Summary

The aim of this study was to examine the response of internal conductance to CO\(_2\) \((g_i)\) to soil water deficit and contrasting light conditions, and their consequences on photosynthetic physiology in two Picea asperata Mast. populations originating from wet and dry climate regions of China. Four-year-old trees were subjected to two light treatments (30% and 100% of full sunlight) and two watering regimes (well watered, drought) for 2 years. In both tested populations, drought significantly decreased \(g_i\) and the net photosynthesis rate \((A)\) and increased carbon isotope composition \((\delta^{13}C)\) values in both light treatments, in particular in the sun. Moreover, drought resulted in a significantly higher relative limitation due to stomatal conductance \((L_s)\) in both light treatments and higher relative limitation due to internal conductance \((L_i)\) and abscisic acid (ABA) in the sun plants. The results also showed that \(L_i\) (0.26–0.47) was always greater than \(L_s\) (0.12–0.28). On the other hand, drought significantly decreased the ratio of chloroplastic to internal CO\(_2\) concentration \((C_c/C_i)\), photosynthetic nitrogen utilization efficiency (PNUE) and total biomass in the sun plants of the wet climate population, whereas there were no significant changes in these parameters in the dry climate population. Our results also showed that the dry climate population possessed higher \(\delta^{13}C\) values with higher ratio of internal conductance to stomatal conductance \((g_i/g_s)\), suggesting that increasing the \(g_i/g_s\) ratio enhances water-use efficiency (WUE) in plants evolved in arid environments. Thus, we propose that the use of the \(g_i/g_s\) parameter to screen P. asperata plants with higher water deficit tolerance is certainly worthy of consideration. Furthermore, \(g_i\) is an important variable, which reflects the population differences in PNUE, and it should thus be included in plant physiological investigations related to leaf economics.

Keywords: carbon isotope discrimination, internal conductance, limitation analysis, photosynthesis, stomatal conductance.

Introduction

Picea asperata Mast., an important tree species used for the production of pulp wood and timber, is a prime reforestation species in western China. It occurs in the alpine and canyon regions of the northwestern Sichuan province and the southeastern Gansu province, both of which are water-limited regions (Wu and Raven 1994). Of an additional concern is the global climate change, which will presumably increase the global temperature, change the distribution of precipitation and intensify drought in arid and semi-arid areas (Li et al. 2000, Wigley and Raper 2001). As a consequence, plants will have to cope with drought stress. Under such circumstances, plant productivity, which is largely dependent on photosynthesis, is inevitably limited (Chaves et al. 2009). For this reason, it is desirable to develop a better understanding of the mechanisms by which water deficit affects the gas exchange in plants. Such knowledge would enable better predictions of photosynthesis, plant productivity and plant responses to climate change.

During photosynthesis, CO\(_2\) diffusion from substomatal cavities to chloroplasts is limited by mesophyll diffusion conductance \((g_i)\) that reduces the CO\(_2\) concentration in chloroplasts \((C_c)\) relative to that in substomatal cavities \((C_i)\). Early gas-exchange studies assumed that \(g_i\) is large and constant and, hence, that \(C_c\) and \(C_i\) are nearly the same (Farquhar et al. 1980). However, \(g_i\) has recently been recognized as an equally important cause for reduced CO\(_2\) diffusion under stress conditions (Warren 2008a). A number of studies have indicated that \(g_i\) may significantly limit photosynthesis, and several sources of variation in \(g_i\) have
been described, including water, nutrient level and salt stresses as well as changes in leaf temperature (for a review, see Flexas et al. 2008). Soil water deficit and light are the main factors affecting leaf gas exchange, and their effects on gᵢ have been observed in many species. However, only a handful of experiments have shown that gᵢ acclimates to the light environment (Niinemets et al. 2006, Warren et al. 2007), but the results are contradictory. The reported difference in gᵢ between sun and shade leaves is large in some species (Piel et al. 2002, Warren et al. 2007) but small in others (Warren et al. 2003).

Additionally, gᵢ affects the economics of water and nitrogen use in photosynthesis. A particular aspect of photosynthetic efficiency, which is enormously important in semi-arid and arid environments, is leaf water-use efficiency (WUE). Carbon isotope composition (δ¹³C), recorded for the tissues of C₃ plants, has been demonstrated to be a useful tool for assessing WUE (for a review, see Farquhar et al. 1989). The fractionation of carbon isotopes during photosynthesis depends on the biochemical and physical phenomena, and is mainly associated with CO₂ diffusion and carboxylation reactions. In fact, gᵢ may affect δ¹³C by > 3 permille (Hanba et al. 2003). Despite this, gᵢ has not been extensively investigated as a possible factor contributing to variation in WUE. Moreover, finite gᵢ has been recognized to reduce photosynthetic nitrogen utilization efficiency (PNUE) (e.g., Warren and Adams 2006, Flexas et al. 2008). However, the relative contribution of gᵢ to variation in PNUE has been quantified in few species (for a review, see Flexas et al. 2008). It is clear that the role of gᵢ in determining PNUE warrants further research. In addition, previous studies on gᵢ had seldom been targeted at gymnosperm plants, and very little information on the acclimation of gᵢ to environmental changes is available for conifers (Warren et al. 2003).

