII. In this study, αβ was calibrated equally as the slope of the relationship between Φ_{PSII} and $4\Phi_{CO_2}$ (the quantum efficiency of CO_2 fixation) obtained from the light-response curves (P_N/PPFD curves), which were measured under low O_2 concentration (< 1%) conditions by injecting pure N_2 (Valentini *et al.* 1995, Xiong *et al.* 2015a).

Estimation of g_m with the variable J method: The g_m was estimated with the 'variable *J* method' described in Harley *et al.* (1992):

$$g_m = \frac{P_N}{C_i - \dfrac{\Gamma^*\left[\mathrm{ETR} + 8\left(P_N + R_D\right)\right]}{\mathrm{ETR} - 4\left(P_N + R_D\right)}} \quad (3)$$

Here, P_N is the net photosynthetic rate [μmol(CO_2) m^{-2} s^{-1}]; C_i is the intercellular CO_2 concentration [μmol(CO_2) mol^{-1}], which was directly obtained from gas-exchange measurements; Γ^* represents the CO_2-compensation point in the absence of respiration [μmol mol^{-1}]; and R_d represents mitochondrial respiration in the light [μmol m^{-2} s^{-1}].

Γ^* and R_d were measured using the Laisk method (Laisk 1977). In this study, three P_N–C_i curves measured under low light [150, 100, and 50 μmol(photon) m^{-2} s^{-1}] and low CO_2 concentrations [from 150 to 40 μmol(CO_2) mol^{-1}] formed a triangle range (Sun *et al.* 2015), and the barycentre of the triangle at the *x*-axis and *y*-axis were considered Γ^* and R_d, respectively. The Γ^* and R_d values are provided by Zhu *et al.* (2019).

Photosynthesis limitations analysis: According to Grassi and Magnani (2005) and Wang *et al.* (2018), the relative changes in light-saturated assimilation were sequentially expressed in terms of parallel relative changes in stomatal to g_{sc} and g_m and in biochemical capacity (*i.e.*, in maximum carboxylation rate, V_{cmax}):

$$\frac{dP_N}{P_N} = SC_L + MC_L + B_L = l_{sc} \times \frac{dg_{sc}}{g_{sc}} + l_m \times \frac{dg_m}{g_m} + l_b \times \frac{dV_{cmax}}{V_{cmax}} \quad (4)$$

$$l_{sc} = \frac{\dfrac{g_{tot}}{g_{sc}} \times \dfrac{\partial P_N}{\partial C_c}}{g_{tot} + \dfrac{\partial P_N}{\partial C_c}} \quad (5)$$

$$l_m = \frac{\dfrac{g_{tot}}{g_m} \times \dfrac{\partial P_N}{\partial C_c}}{g_{tot} + \dfrac{\partial P_N}{\partial C_c}} \quad (6)$$

$$l_b = \frac{g_{tot}}{g_{tot} + \dfrac{\partial P_N}{\partial C_c}} \quad (7)$$

Here, l_{sc}, l_m, and l_b are the relative limitations imposed by g_{sc}, g_m, and biochemical capacity, respectively ($0 < l_i < 1$, i = sc, m, b); g_{tot} is total conductance to CO_2 between the leaf surface and carboxylation sites; SC_L, MC_L, and B_L are the contributions of g_{sc}, g_m, and biochemical capacity, respectively, to dP_N/P_N; and the sum of contributions due to g_{sc} and g_m was defined as diffusional limitation ($D_L = SC_L + MC_L$), whereas the sum of g_m and biochemical capacity was defined as nonstomatal limitation ($NS_L = MC_L + B_L$) (Grassi and Magnani 2005). In the current study, V_{cmax} was replaced with ETR, which is coupled with V_{cmax} (Galmés *et al.* 2007, Galle *et al.* 2009, Wang *et al.* 2018, Han *et al.* 2019), and the fitted photosynthesis parameters at Ψ_{pd} = –0.25 MPa (CK) were used as the reference values (Wang *et al.* 2018). Thus,

$$\frac{dx}{x} = \frac{x_{ref}{}^{-x}}{x_{ref}} = \frac{x_{0.25}{}^{-x}}{x_{0.25}} \quad (8)$$

where *x* represents the fitted g_{sc}, g_m or ETR and $x_{0.25}$ (*i.e.*, x_{ref}) represents the *x* value at Ψ_{pd} = –0.25 MPa (CK).

The g_{tot} was the total conductance to CO_2, and it was calculated by Eq. 9:

$$g_{tot} = \frac{1}{\dfrac{1}{g_{sc}} + \dfrac{1}{g_m}} \quad (9)$$

$$g_{sc} = g_{sw}/1.6 \quad (10)$$

where g_{sw} is the stomatal conductance to water vapour [mol(H_2O) m^{-2} s^{-1}], and it was directly obtained from gas-exchange measurements; 1.6 is the ratio of the diffusivities of CO_2 and water in air.

