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Mesophyll conductance constrains photosynthesis in three common sclerophyllous species in Central Chile

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Abstract

Background: *Quillaja saponaria* Mol., *Cryptocarya alba* Mol. Looser, and *Lithraea caustica* Molina Hook *et* Arn., are common sclerophyllous species in Mediterranean Central Chile. Mesophyll conductance, g_m, may strongly limit photosynthesis in these semiarid environments.

Results: Simultaneous measurements of gas exchange and chlorophyll fluorescence were carried out in 45 nursery plants from these species to determine diffusional and biochemical limitations to photosynthesis. Values of stomatal conductance, g_s, were greater than those of mesophyll conductance, g_m, while their ratio (g_m/g_s) was not influenced by species being on average 0.47. Relative limitations posed by mesophyll conductance to photosynthesis, L_m, (0.40 ± 0.02) were high compared to those imposed by stomata, L_s (0.07 ± 0.01). The average CO_2 concentration in the intercellular air spaces (C_i) was 32 µmol mol^{-1} lower than in the atmosphere (C_a), while the average CO_2 concentration in the chloroplasts (C_c) was 131 µmol mol^{-1} lower than C_i independent of species. Maximal rates of Rubisco carboxylation, V_{cmax}, and maximal electron transport rates driving regeneration of RuBP, J_{max}, ranged from 13 to 66 µmol CO_2 m^{-2} s^{-1} and from 33 to 148 µmol electrons m^{-2} s^{-1}, respectively, and compare well to averages for C_3 plants.

Conclusions: Photosynthetic performance was in the series: *Q. saponaria* > *C. alba* ≥ *L. caustica*, which can be attributed first to mesophyll conductance limitations, probably mediated by leaf anatomical traits and then to species specific foliage N partitioning strategies.

Keywords: Chloroplastic CO_2 concentration; Electron transport; Mesophyll conductance; Rubisco carboxylation; Sclerophyllous species

Background

The biochemical model of leaf photosynthesis originally proposed by Farquhar et al. (1980) and later improved (von Caemmerer and Farquhar, 1981; Sharkey, 1985; Harley and Sharkey, 1991) is widely used in ecophysiological research for describing CO_2 exchange processes and to scale carbon exchange from leaves to canopies (Baldocchi and Harley 1995; McMurtrie et al. 1992; Whitehead et al. 2004). In this model, photosynthesis is considered to be limited by the maximal rate of ribulose-1,5-*bis*phosphate (RuBP) carboxylase-oxygenase (Rubisco) carboxylation, V_{cmax}, and by the maximal electron transport rate driving

regeneration of RuBP, J_{max}, and by triose phosphate utilization (TPU) (Farquhar et al. 1980; von Caemmerer 2000; von Caemmerer and Farquhar 1981). These parameters are fitted to the response of photosynthesis, A, to the CO_2 concentration in the intercellular air spaces, C_i, known as A/C_i curves. Values of C_i, are estimated from stomatal conductance to CO_2 transfer, g_s, and the ambient CO_2 concentration external to the leaf, C_a. This model has been used to explain the mechanisms of photosynthetic acclimation to elevated atmospheric CO_2 (Griffin et al. 2000; Hogan et al. 1996; Kellomaki and Wang 1996; Murray et al. 2000; Turnbull et al. 1998), the influence of global warming on plant carbon budgets (Turnbull et al. 2002), and for identifying factors limiting photosynthesis under water stressed conditions in Mediterranean plants

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(Grassi and Magnani 2005; Gulias et al. 2002; Niinemets et al. 2009b), among others.

In most studies, the mesophyll conductance of CO_2 from the intercellular air spaces to the sites of Rubisco carboxylation in the chloroplasts, g_m, has been considered to be sufficiently large to be negligible (Farquhar et al. 1980). However, there is a growing awareness that the significance of g_m in limiting photosynthesis can be similar to the limitation imposed by stomata (Flexas et al. 2008; Harley et al. 1992; Loreto et al. 1992; von Caemmerer 2000; Warren and Adams 2006; Warren et al. 2003). As a result, C_i is greater than the CO_2 concentration in the chloroplasts, C_c, and values of V_{cmax} and J_{max} are underestimated when fitted to estimates of C_i rather than C_c.