In this investigation, we studied the effects of water deficit and light on gᵢ and its relationship with photosynthesis in two contrasting P. asperata populations. A novel A–Cᵢ curve (net photosynthesis rate ‘A’ as a function of its intercellular CO₂ concentration ‘Cᵢ’) analysis method (Ethier and Livingston 2004, Ethier et al. 2006) was used to estimate gᵢ. The objectives of this study were to discover, (i) whether water deficit, light and their combination affect gᵢ, mesophyll limitation and consequently, photosynthetic physiology and (ii) whether gᵢ varies between dry and wet climate populations.

Materials and methods

Plant materials and experimental design

A dry climate population (Diebu, 34°03’ N and 103°05’ E) and a wet climate population (Heishui, 32°39’ N and 103°06’ E) of P. asperata were selected for this study. The mean annual precipitation equals 547 and 833 mm, and the mean annual temperature is 0.7 and 9.0 °C at the origin of the dry climate population and the wet climate population, respectively. Four-year-old healthy seedlings of uniform height (25–30 cm) of both populations were grown in 5-l plastic pots filled with homogenized soil at the Maoxian Field Ecological Station of the Chinese Academy of Sciences. Experimental treatments started 30 days after the seedlings were transplanted. The plants were subjected to the water deficit and light treatments for 2 years. About 20 g of fertilizer (13% N, 10% P and 14% K), released evenly over the experimental period, was added to each pot.

Two water conditions were included in the experiments. (i) Well-watered condition: 40 pots of each population were watered to 100% of field capacity by supplying an amount of water equal to transpiration losses every 4 days. In this case, the soil water content was always kept at around 33% in volume. (ii) Water deficit condition: 40 pots of each population were maintained at 25% of field capacity by watering every 4 days. In this case, the soil water content was always kept at around 8.3% in volume. The volumetric water content of each pot was measured to check the homogeneity of water availability across light treatments. Drought treatments were equivalent in sun and shade, as evidenced in Figure 1. The experimental shade treatments simulated field conditions in the forest understorey (middle shade). In each population and treatment, half of the seedlings were grown in a shade house covered with neutral shade cloths, which have a neutral effect on light quality (Yates 1989). In the shade house, irradiance was about 30% of full sunlight. The rest of the seedlings was grown in full sunlight in a greenhouse. Mean photosynthetic photon flux densities (PPFD) were 905 ± 60 and 301 ± 10 μmol m⁻² s⁻¹ in the sun and shade treatments.

Figure 1. Variation of the soil water content in volume (%) during the experiment in plants of P. asperata. Data combined from the wet climate population and the dry climate population plants. The symbols represent different treatments: (□), shade + well watered; (○), full sunlight + well watered; (△), shade + water deficit and (●), full sunlight + water deficit. The values are shown for individual replicates. (P(W), water effect; (P(L), light effect and P(W × L), water × light interaction effect. ns, P > 0.05.)
respectively. Mean air temperature and relative humidity for the sun and shade treatments were 31.4 ± 4.2 and 29.8 ± 3.9 °C and 50.1 ± 6.9% and 53.1 ± 8.7%, respectively. The values of PPFD, temperature and relative humidity were measured at midday in 24 clear days during the experiments. Thus, a total of four treatments were included: (i) well watered, full sunlight (control); (ii) well watered, shade; (iii) water deficit, full sunlight and (iv) water deficit, shade. In each treatment, there were 20 seedlings, which were arranged in four blocks (five seedlings per population and treatment in each block). Moreover, the locations of the four blocks in the greenhouse were randomized every 2 weeks to eliminate block effects. Measurements of various morphological, physiological and biochemical parameters were done at the end of the experiments. As the soil never became waterlogged and was porous enough to allow oxygen to diffuse freely, there was no indication of root rot or root death from the lack of oxygen at the end of the experiments.

**Relative water content of needles, growth traits and nitrogen concentrations**

For each population and treatment, current-year needles of 12 plants from four blocks (three plants from each block) were randomly selected. The relative water content (RWC) of the needles was calculated as RWC (%) = (FW – DW)/(TW – DW) × 100, where FW is the fresh weight, TW is the turgid weight after rehydrating samples sealed in saturation and DW is the dry weight after oven-drying samples to constant weight at 80 °C for 24 h.

For each population and treatment, eight plants from four blocks (two plants from each block) were randomly selected and harvested at the end of the experiment, and were divided into needles, stem and roots. Leaf area was measured using a Portable Leaf Area Meter (CI-203, CID Inc., Camas, WA). The leaf mass per area (LMA) (the leaf dry weight divided by the projected leaf area of the whole seedling) was then calculated. Measurements were done for new, fully matured current-year leaves.

The samples of leaves were ground and passed through a 20 mesh screen after being dried at 80 °C for 36 h. The nitrogen (N) concentrations were determined by the semimicro Kjeldahl method.