Calculation of leaf WUE_i: At the leaf level, the WUE_i was defined as the ratio of leaf P_N to g_{sw} (Osmond *et al.* 1980), which was calculated as:

$$WUE_i = P_N/g_{sw} \quad (11)$$

Statistical analysis: After testing for normality and homogeneity of variance, using *SPSS 17.0* (*SPSS*, *Inc*., Chicago, IL, USA), we performed one-way analysis of variance (*ANOVA*) to detect differences in l_{sc}, l_m, and l_b between the different treatments. Furthermore, regression analyses of P_N, g_{sc}, and g_m and analyses of the correlations between WUE_i and l_{sc}, l_m, and l_b were performed. The mean values were compared using the least significant difference (LSD) multiple comparison test (P<0.05 or P<0.01).

Results

Responses of relative l_{sc}, l_m, and l_b to soil drought and rewatering: The responses of relative photosynthesis limitations (l_{sc}, l_m, and l_b) to soil drought and rewatering were shown in Fig. 3. l_{sc}, l_m, and l_b all showed similar changes during soil drought between July and August; specifically, both l_{sc} and l_m increased with water stress, while l_b decreased significantly. Biochemical capacity was found to be the major limiting factor for photosynthesis in well-irrigated saplings in both species, as l_b contributed more than 50% of the photosynthesis limitations. However, the major limiting factor was the conversion of l_m and l_{sc} during drought, which increased from 10–20% to 30–40% after water stress was established, whereas l_b decreased by 30–50%. In addition, l_m was larger than l_{sc} in the different treatments in Manchurian ash but lower than l_{sc} in Mongolian oak overall. Thus, the reductions in plant photosynthesis during soil drought in Manchurian ash might result mainly from the increase in l_m, whereas that in Mongolian oak might be largely caused by the increase in l_{sc}.

After rewatering, with the increases in soil RWC and leaf Ψ_{pd} (data available in Zhu *et al.* 2019), the photosynthesis limitations changed, with both l_m and l_{sc} decreasing and l_b increasing. Furthermore, these changes showed some species differences. Average decreases of 7.6% (MW) and 5.1% (SW) in l_m relative to the values before rewatering occurred in Manchurian ash, whereas l_m decreased from pre-watering levels by 9.4% (MW) and 19.6% (SW) on average in Mongolian oak (Fig. 3). In contrast, l_{sc} in Manchurian ash increased by approximately 1.0% (MW) and 3.4% (SW), whereas that in Mongolian oak increased by 1.6% in the MW treatment and decreased by 6.4% in the SW treatment, on average. In contrast to l_m and l_{sc}, l_b increased after rewatering, by 7.0% (MW)

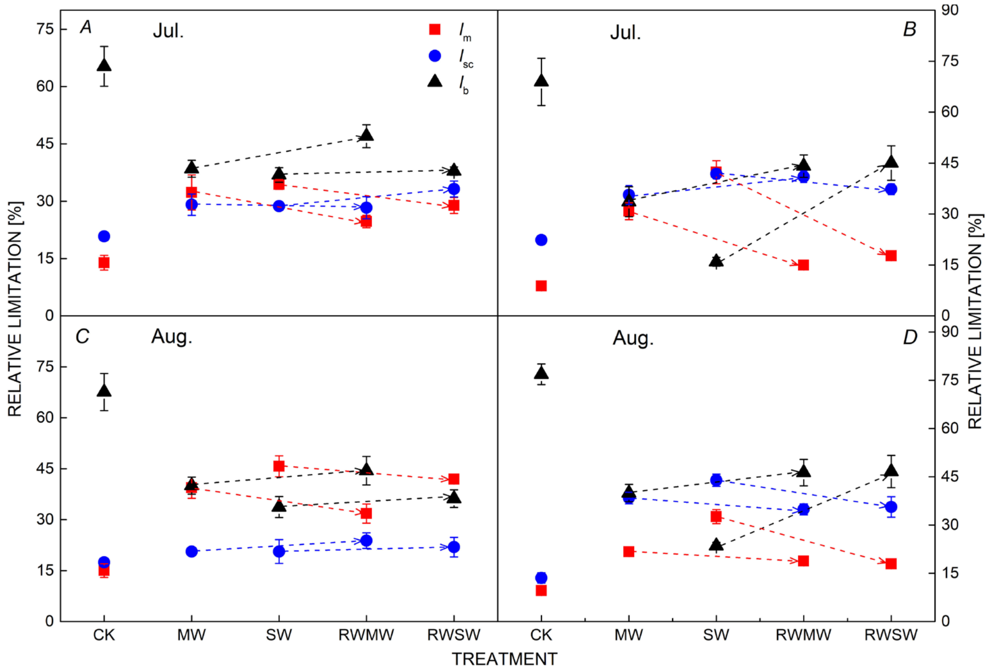

Fig. 3. Effects of soil drought and rewatering on the relative limitations in Manchurian ash (*A*,*C*) and Mongolian oak saplings (*B*,*D*) in July (Jul.) and August (Aug.). CK – control; MW – medium water stress; SW – severe water stress; RWMW – rewatering after initial medium water stress; RWSW – rewatering after initial severe water stress. l_b – biochemical limitation; l_m – mesophyll limitation; l_{sc} – stomatal limitation.