Arid and semiarid lands account for 41% of Chile's continental territory, covering about 31 million ha (Benites et al. 1994). Within this area, semiarid sclerophyllous (i.e. a woody plant with small coriaceous evergreen leaves dominant of the Mediterranean region) shrublands and forests extends from 32-36° S latitude (~345,000 ha) in Central Chile (Armesto et al. 2007; CONAF 1999); exhibiting high levels of endemism, and are therefore considered a priority for biodiversity conservation (Arroyo et al. 2004). These ecosystems are subjected to high radiative and water stresses that limit their development and reproduction (Cabrera 2002); and, additionally, have a long history of degradation by human activity, which may represent an important adaptive pressure (Galmes et al. 2007). Therefore, understanding how environmental limitations are imposed on the carbon budget is relevant to accurately estimate carbon uptake and water use by sclerophyllous species.

There is extensive work on comparisons of g_m between species and plant functional groups (Loreto et al. 1992; De Lucia et al. 2003; Hassiotou et al. 2009; Niinemets et al. 2009b). However, little is known about the extent to which g_m regulates the rate of photosynthesis in sclerophyllous species of central Chile. Consequently we chose to work with three native sclerophyllous species: *Quillaja saponaria* Mol., *Cryptocarya alba* Mol. Looser and *Lithraea caustica* Molina Hook *et* Arn., to determine stomatal, mesophyll and biochemical limitations to photosynthesis. Specifically, we assessed whether mesophyll conductance to CO_2 induces similar constrains to photosynthesis across different sclerophyllous species.

Methods
Plant material
Plant material consisted of 15 randomly selected plants from each of the following species: *Q. saponaria, C. alba and L. caustica*, from a nursery of the Faculty of Forestry and Nature Conservation at University of Chile, Santiago, Chile. Seeds were sown in the winter 2008, and transferred during spring 2008 and spring 2009 to 12×15 cm (0.25 L)

and 20×30 cm (2 L) black polyethylene bags filled with an even mixture of composted plant residues, soil and sand. No fertilizers were applied. Plants developed under 46% shading (in order to mimic the light environment experienced by saplings in their natural habitat) and received weekly irrigation during winter, and daily irrigation to field capacity in other seasons. At the time of measurements, we selected fifteen homogenous plants of *Q. saponaria, C. alba* and *L. caustica* which exhibited an average (±1 SD) plant height of 82.9 ± 14.4 cm, 63.4 ± 18.9 cm, and 47.6 ± 15.3 cm; and a collar diameter of 7.5 ± 0.8 mm, 9.1 ± 1.4 mm, and 11.0 ± 2.6 mm, respectively. All gas exchange and chlorophyll fluorescence measurements were taken in a laboratory without thermal regulation, with open windows to allow circulation of fresh air. Measurements took 45 days; with only one plant measured per day, spread in the summer period from December 20, 2010 to March 15, 2011; six months after plants were transferred from small to large containers. Gas exchange measurements for each individual plant typically took all day between 10 am and 6 pm; and followed the exact same sequence of measurements for all plants (see below). We alternated species each day in order to avoid differences brought about by day to day meteorological changes.

Gas exchange measurements
Simultaneous measurements of gas exchange and chlorophyll *a* fluorescence were carried out on the 45 selected plants, using a portable photosynthesis system (CIRAS-2, PP Systems, MA, USA) equipped with an integrated chlorophyll fluorescence and gas exchange chamber (PLC6-U Auto Leaf Cuvette, PP System, MA, US). Plants were shifted from the nursery to the laboratory the day before measurements were undertaken.

For each plant, a fully-developed leaf in the upper third of the plant was chosen and placed inside a circular 2.5 cm^2 (18 mm in diameter) cuvette. Temperature in the cuvette (block) was maintained at 25ºC while leaf-to-air vapour pressure deficit (*VPD*) was maintained below 1 kPa. Each leaf was left to equilibrate inside the cuvette for 10 min at about 368 ± 3 µmol mol^{-1} CO_2 concentration and saturating irradiance (2,000 µmol photons m^{-2} s^{-1}), before measuring the response of net assimilation (*A*) to intercellular CO_2 concentration (C_i). External CO_2 concentration (C_a) was supplied with a CO_2 mixer across the sequence 25, 50, 75, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200 and 1500 µmol mol^{-1}, with saturating irradiance, *Q* (400–700 nm), maintained at 2,000 µmol m^{-2} s^{-1}. Measurements were recorded after values of *A*, C_i and g_s became stable but with a minimum waiting time of 3 min at each step within the sequence. At each value of C_i, measurements of fluorescence for the light-adapted leaf were made simultaneously to the gas exchange measurements. Values of *F* and F_m' (the steady and maximal fluorescence