**Gas-exchange measurements and estimation of internal conductance by a curve-fitting method**

Within the chamber (6400-05 Li-Cor Conifer Chamber), a section of a lignified shoot clamped together with fully matured, current-year needles were acclimated at a light-saturating PAR (about 900 μmol m⁻² s⁻¹) until photosynthesis and transpiration rates were steady. At that point, the net photosynthesis rate (A) was recorded. Measurements were done between 10 am and 12 noon using an open gas-exchange system Li-6400 (Li-Cor Inc., Lincoln, NE). The measurements took place under optimal summer conditions: the air in the leaf chamber was maintained at 25 °C. The molar flow rate of air through the chamber varied between 200 and 250 μmol s⁻¹. The measurements were done for two randomly selected seedlings per block, a total of eight plants per population/watering/light. After inducing steady-state photosynthesis, the photosynthesis responses to varying intercellular CO₂ concentrations (Cᵢ) were measured. The concentration of atmospheric CO₂ (Cᵢ) was lowered stepwise from 360 to 50 μmol mol⁻¹, and then returned to 360 μmol mol⁻¹ to reestablish the initial steady-state value of photosynthesis. Cᵢ was then increased stepwise from 360 to 1500 μmol mol⁻¹. At each Cᵢ, photosynthesis was allowed to stabilize for at least 4 min until gas exchange was steady. The ratio of A to Nₑ (leaf nitrogen content on area basis in g m⁻²), measured for the leaves at 360 μmol mol⁻¹, was regarded as the PNUE. Ambient CO₂ leakage inside the shoot chamber was prevented by wrapping the chamber’s foam gasket junction with Teflon tape. Data were not corrected for CO₂ diffusion into and out of the leaf chamber, because the effect was insignificant for the experimental set-up. Preliminary experiments had established that with a molar flow rate of 200 μmol s⁻¹ (the lowest flow rate used in this experiment), there was not > 1.5 μmol mol⁻¹ diffusion of CO₂ into an empty chamber maintained at 50 μmol mol⁻¹ CO₂.

A curve-fitting method was used to estimate gᵣ (Ether and Livingston 2004, Ethier et al. 2006). This is based on the hypothesis that gᵣ reduces the curvature of the Rubisco-limited portion of an A–Cᵢ response curve. Following Ether and Livingston (2004), A–Cᵢ curves were fitted with a non-rectangular hyperbola version of the biochemical model of Cᵢ leaf photosynthesis by Farquhar et al. (1980) which accounts for new, fully matured current-year leaves.

\[
A = \min \{A_c, A_j\},
\]

\[
A_c = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a},
\]

\[
a = -1/g_i
\]

\[
b = (V_{cmax} - R_d)/g_i + C_i + K_c(1 + O/K_o)
\]

\[
c = R_d(C_i + K_c(1 + O/K_o)) - V_{cmax}(C_i - \Gamma^*)
\]

\[
A_j = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a},
\]

\[
a = -1/g_i
\]

\[
b = (J/4 - R_d)/g_i + C_i + 2\Gamma^*
\]

\[
c = R_d(C_i + 2\Gamma^*) - J/4(C_i - \Gamma^*)
\]
where $A_c$ and $A_l$ are the RuBP-saturated and RuBP-limited net CO$_2$ assimilation rates, respectively, $V_{\text{max}}$ is the maximal CO$_2$ carboxylation rate, $J$ is the photochemical electron transport rate under RuBP-limited conditions, $R_d$ is the mitochondrial respiration in the light, $I^*$ is the CO$_2$ compensation point in the absence of mitochondrial respiration in the light, $K_c$ and $K_o$ are the Michaelis–Menten constants for RuBP carboxylation and oxygenation, respectively and $O$ is the oxygen concentration. For $[K_c (1 + O/K_o)]$, a value of 736 μmol mol$^{-1}$ was used (von Caemmerer et al. 1994), while $I^*$ was calculated using the Laisk method (Laisk 1977). The accuracy of the photosynthesis model depends on the proper representation of the kinetic properties of Rubisco. It may be argued that the estimation of these parameters is strongly affected by the choice of kinetic parameters involved in the equations. However, as these parameters have not yet been described for P. asperata, any choice of parameters published for other species could be considered arbitrary. Consequently, we have selected a general set of kinetic parameters but with caution (Tcherkez et al. 2006). This approach is justified by the belief that the kinetic properties of Rubisco among C$_3$ plants have been shown to be relatively conserved.

The $C_i$ cut-off point was determined based on the method proposed by Ethier et al. (2006). This method has been successfully used in several studies, showing a good agreement with other independent estimates of $g_i$ (Niinemets et al. 2006). Detailed derivations of Eqs. (2) and (3), as well as a thorough evaluation of errors resulting from assuming an infinite $g_i$ when fitting the Farquhar et al. (1980) model equations to $A–C_i$ curves, are given in Ethier and Livingston (2004). Fitting the $A–C_i$ curves involved optimizing the parameter values by adjusting them so as to minimize the sums of residuals between the observed and modelled assimilation values over a range of $C_i$. The estimates were weighted by the reciprocal of their squared standard error (Murtaugh 2007) to minimize the influence of poorly estimated parameters. $A–C_i$ curves that were noisy (for most of them, convergence problems occurred during the fit) were therefore discarded, and the rest of them were kept when the standard error of the $g_i$ estimate was < 50% of the estimated value. A total of 32 of 64 $A–C_i$ curves were finally retained.