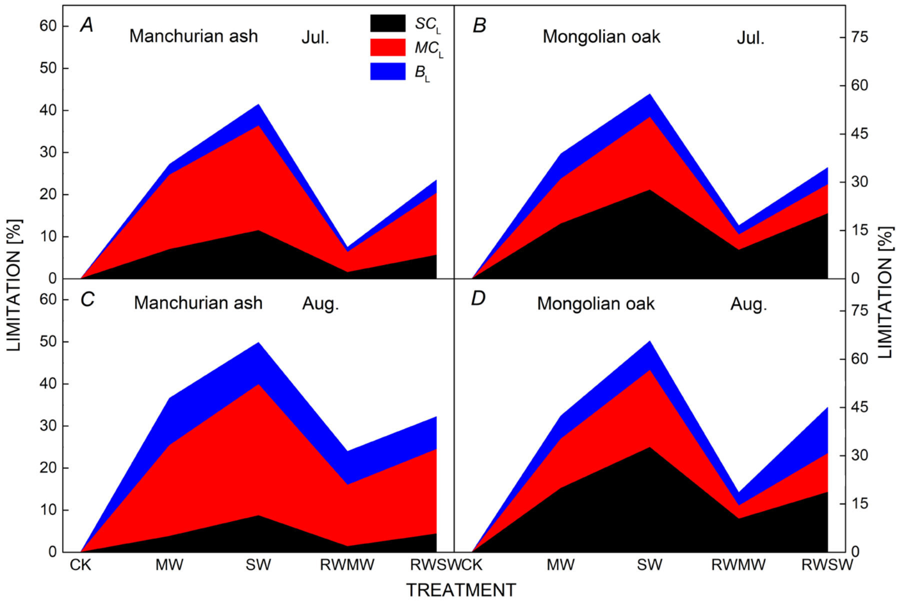

Fig. 4. Contributions of stomatal conductance to CO_2 (g_{sc}) (SC_L), mesophyll conductance (g_m) (MC_L), and biochemical capacity (B_L) to photosynthesis change (dP_N/P_N) for controlled, water-stressed, and rewatered saplings during soil and rewatering in Manchurian ash and Mongolian oak in July (Jul.) and August (Aug.). CK – control; MW – medium water stress; SW – severe water stress; RWMW – rewatering after initial medium water stress; RWSW – rewatering after initial severe water stress.

and 1.8% (SW) on average in Manchurian ash and greater extents, 8.5% (MW) and 26.1% (SW), in Mongolian oak.

Photosynthesis contributions of g_{sc}, g_m, and biochemical capacity during soil drought and rewatering: Fig. 4 showed the contributions of g_{sc}, g_m, and biochemical capacity (SC_L, MC_L, and B_L) to photosynthesis change (dP_N/P_N) in this study. SC_L, MC_L, and B_L all increased compared to their corresponding values in the well-irrigated treatment after water stress was established. After rewatering, the values of SC_L, MC_L, and B_L decreased in both species, and MC_L was still the largest in Manchurian ash, whereas SC_L was the largest in Mongolian oak. These results indicated that photosynthesis recovery was mostly affected by l_m in Manchurian ash and by l_{sc} in Mongolian oak. Significant differences between treatments were found for SC_L, MC_L, and B_L in both species. Upon rewatering, all three limitations became much more similar to each other than they were under stress. In addition, the contributions of diffusional limitations (SC_L +MC_L) were much larger than those of biochemical limitation (B_L) over the two periods of water stress and rewatering, indicating that CO_2 diffusion inside leaves had a larger effect on plant photosynthesis than did other biochemical processes.

Changes in leaf WUE$_i$: WUE$_i$ (Fig. 5) decreased in both species with water stress, but after rewatering, it increased by 13.9% (RWMW) and 19.8% (RWSW) in July and by 25.2% (RWMW) and 22.5% (RWSW) in August in Manchurian ash and by 4.4% (RWMW) and 9.2% (RWSW) in July and by 8.4% (RWMW) and 11.1% (RWSW) in August in Mongolian oak. Overall, some differences in WUE$_i$ were observed depending on the extent of stress and rewatering treatment. Mongolian oak maintained a much larger WUE$_i$ than that of Manchurian ash during water stress and rewatering in July and August.

Relationships between leaf WUE$_i$ and photosynthesis limitations: We explored the relationships between WUE$_i$ and relative photosynthesis limitations (l_m, l_{sc}, and l_b) in both species (Fig. 6). The results showed that l_m and l_{sc} each maintained a negative correlation with WUE$_i$, whereas l_b maintained a positive correlation with WUE$_i$. The correlation coefficient (r) values between WUE$_i$ and l_m, l_{sc}, and l_b were –0.525, –0.386, and 0.448, respectively, in Manchurian ash and –0.449, –0.395, and 0.509, respectively, in Mongolian oak.