respectively), were used to calculate photochemical efficiency of photosystem II, Φ_{PSII}. The response of net assimilation to irradiance (*A/Q* curves) was measured immediately after each *A/C$_i$* curve ended, across the *Q* sequence: 0, 50, 100, 150, 200, 300, 400, 500, 800, 1000, 1200, 1400, 1600, 1800 and 2000 µmol m^{-2} s^{-1}, with ambient CO_2 concentration maintained at 368 ± 3 µmol mol^{-1} using a CO_2 mixer. The *A/C$_i$* and *A/Q* curves were measured on the same foliage sample.

Models fitted

The biochemical model of leaf photosynthesis by Farquhar et al. (1980) describes the rate of photosynthesis (*A*) as:

$$A = \min \{A_c , A_q\} - R_d \qquad (1)$$

where A_c and A_q are the photosynthetic rates limited by Rubisco carboxylation and by electron transport rate respectively, and min {} indicates the minimum of these two rates. R_d is the rate of daytime respiration resulting from processes other than photorespiration. The photosynthetic rate limited by Rubisco carboxylation (A_c) is given by:

$$A_c = V_{cmax} \frac{C_i - \Gamma^*}{C_i + K_c (1 + O_i / K_o)} \qquad (2)$$

where V_{cmax} is the maximum rate of Rubisco carboxylation under saturating RuBP and CO_2, C_i and O_i are the intercellular CO_2 and O_2 concentrations, Γ^* is the CO_2 compensation concentration in the absence of day respiration, and K_c and K_o are the Michaelis constants for CO_2 and O_2, respectively.

The photosynthetic rate limited by RuBP regeneration driven by electron transport (A_q) is given by:

$$A_q = \frac{J (C_i - \Gamma^*)}{4 (C_i + 2\Gamma^*)} \qquad (3)$$

where *J* is the rate of electron transport at a given irradiance *Q*.

Prioul and Chartier (1977) described the response of *A* to *Q* by a non-rectangular hyperbola as:

$$A = \frac{\alpha Q + A_{sat} - \sqrt{(\alpha Q + A_{sat})^2 - 4\alpha\theta Q A_{sat}}}{2\theta} - R_{dark} \qquad (4)$$

where θ is the convexity of the non-rectangular hyperbola, α is the initial slope of the *A/Q* curve (often referred as 'apparent maximum quantum efficiency'), A_{sat} is the light-saturated photosynthetic capacity and R_{dark} the respiration rate at zero irradiance. Individual *A/Q* response curves were fitted using Eq. 4 in order to estimate α, A_{sat} and R_{dark}. The light saturation point, Q_{sat}, was calculated as the irradiance for which 90% of A_{sat} was achieved.

Values of the rate of mitochondrial respiration in the light, R_d^*, and the chloroplastic CO_2 compensation point, Γ^*, were estimated for each sample using the Laisk method (von Caemmerer 2000). Briefly, the *A/C$_i$* response was measured at three levels of low irradiance (*Q* = 50, 200 and 400 µmol m^{-2} s^{-1}) for six increasing values of C_a from 25 to 400 µmol CO_2 mol^{-1}. Linear relationships between *A* and C_i were fitted and the point of intersection of the three lines was taken as R_d^* (*y*-axis) and Γ^* (*x*-axis) (Figure 1).