Estimation of $g_i$ by the photocompensation point method

The same gas-exchange system was used to determine $g_i$ via the photocompensation point method. The substomatal CO$_2$ photocompensation point ($C_i^*$) and $R_d$ were estimated using the Laisk (1977) method. $C_i^*$ and $R_d$ were estimated from three partial CO$_2$-response curves (50–150 μmol mol$^{-1}$ CO$_2$) measured at lower PPFDs (500, 300 and 150 μmol m$^{-2}$ s$^{-1}$). These light intensities were chosen following preliminary trials to ensure maximum separation of the response curves while maintaining a similar degree of inhibition of dark respiration in the light. The intersection of the three lines identified $C_i^*$ (x axis) and $R_d$ (y axis). The good agreement between both methods suggests that the data were sufficiently reliable. Therefore, in the Results section, we demonstrate only the statistical relationships with $g_i$ from the novel fitting of $A–C_i$ curves.

Calculation of CO$_2$ drawdown

$C_i$ was measured at ambient CO$_2$ concentrations (average ambient CO$_2$ concentration 350–400 μmol mol$^{-1}$). Using estimated $g_i$ and measured $A$ and $C_i$, the CO$_2$ concentration in chloroplasts ($C_c$) was calculated as $C_c = C_i - (A/g_i)$. From these simulated values, actual average CO$_2$ drawdown from the substomatal cavities to chloroplasts, $C_t - C_c$, and the ratio of $C_c$ to $C_i$ were calculated.

Relative limitation on photosynthesis imposed by internal conductance ($g_i$) and stomatal conductance ($g_s$)

The limitation of $A$ imposed by finite $g_i$ and $g_s$ was based on the estimates of the potential rate of photosynthesis, assuming that these conductances were either infinite or as measured (Farquhar and Sharkey 1982, Warren et al. 2003). The estimates of $A$ were based on the CO$_2$-response curves, and measured as $g_i$ and $g_s$. The net photosynthesis rate was estimated assuming that $g_i$ and $g_s$ were as measured ($A$, the light-saturated rate of photosynthesis at $C_a = 360$ μmol mol$^{-1}$), assuming $g_i$ being infinite and $g_s$ as measured ($A_{il}$, the light-saturated rate of photosynthesis at $C_c = C_i$), or assuming $g_i$ as measured and $g_s$ being infinite ($A_{il}$, the light-saturated rate of photosynthesis at $C_t = 360$ μmol mol$^{-1}$). The relative limitations due to internal conductance resistances ($L_i$) and stomatal resistances ($L_a$) were estimated as

$$L_i = (A_{il} - A)/A_{il},$$

$$L_a = (A_{il} - A)/A_{il}.$$

Carbon isotope composition

For each population and treatment, current-year needles of 12 plants from four blocks (three plants from each block) were randomly selected for the carbon isotope analysis. The samples of 100 mg DW of plant material, oven-dried at 80 °C for 24 h, were homogenized by grinding in a ball mill. The stable carbon isotope abundance in the combusted samples was measured with a mass spectrometer (Finnegan MAT Delta-E, Bremen, Germany) as described by Li et al. (2004). The overall precision of the δ-values was better than 0.1‰, as determined from repeated samples.

Quantitative analysis of abscisic acid

For each population and treatment, current-year needles of 12 plants from four blocks (three plants from each block) were randomly selected and immediately immersed in liquid
nitrogen and stored at −70 °C before being used for the determination of abscisic acid (ABA). Plant tissues (0.5 g) were homogenized and extracted twice in a total of 4 ml of 80% methanol at 4 °C for 2 h. To remove plant pigments and other non-polar compounds, which could interfere in the immunoassay, extracts were first passed through a polyvinylpyrrolidone column and C18 cartridges. The eluates were dried by vacuum evaporation and resuspended in Tris-buffered saline before enzyme-linked immunosorbent assay (ELISA). ABA was quantified by ELISA using assay kits (made by China Agricultural University), according to the manufacturer’s instructions and those described by Asch (2000). A standard curve was established for each microtitre plate. The ABA levels were calculated in μg g⁻¹ FW.

Statistical analyses

All measurements were tested by a three-way ANOVA for the effects of light, water deficit and population. Before ANOVA, these data were checked for normality and the homogeneity of variances and log transformed to correct deviations from these assumptions when needed. The analyses were performed with the general linear ANOVA model procedure of SPSS 11.0 (SPSS Inc., Chicago, IL). Post hoc comparisons were tested using Tukey’s test at a significance level of \( P < 0.05 \). Pearson’s correlation coefficients were calculated to determine the relationships between variables.

A path analysis (Grace and Pugesek 1998) was used to quantify the relative contributions of the direct and indirect effects of the ratio of internal conductance to stomatal conductance \((g_l/g_s)\), leaf nitrogen content on area basis \((N_a)\) and LMA on the \( \delta^{13}C \) values.