Relationships between leaf P_N and g_m, g_{sc}, g_{tot}, and l_b: Leaf P_N in the two varieties both showed a regression relationship with g_m, g_{sc}, and g_{tot} during the drought and recovery treatments (Fig. 7), and this regression weakened after rewatering, reflected by the declined slope during recovery. But the P_N did not show any regression with the l_b in ash and oak saplings.

Relationships between leaf WUE$_i$ and g_m, g_{sc}, and g_m/g_{sc}: Leaf WUE$_i$ did not correlate with g_m and g_{sc}, but a strong correlation between WUE$_i$ and g_m/g_{sc} was observed in these two species (Fig. 8, $P<0.05$).

Discussion

Photosynthesis roles of l_{sc}, l_m, and l_b changing with soil drought and rewatering: Our data showed that photosynthesis limitations imposed by changes in g_{sc} and g_m and biochemical capacity (*i.e.*, l_{sc}, l_m, and l_b) during soil drought and rewatering vary with soil water fluctuations. Biochemical capacity limitation plays a primary role in well-irrigated saplings, with l_b being the largest among the studied limitations, but this limitation gradually changes as soil water availability decreases. Once water stress is established, both l_{sc} and l_m markedly increase while l_b

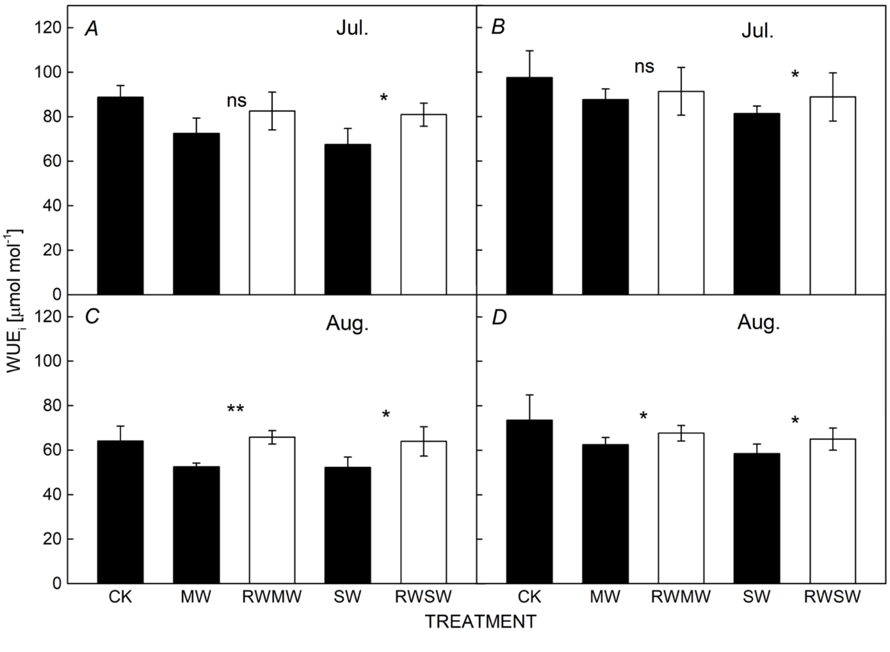

Fig. 5. Changes of leaf intrinsic water-use efficiency (WUE$_i$) during soil drought and rewatering in Manchurian ash (*A*,*C*) and Mongolian oak saplings (*B*,*D*) in July (Jul.) and August (Aug.). Values were mean ± SE (n = 5). The associated probabilities were shown (ns, not significant; * $P<0.05$; ** $P<0.01$). CK – control; MW – medium water stress; SW – severe water stress; RWMW – rewatering after initial medium water stress; RWSW – rewatering after initial severe water stress.