Results

Water deficit and shade affected photosynthetic \( CO_2 \)-response characters

In both populations tested, drought significantly decreased \( g_l \), \( A \) and \( J_{max} \) in both light treatments, in particular, in the sun (Table 1). Moreover, drought resulted in significantly higher \( L_s \) and \( C_l–C_i \) in both light treatments, but higher \( L_i \) only in the sun plants. The results also showed that \( L_i \) (0.26–0.47) was always greater than \( L_s \) (0.12–0.28). On the other hand, drought significantly decreased \( C_l/C_i \) and \( V_{cmax} \) in the sun plants of the wet climate population, whereas there were no significant changes in these parameters in the dry climate population. Additionally, drought significantly increased \( g_l/g_s \) in the sun plants of the dry climate population, whereas there was no significant change in the wet climate population. No significant population differences were observed in \( L_s \) and \( C_l–C_i \) in any treatments. Compared to the wet climate population, the dry climate

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Population</th>
<th>Well watered</th>
<th>Water deficit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shade</td>
<td>Sun</td>
<td>Shade</td>
</tr>
<tr>
<td>( g_l ) (mol m⁻² s⁻¹)</td>
<td>Heishui</td>
<td>0.10 ± 0.00 a</td>
<td>0.11 ± 0.01 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>0.06 ± 0.01 c</td>
<td>0.04 ± 0.00 d</td>
</tr>
<tr>
<td>( g_l/g_s )</td>
<td>Heishui</td>
<td>0.25 ± 0.01 ab</td>
<td>0.29 ± 0.02 ab</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>0.14 ± 0.02 a</td>
<td>0.16 ± 0.02 ab</td>
</tr>
<tr>
<td>( A ) (μmol m⁻² s⁻¹)</td>
<td>Heishui</td>
<td>7.08 ± 0.03 a</td>
<td>6.96 ± 0.05 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>3.37 ± 0.12 d</td>
<td>3.45 ± 0.01 d</td>
</tr>
<tr>
<td>( C_l/C_i )</td>
<td>Heishui</td>
<td>0.63 ± 0.01 ab</td>
<td>0.65 ± 0.01 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>0.51 ± 0.02 c</td>
<td>0.54 ± 0.02 bc</td>
</tr>
<tr>
<td>( C_l–C_i ) (μmol mol⁻¹)</td>
<td>Heishui</td>
<td>52 ± 2 a</td>
<td>58 ± 2 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>56 ± 1 a</td>
<td>66 ± 4 a</td>
</tr>
<tr>
<td>( V_{cmax} ) (μmol m⁻² s⁻¹)</td>
<td>Heishui</td>
<td>41.4 ± 1.1 a</td>
<td>38.7 ± 0.7 ab</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>20.9 ± 1.1 c</td>
<td>20.0 ± 1.1 c</td>
</tr>
<tr>
<td>( J_{max} ) (μmol m⁻² s⁻¹)</td>
<td>Heishui</td>
<td>44.2 ± 0.06 a</td>
<td>42.5 ± 0.04 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>30.4 ± 0.00 b</td>
<td>30.2 ± 0.01 b</td>
</tr>
<tr>
<td>( L_s )</td>
<td>Heishui</td>
<td>0.12 ± 0.01 a</td>
<td>0.11 ± 0.01 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>0.15 ± 0.01 ab</td>
<td>0.13 ± 0.01 a</td>
</tr>
<tr>
<td>( L_i )</td>
<td>Heishui</td>
<td>0.29 ± 0.01 ab</td>
<td>0.26 ± 0.01 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>0.39 ± 0.03 c</td>
<td>0.37 ± 0.02 c</td>
</tr>
</tbody>
</table>

Table 1. The effects of water deficit and light on internal conductance, photosynthesis and other parameters in P. asperata trees originating from dry and wet areas.

Heishui, a population from a wet climate region and Diebu, a population from a dry climate region. The values within a row or column not sharing the same letters are significantly different \( (P < 0.05) \) according to Tukey’s test. Each value represents the means ± SE of four replicates.

g₁, Internal conductance to \( CO_2 \); \( g_l/g_s \), the ratio of internal conductance to stomatal conductance; \( A \), net photosynthesis rate; \( C_l/C_i \), the ratio of chloroplastic to internal \( CO_2 \) concentration; \( C_l–C_i \), the \( CO_2 \) drawdown from the substomatal cavities to chloroplasts; \( V_{cmax} \), maximal carboxylation rate; \( J_{max} \), maximal photochemical electron transport rate; \( L_s \), relative limitation to net photosynthesis posed by stomatal conductance and by \( L_i \), internal conductance.

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population had lower $g_i$, $A$, $V_{\text{max}}$ and $J_{\text{max}}$ in all treatments, lower $C_4/C_i$ in the well-watered treatment irrespective of light treatments, whereas it possessed a higher $g_i/g_s$ in drought plus sun treatment and a higher $L_i$ across all treatments, except in drought plus sun treatment (Table 1).

**Water deficit and shade affected LMA, total biomass and carbon isotope composition**

Water deficit resulted in a significantly lower RWC in both sun and shade plants of the wet climate population, but only in the sun plants of the dry climate population (Table 2). For both populations that were tested, drought significantly increased ABA only in the sun plants, whereas it increased $\delta^{13}C$ values in both light treatments. Neither light nor water deficit had significant effects on $N_i$ (Table 2). Water deficit marginally significantly decreased the biomass of whole individuals (total biomass) in the sun plants of the wet climate population, but there was no significant difference in total biomass across the treatments in the dry climate population, though there was a tendency for the values to be lower in the drought-stressed sun plants (Table 2). Additionally, drought significantly decreased PNUE in the sun plants of the wet climate population, whereas there was no such change in the dry climate population. No significant population differences were observed in $N_i$ in any treatments. Compared to the wet climate population, the dry climate population showed a significantly lower RWC and total biomass under well-watered conditions in both light regimes, whereas it possessed a higher PNUE in all treatments, a higher ABA under well-watered conditions, higher $\delta^{13}C$ values under water deficit conditions, as well as higher LMA in all treatments, except in water deficit plus sun treatments (Table 2).