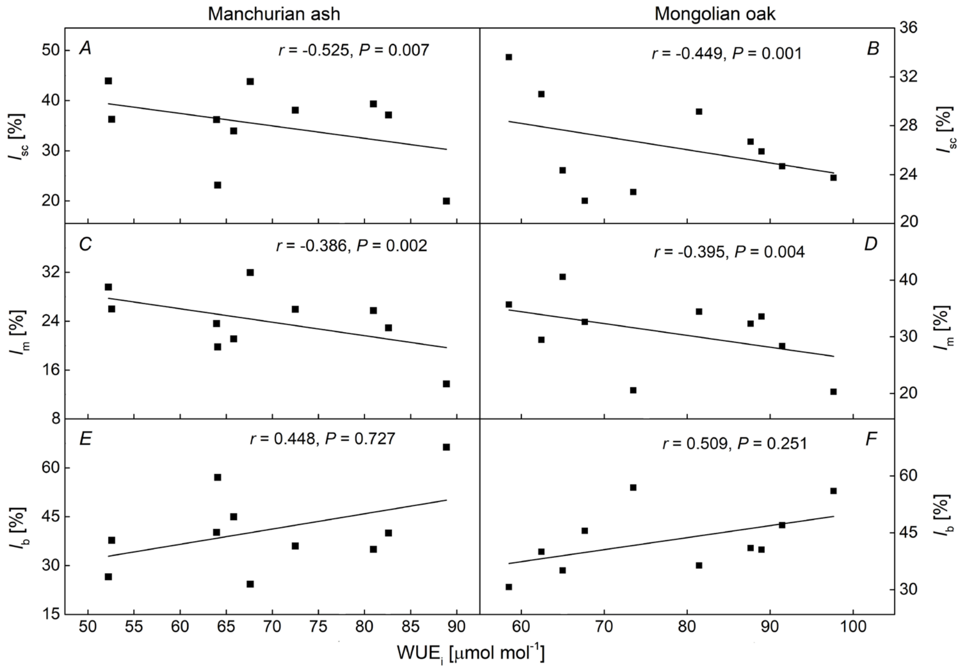

Fig. 6. Relationships between leaf intrinsic water-use efficiency (WUE$_i$) and mesophyll limitation (l_m), stomatal limitation (l_{sc}), and biochemical limitation (l_b) in Manchurian ash (*A*,*C*,*E*) and Mongolian oak saplings (*B*,*D*,*F*).

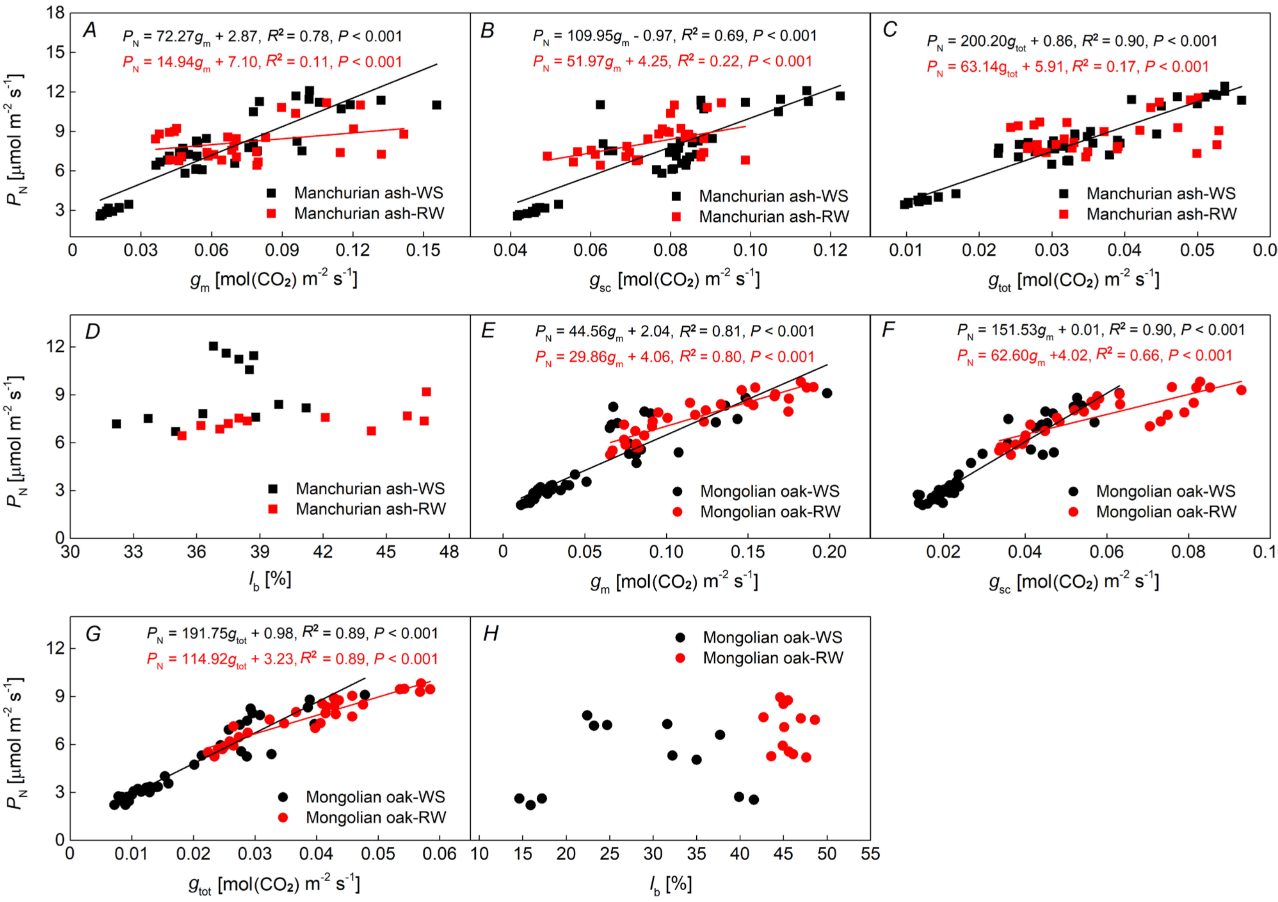

Fig. 7. Regression relationships between net photosynthetic rate (P_N) and mesophyll conductance (g_m) (*A*,*E*), stomatal conductance to CO$_2$ (g_{sc}) (*B*,*F*), total conductance to CO$_2$ (g_{tot}) (*C*,*G*), and biochemical limitation (l_b) (*D*,*H*) in Manchurian ash and Mongolian oak saplings during soil drought and rewatering. WS – water stress treatment; RW – rewatering treatment.

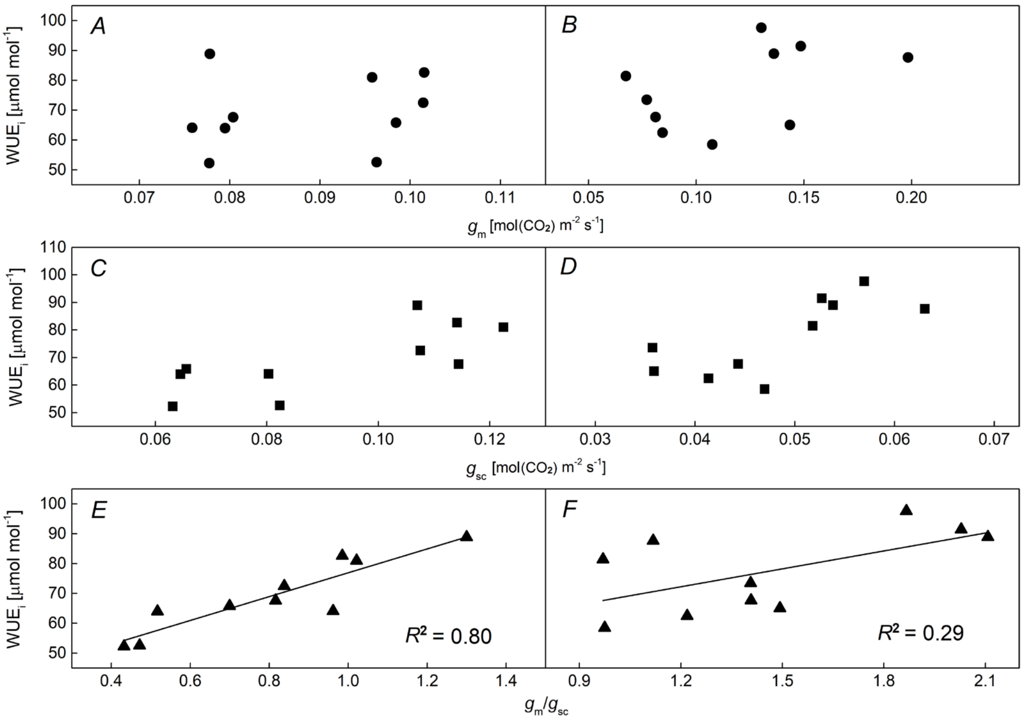

Fig. 8. Relationships between leaf intrinsic water-use efficiency (WUE$_i$) and mesophyll conductance (g_m), stomatal conductance to CO$_2$ (g_{sc}), and g_m/g_{sc} in Manchurian ash (*A*,*C*,*E*) and Mongolian oak saplings (*B*,*D*,*F*) ($P<0.05$).

decreases, indicating that CO$_2$ diffusional conductances decline while plant metabolic activities strengthened. The primary limitation factor is converted to l_m in Manchurian ash and l_{sc} in Mongolian oak during soil drought. Thus, diffusive (stomatal and mesophyll) limitations are the main limiting factors for photosynthesis in ash and oak; this finding is partially consistent with the study of Peguero-Pina *et al.* (2018b). The declines in g_{sc} and g_m have larger negative effects than the changes in metabolic activity on photosynthesis and ultimately lead to a decrease in P_N. Furthermore, with water stress aggravation, l_b continues to decrease, causing B_L to become less than 10% (Fig. 4), *i.e.*, nearly negligible, and the total contributions of diffusional limitations ($D_L = SC_L + MC_L$) gradually account for all photosynthesis limitations. Diffusional processes appear to have a prominent role in photosynthesis during soil drought, an observation that is strongly supported by the studies of Grassi and Magnani (2005) and Galmés *et al.* (2007), who identified the primary role of diffusional limitations (l_{sc} + l_m) in photosynthesis. This prominent role of diffusional processes may be mainly due to the common stomatal closure in pot experiments under water stress conditions, wherein plants experience more rapid dehydration than those under field conditions (Gunasekera and Berkowitz 1992, Kubiskem and Abrams 1993).