**Relationships between variables**

The $A-\Gamma_i$ curves were not statistically different in both the initial slope and in the saturating portion of the curve at high CO$_2$ in sun plants and in shade plants under well-watered conditions. On the contrary, the shape of the $A-\Gamma_i$ curves was dramatically affected by water deficit, especially in the sun (Figure 2). $\Gamma_*^i$ was strongly negatively correlated with $R_g$ (Figure 3A). From the regression equation $\Gamma_*^i = \Gamma^i - R_g/g_i$, the mean $\Gamma_*^i$ was estimated as 39.9 mol mol$^{-1}$. Furthermore, the alternative estimates of $g_i$ were strongly correlated ($r = 0.87$, $P < 0.001$; Figure 3B). There were strong positive relationships between $g_i$ and $g_s$ in both populations (for the dry climate population, $g_i = 0.03 \times g_s + 0.02$, $r = 0.92$, $P < 0.001$ and for the wet climate population, $g_i = 0.06 \times g_s + 0.03$, $r = 0.89$, $P < 0.001$; Figure 4A). The $g_i$ values were positively correlated with LMA under well-watered conditions in the wet climate population ($r = 0.72$, $P = 0.04$). However, a negative correlation was observed between $g_i$ and LMA under water deficit conditions ($r = -0.65$, $P = 0.08$ for the dry climate population and $r = -0.87$, $P = 0.01$ for the wet climate population; Figure 4B). A significant correlation was observed between $g_i$ and RWC ($r = 0.67$, $P = 0.01$ for the dry climate population; $r = 0.82$, $P < 0.001$ for the wet climate population) and between $g_i$ and ABA ($r = -0.52$, $P = 0.04$ for the dry climate population and $r = -0.79$, $P < 0.001$ for the wet climate population; Figure 5). The $g_i$ was positively correlated with $A$ ($r = 0.81$, $P < 0.0001$ for

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Population</th>
<th>Well watered</th>
<th>Water deficit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Shade</td>
<td>Sun</td>
</tr>
<tr>
<td>RWC (%)</td>
<td>Heishui</td>
<td>87.3 ± 1.8 a</td>
<td>86.7 ± 1.3 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>77.6 ± 2.2 b</td>
<td>76.7 ± 1.7 b</td>
</tr>
<tr>
<td>ABA (µg g$^{-1}$FW)</td>
<td>Heishui</td>
<td>0.75 ± 0.05 a</td>
<td>0.98 ± 0.01 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>1.14 ± 0.02 b</td>
<td>1.14 ± 0.08 b</td>
</tr>
<tr>
<td>$N_i$ (g m$^{-2}$)</td>
<td>Heishui</td>
<td>1.41 ± 0.05 a</td>
<td>1.37 ± 0.07 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>1.22 ± 0.04 a</td>
<td>1.21 ± 0.01 a</td>
</tr>
<tr>
<td>PNUE (µmol g$^{-1}$ s$^{-1}$)</td>
<td>Heishui</td>
<td>5.03 ± 0.16 a</td>
<td>5.11 ± 0.29 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>2.76 ± 0.09 c</td>
<td>2.83 ± 0.02 c</td>
</tr>
<tr>
<td>$\delta^{13}C$ (%o)</td>
<td>Heishui</td>
<td>-27.67 ± 0.16 a</td>
<td>-27.45 ± 0.17 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>-27.55 ± 0.09 a</td>
<td>-27.50 ± 0.01 a</td>
</tr>
<tr>
<td>LMA (g m$^{-2}$)</td>
<td>Heishui</td>
<td>98.5 ± 6.0 a</td>
<td>103.7 ± 1.9 ab</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>122.6 ± 4.9 cd</td>
<td>128.4 ± 2.9d</td>
</tr>
<tr>
<td>Total biomass (g)</td>
<td>Heishui</td>
<td>31.9 ± 1.6 ab</td>
<td>33.3 ± 0.9 a</td>
</tr>
<tr>
<td></td>
<td>Diebu</td>
<td>22.9 ± 0.6 cd</td>
<td>25.5 ± 1.1 bcd</td>
</tr>
</tbody>
</table>

Heishui, a population from a wet climate region and Diebu, a population from a dry climate region. The values within a row or column not sharing the same letters are significantly different ($P < 0.05$) according to Tukey's test. Each value represents the means ± SE of four replicates.

RWC, Relative water content of needles; ABA, abscisic acid; $N_i$, leaf nitrogen content on area basis; PNUE, photosynthetic nitrogen utilization efficiency; $\delta^{13}C$, carbon isotope composition and LMA, the biomass of whole individual (total biomass).
and intercellular concentration ($C_i$) well watered; ($f$), full sunlight + well watered; ($r$), shade + water deficit; ($g$), shade + water deficit and ($h$), full sunlight + water deficit.

The symbols represent different treatments: ($C$), shade + well watered; ($L$, full sunlight + well watered; ($L$, shade + water deficit and ($h$), full sunlight + water deficit.