In the present study, l_m is larger than l_{sc} in Manchurian ash, whereas l_{sc} is much larger than l_m in Mongolian oak (Fig. 3), indicating that during soil drought, Mongolian oak maintains a lower g_{sc} than g_m while Manchurian ash maintains a larger g_{sc} than g_m. Drought-tolerant species, such as Mongolian oak, close their stomata earlier than drought-intolerant species to avoid leaf dehydration and the generation of excessive tension in the water column inside the xylem under drought (Aranda *et al.* 2012b). However, for less drought-tolerant species, such as Manchurian ash, the tendency to resist soil drought may be to minimize photosynthesis evapotranspiration. In a previous study, ash did not close its stomata to the same extent as oak under water stress, although the g_{sw} of well-watered plants was higher in ash than that in oak (Zhu *et al.* 2019). The stomatal control of water loss is more effective in oak than that in ash. The lack of effective stomatal regulation of water loss leads to lower leaf water potential in ash than in oak, which induces damage to the mesophyll cells in ash (as observed from photographs of mesophyll cells and aquaporin (AQP) activity data in Zhu *et al.* 2019) and reduces g_m more in ash than that in oak. Water and CO$_2$ diffusion in leaves share common pathways (Xiong *et al.* 2015b), and CO$_2$ diffusion in Manchurian ash may be seriously weakened simultaneously with water diffusion under water stress, as ash was more susceptible than oak to soil water availability (Grassi and Magnani 2005).

Soil rewatering triggers a great recovery in photosynthesis in this study, and we believe the declines in l_{sc} and l_m caused by the improvements in CO$_2$ diffusion are the main reasons for this recovery. Furthermore, plants are able to repair embolized xylem conduits by pushing water from living conduit-associated parenchyma cells into gas-filled lumina when the bulk of water-transporting xylem is still under tension (Bucci *et al.* 2003, Hacke and Sperry 2003). The great photosynthesis recovery in the two species may be also mediated by the development of new xylem or by restoring the function of previously embolized vessels (Resco *et al.* 2009, Brodribb *et al.* 2010). Soil rewatering does not change the photosynthesis roles of l_{sc}, l_m, and l_b, and it strengthens the diffusional contributions to photosynthesis recovery, as evidenced by the significantly larger D_L than NS_L during recovery and by the absence of a significant regression relationship of l_b with P_N before and after soil rewatering in the two varieties (Fig. 7*D,H*). When D_L is further partitioned into its components, we found that over the whole experiment, MC_L played the largest role in Manchurian ash, whereas SC_L played the largest role in Mongolian oak. Nevertheless, soil rewatering significantly weakens the primary roles of l_m and l_{sc}: the slopes of the regression relationships between P_N and g_m, g_s, and g_{tot} are weaker in both species (Fig. 7) during recovery than during soil drought.

Overall, the contributions of g_{sc} (SC_L) and g_m (MC_L) and biochemical capacity (B_L) to photosynthesis change (dP_N/P_N) during drought and recovery were larger in August, *i.e.*, upon the second drought, than that in July, indicating that the effects of g_{sc} and g_m and biochemical capacity on photosynthesis were strengthened with plant growth. The above changes in SC_L, MC_L, and B_L from July to August should occur largely due to the changes in AQP and carbonic anhydrase (CA) activities, as the activities of these proteins both decreased during drought and recover after rewatering (Zhu *et al.* 2019), although we did not measure their activities from July to August. In addition, Manchurian ash and Mongolian oak have a less than four month growing cycle (from June to early September) in the Changbai Mountain areas, and their metabolic parameters, such as P_N, begun to weaken in August (Zhu *et al.* 2019). Consequently, the contributions of g_{sc} and g_m and biochemical capacity to dP_N/P_N can be expected to be enlarged in August.

Our results agree with those of Galmés *et al.* (2007) and Cai *et al.* (2015), who confirmed the primary role of l_m in photosynthesis recovery for species in the Balearic Islands and *Rhododendron delavayi* Franch. In addition, the studies of Flexas *et al.* (2009) and Perez-Martin *et al.* (2014) emphasized the dominant role of l_{sc} in *Vitis berlandieri* × *Vitis rupestris* and *O. europaea* L. var. Manzanilla, consistent with our results. However, in the present study, the role of B_L was nearly negligible; in contrast, Ennahli and Earl (2005) suggested that photosynthesis recovery in *Gossypium hirsutum* L. was mostly affected by l_b, whereas influences of l_{sc} and l_m were almost absent. Another difference between the present results and previous results is that in the present study, the primary roles of l_m and l_{sc} were constant over periods of soil drought and rewatering, whereas in other studies, they varied with drought intensity and rewatering treatment (Grassi and Magnani 2005, Galmés *et al.* 2007). Genotypic variation may be an important reason for this difference, as Mongolian oak maintains a better water status and higher WUE_i than ash; the reasons need to be explored. Furthermore, the small bias resulting from the calculation of l_b using ETR (Wang *et al.* 2018) rather than V_{cmax} (Flexas *et al.* 2009, Cano *et al.* 2014) may influence the measurements of changes in l_{sc} and l_m during water stress and rewatering. Given that V_{cmax} (or ETR here) is directly proportional to dP_N/dC_c, the use of ETR instead of V_{cmax} may have artificially increased l_b and decreased l_{sc} and l_m. Nevertheless, this potential bias should not affect the relative strengths of l_{sc} and l_m.

Species dependence on the photosynthesis roles of l_{sc}, l_m and l_b and leaf WUE_i: The above discussion of previous studies suggests that the primary factors among l_{sc}, l_m, and l_b may be species dependent, which is consistent with our results that Manchurian ash has l_m as the primary factor influencing photosynthesis in water-limited conditions, whereas in Mongolian oak, l_{sc} maintains a primary role. Different species, provenances and cultivars differ in their ability to adapt g_{sc} or leaf biochemical capacity for carbon fixation to optimize carbon gain with respect to water loss (Hommel *et al.* 2014). Such differences in optimization strategies could strongly affect synecological interactions, especially at sites exposed to periodic drought (Grams *et al.* 2007, Niinemets *et al.* 2009). As a less drought-tolerant species (Epron and Dreyer 1993, Grassi and Magnani 2005), Manchurian ash does not close many stomata despite reaching lower predawn water potential than Mongolian oak. This pattern may be due to the higher sensitivity of g_m to changes in SWC than g_{sc} in ash, as g_m decreases much more than g_{sc} under water stress conditions (Zhu *et al.* 2019). Soil rewatering may not change the primary role of diffusional limitations, but it still influences the roles of g_m, g_{sc} and biochemistry in limiting P_N. After rewatering, the l_{sc} of the two varieties is significantly reduced under the initial SW treatment, whereas a significant reduction in l_m occurs under the two drought treatments.

Barbour and Kaiser (2016) suggested that the combination of high l_{sc} and low l_m should produce high leaf water-use efficiency, reflecting the close correlations between water-use efficiency and l_{sc} and l_m. In the present study, l_{sc} and l_m each shows a negative correlation with WUE_i (Fig. 6), and a strong correlation is observed between WUE_i and g_m/g_{sc} (Fig. 8*E,F*) but not g_m or g_{sc} (Fig. 8*A–D*). These results suggest that WUE_i correlates with neither g_m nor g_{sc} but is strongly affected by the ratio of g_m/g_{sc} (Flexas *et al.* 2013, Han *et al.* 2016), possibly because stomata control CO_2 gain and water loss, whereas g_m is related only to CO_2 diffusion inside leaves. The l_b in this study shows a positive correlation with WUE_i (Fig. 6), further demonstrating the regulation *via* biochemical mechanisms of the variability of WUE_i under moderate and severe drought conditions. Hence, the differences in water-use efficiency are explained by changes in the balance between photosynthesis limitations.

Our results show a generally higher WUE_i in Mongolian oak than that in Manchurian ash, which is related to the larger S_L and lower MC_L in the former species. Since the investment of large amounts of reserves in the development of a large and deep root system might be crucial for trees in water-limited habitats (Bloom *et al.* 1985), the difference in WUE_i between the two varieties may be largely due to their differences in physiological mechanisms. Mongolian oak has a more developed root system and much larger leaf hydraulic conductance (K_{leaf}) and stem vessel diameter

(D_{stem}) than Manchurian ash (Zhang *et al.* 2020); because K_{leaf} is highly coordinated with both g_{sc} and P_N, Mongolian oak can thus absorb and use much more water from deeper parts of the soil than Manchurian ash (Aasamaa *et al.* 2001, Brodribb and Holbrook 2006). Such ability would be of major importance under water stress conditions (Shatil-Cohen *et al.* 2011). Hence, a species' dependence on the photosynthesis roles of l_{sc} and l_m should be strongly related to plant water status and water-use capacity; this expectation is indirectly supported by the finding of Tomás *et al.* (2014) that g_{sc} and g_m greatly affected leaf water-use efficiency.

In addition, we obtained the unexpected result that the WUE$_i$ under the initial MW and SW conditions was lower than that under the CK condition in both species (Fig. 5). This result may be largely due to the failure to consider the cuticular transpiration (g_{cw}) in this study, which is used to correct C_i and recalculate g_{sw} (Warren *et al.* 2011, Cano *et al.* 2014) and hence affects WUE$_i$. The failure to consider g_{cw} could give rise to an apparent biphasic response of C_i (or WUE$_i$) to water stress, whereby C_i initially decreased under moderate stress but then increased under severe stress (Medrano *et al.* 2002). Furthermore, in this study, there was no separation of the treatment and ontogenetic effects on multiple estimations to well-watered plants, which may result in an unclear meaning of CK.

Conclusion: The l_{sc} and l_m increased while l_b decreased with water stress during soil drought in both Manchurian ash and Mongolian oak. Soil rewatering decreased both l_{sc} and l_m but increased l_b. The primary limiting factor for photosynthesis in well-irrigated saplings is biochemical capacity (*i.e.*, l_b), but when water stress was established, the primary limiting factor was gradually converted to increased mesophyll resistance (*i.e.*, l_m) in Manchurian ash and stomatal closure (*i.e.*, l_{sc}) in Mongolian oak. Soil rewatering did not change the primary roles of l_{sc} and l_m and made the largest g_m contribution (MC_L) to photosynthesis recovery in Manchurian ash and the largest g_{sc} contribution (SC_L) to that in Mongolian oak. Mongolian oak maintained higher WUE$_i$ than Manchurian ash during soil drought and rewatering. Therefore, leaf WUE$_i$ can be directly influenced by photosynthesis limitations, becoming larger as biochemical limitations increase at the expense of diffusional limitations (l_{sc} and l_m).

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