In this study, the $g_i$ of current-year needles of 4-year-old $P. asperata$ trees was determined by novel fitting of $A$–$C_i$ curves. As outlined by Warren and Adams (2006), estimating $A$–$C_i$ curves might be delicate, as small or noisy data sets can lead to significant estimation problems, and thus, high-quality data are required (Pons et al. 2009). To minimize the influence of poorly estimated parameters, those $A$–$C_i$ curves that displayed a large data scatter were discarded and the estimates were weighted by the reciprocal of their squared standard error (Murtaugh 2007). We also used the photocompensation point method to estimate $g_i$, although these two methods are not totally independent, because they share a few common assumptions. This approach is justified by the belief that two methods (that are as independent as possible) should be used for the estimation of $g_i$, when possible, to increase confidence in the results, as no truly independent and assumption-free method exists (Warren 2006). On the other hand, estimates of $g_i$ are very sensitive to variation in the CO$_2$ compensation point in the absence of mitochondrial respiration in the light ($I^*$). The $I^*$ value detected in this study (39.9 μmol mol$^{-1}$) is very similar to the value (37.4 μmol mol$^{-1}$) reported by Bernacchi et al. (2002), but less than the widely used 45 μmol mol$^{-1}$ (Jordan and Ogren 1984). It was reasoned that $I^*$ is an intrinsic property of Rubisco and thus, varies little among species or growing conditions (von Caemmerer 2000). We then conducted recalculations assuming $I^* = 45 \mu$mol mol$^{-1}$, and $g_i$ increased by 11.1–15.6%, while the treatment differences were unaffected. The precautions taken when using each of the two methods, as well as the good agreement between them, suggest that these data were sufficiently reliable. The precision of the present estimates based on the relative standard deviations (RSD) was between 15% ($A$–$C_i$ curves) and 19% (photocompensation method), similarly to the commonly reported values (10–15% RSD, e.g., Warren and Adams 2006).

This study showed that $g_i$ was similar in shade and sun leaves of the wet climate population under unstressed conditions, whereas a modest 33% difference was observed in the dry climate population. Our results contrast to those
reported for *Juglans regia* L. (Piel et al. 2002) and *Fagus sylvatica* L. (Warren et al. 2007), where $g_i$ was approximately doubled in shade leaves when compared to sun leaves. A possible explanation is that in this study the shade treatment was not strong enough. For instance, Piel et al. (2002) used more extreme light treatments (10 ± 1% of incident radiation), whereas here the shade treatment used about 30% of full sunlight. Future studies may also include more extreme shade treatments for the entire plant. The range of $g_i$ values, 0.02–0.10 mol m$^{-2}$ s$^{-1}$, for the current-year leaves of *P. asperata* agrees with the values that were previously reported for other coniferous species (De Lucia et al. 2003), but they are somewhat lower than the values reported for Douglas-fir (0.14–0.20 mol m$^{-2}$ s$^{-1}$, Warren et al. 2003). However, there is no clear explanation for the low $g_i$ value in *P. asperata*, and it may be related to the leaf anatomy, age, position and size of chloroplasts or to the activity of leaf carbonic anhydrase or other biochemical attributes (Flexas et al. 2008). The $g_i$ variable was inhibited by water deficit, but to a variable extent in different populations. In the wet climate population, decreases in $g_i$ were 0.06 times as large as those in $g_s$ whereas in the dry climate population, $g_i$ decreased 0.03 times the rate of $g_s$, confirming that these relationships are not constant (Warren 2008b). In an agreement with our hypothesis, a lower $g_i$ was observed in the dry climate population than in the wet climate population. Yet, currently, we have no evidence explanation for the factors that contribute to the low $g_i$ in the dry habitat population. We suggest that the high LMA value might indicate that low mesophyll porosity, and the thick mesophyll cell walls contribute to low $g_i$ in the dry habitat population. However, some previous studies (Kogami et al. 2001) have showed that a higher LMA does not necessarily result in lower $g_i$. Decreases in $g_i$ were found to be accompanied by increases in the drawdown from $C_i$ to $C_c$. The mean drawdown from $C_i$ to $C_c$ of 52–100 μmol mol$^{-1}$ was very similar to the mean value derived from the
literature review by Warren (2008a). Nevertheless, Cᵢ–Cₑ was larger in water-limited plants, which demonstrated that the photosynthesis is generally more limited by gᵢ in water-stressed plants.

It is interesting to note that gᵢ decreases with increasing LMA under water deficit conditions. In contrast, a positive correlation between LMA and gᵢ was observed under well-watered conditions in the wet climate population. The gᵢ value often decreases with increasing LMA (Terashima et al. 2005). In contrast, some studies have found a positive correlation between LMA and gᵢ (Evans and Loreto 2000, Piel et al. 2002). This discrepancy among the studies occurs because increases in LMA may either reflect the accumulation of cell wall compounds with concomitant increases in tissue density, or it may be associated with greater number of mesophyll layers and, accordingly, greater chloroplast to total leaf surface area ratios (Warren 2004). We agree that it is likely that the positive effect of well-watered conditions on LMA and gᵢ observed in this study relates to the development of a greater relative chloroplastic surface area. However, we cannot ascribe that the observed correlation between LMA and gᵢ reflects a direct causal link, as there is uncertainty of how leaf properties actually affect gᵢ. Also, the correlation between these two variables was dependent on the functional traits of leaves. Future studies should focus on the structural parameters of leaves, such as cell wall thickness and exposed chloroplast surface area, and how they influence gᵢ. While anatomical traits were not measured in P. asperata, it is noteworthy that gᵢ significantly decreased from full sunlight/well-watered conditions (control) to full sunlight/water deficit conditions. Assuming a linear relationship between gᵢ and cell wall thickness, we expect that LMA would vary between control plants and drought-stressed sun plants, given the relationships among gᵢ, anatomy and morphology, but this was not the case in this study. The result suggests that in P. asperata biochemical (carbonic anhydrase and aquaporins) processes were at least partially responsible for reduced gᵢ.
This study demonstrated that the $g_i$ variable was inhibited by water deficit more in the sun than in the shade plants. Yet, there is little doubt that the soil water content was equivalent in sun and shade, as evidenced in Figure 1. Two possible reasons for the more pronounced water deficit effect on $g_i$ detected in the sun plants than in the shade plants are as follows: (i) the $g_i$ responses to soil water deficit in the sun are a consequence of changes in leaf water status which decrease the activity of carbonic anhydrase or aquaporins (Flexas et al. 2006). We observed that changes in the RWC are indeed, on average, larger in sun than in shade during water deficit. (ii) As our results demonstrated that there is a significant correlation between $g_i$ and ABA (Figure 5B), we suggest that the dramatically reduced $g_i$ in plants exposed to full sunlight under water deficit could be connected with the accumulation of ABA, whereas no enhanced ABA content was detected in drought-stressed shaded plants. Similarly, water stresses often result in simultaneous decreases in $g_i$ and $g_s$ (Flexas et al. 2008), and there is some indication that this could be related to ABA (Flexas et al. 2006, 2008). However, the effects of ABA on $g_i$ are far from clear (Vráb et al. 2009).

We observed significant linear correlations between the photosynthetic capacity and $g_i$ under both well-watered and water deficit conditions. This linear relationship is similar to that reported by Evans and Loreto (2000). The limitation analysis showed that a stronger relative limitation of photosynthesis by CO$_2$ diffusion occurred under full sunlight than under shade conditions. In all cases, the relative limitation due to $g_i$ (0.26–0.47) was larger than that due to $g_s$ (0.12–0.28), confirming the importance of including this component into any detailed study of gas-exchange response to water deficits (Flexas et al. 2008). Furthermore, in both populations, the treatment-related PNUE decline was not a consequence of changes in $N_a$, as $N_a$ did not appreciably change during the experiment. Rather, mesophyll resistance could be a factor contributing to the variation in PNUE, and this could be the reason why a relationship was found between PNUE and $C_T$-$C_a$. In addition, PNUE was lower in the dry climate population than in the wet climate population, which possessed lower $g_i$. Thus, the poor PNUE of the dry climate population relates to the limitation due to greater $g_i$, and is accompanied by a greater drawdown of $C_T$-$C_a$.

On the other hand, these data showed a more pronounced water deficit effect on $\delta^{13}C$ in the sun than in the shade. We used the path analysis to determine the relative influences of $g_i/g_s$, LMA and $N_a$ on $\delta^{13}C$ across all treatments. The path analysis showed that a direct positive effect of $g_i/g_s$ on $\delta^{13}C$ was greater compared to other characters, and there was an indirect effect on $\delta^{13}C$ through its association with $g_i/g_s$. Thus, the $g_i/g_s$ is the factor with the greatest effect on $\delta^{13}C$, and the other factors affect $\delta^{13}C$ via $g_i/g_s$. This was in accordance with the previous studies (Lauter et al. 1997) showing consistently a positive relationship between WUE and the $g_i/g_s$ ratio. In addition, we observed that the dry climate population showed a higher $\delta^{13}C$ with a higher $g_i/g_s$ under water deficit conditions. This information has important implications for developing drought-screening tools, and it provides new insights into the biological effects of drought stress at both the cellular and whole-plant levels.

In conclusion, this is one of the very few published studies that have provided estimates of the combined effects of water and shade on $g_i$ for a coniferous species with the novel $A$–$C_i$ curve analysis method. The more pronounced water deficit effect on $g_i$ in the sun plants than in the shade plants relates to water deficit having different effects on RWC and ABA contents in sun and shade plants. Moreover, $g_i$ limited photosynthesis by about 26–47% as compared to about 12–28% of stomatal limitation. Furthermore, $g_i$ is also important in contributing to population differences in PNUE, which suggests a profound effect of $g_i$ on photosynthesis and the importance of including this component in detailed plant physiological studies in a climate change scenario. In addition, the path analysis showed that the different factors have the following ordinal influence on the change of $\delta^{13}C$: $g_i/g_s$, LMA and $N_a$. In this study, the dry climate population showed a higher $\delta^{13}C$ with a higher $g_i/g_s$, suggesting that an increase in the ratio $g_i/g_s$ enhances WUE in plants evolved under arid environments. Thus, we propose that the use of the $g_i/g_s$ parameter to screen plants with higher water deficit tolerance is certainly worthy of consideration in *P. asperata*.

Acknowledgments

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