The photosynthesis model described by Farquhar et al. (1980) (Eq. 1) was fitted to the *A/C$_i$* and *A/C$_c$* curves by non-linear least squares regression (SigmaPlot, Version 12.1, SPSS, Chicago, IL). Values of V_{cmax} and R_d were estimated from the lower part of the *A/C$_i$* or *A/C$_c$* curve (C_i or C_c < 220 µmol mol^{-1}) (Eq. 1 and 2), and these values were then used to estimate J_{max} over the entire range of measured C_i or C_c (Eq. 1,2 and 3). Michaelis-Menten constants of Rubisco for CO_2 and O_2, K_c and K_o, used in the fitting (25 °C) were 404.9 µmol mol^{-1} and 278.4 mmol mol^{-1}, respectively as described by Bernacchi et al. (2001).
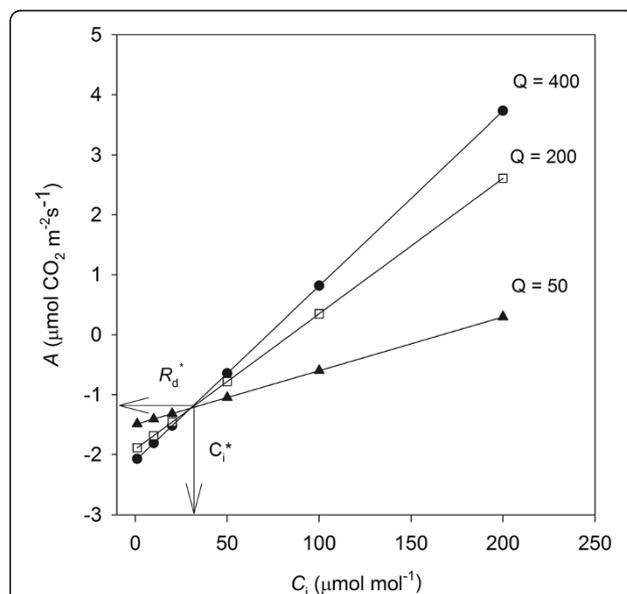

Figure 1 The response of net photosynthesis (*A*) to intercellular CO_2 concentration (*C$_i$*) at three different irradiances (*Q* = 400, 200 and 50 µmol m^{-2} s^{-1}, 400–700 nm) for a representative foliage sample. Linear relationships between *A* and C_i were fitted for each *Q* level and their intersections averaged to yield a point which when projected to the *A* axis was taken as the rate of day respiration (R_d^*) and when projected to the C_i axis was taken as the intercellular CO_2 compensation concentration in the absence of day respiration (C_i^*). The mitochondrial CO_2 compensation concentration (Γ^*) was calculated as $\Gamma^* = C_i^* + R_d^* / g_m$ (von Caemmerer 2000), where g_m is mesophyll conductance to CO_2 transfer. Method originally proposed by Laisk, A. (von Caemmerer 2000) to determine C_i^* and R_d. This figure is equivalent to the one presented by De Lucia et al. (2003) but with data drawn from this study.

Calculations of mesophyll conductance

Mesophyll conductance, g_m, was estimated using the "constant J" method (Harley et al. 1992; Loreto et al. 1992) where J is the rate of electron transport (Figure 2b). This method uses data in the RuBP-regeneration limited portion of the A/C_i curve, where rates of electron transport are constant. Within this region, further increases in photosynthesis with increasing C_i are due to suppression of photorespiration as the rate of carboxylation progressively substitutes the rate of oxygenation. Thus, photosynthesis is related to C_c and the relative CO_2/O_2 specificity of Rubisco, normally described by the chloroplastic CO_2 compensation point, Γ^*. The constant J method is sensitive to errors in both the rate of mitochondrial respiration in the light, R_d^*, and Γ^* and the approach assumes that both J and g_m are constant across the range of C_a concentrations used for the measurements (Harley et al. 1992; Pons et al. 2009). The relationship of g_m with Γ^*, R_d^* and intercellular CO_2 compensation point in the absence of day respiration, C_i^* is given by $\Gamma^* = C_i^* + R_d^*/g_m$ (Peisker and Apel 2001; von Caemmerer 2000).

The photochemical efficiency of photosystem II (Φ_{PSII}) was estimated from chlorophyll fluorescence measurements as $(F_m' - F) / F_m'$, where F and F_m' are the steady and maximal fluorescence in the light-adapted sample respectively (Schreiber et al. 1994). The values of Φ_{PSII} are directly proportional to the rate of electron transport through photosystem II, and therefore can be used to determine the portion of the A/C_i curve where the rate of electron transport is constant (Genty et al. 1989) (Figure 2a). Optimal values for g_m were resolved iteratively from three or more measurements of photosynthesis at high values of C_i that correspond with constant rates of electron transport (Singsaas et al. 2004; Warren 2006). This was done using the Generalized Reduced Gradient Nonlinear Solving Method for nonlinear optimization included in the Solver Tools of Microsoft Excel with measurements of A, C_i, Γ^*, R_d^* to resolve the value of g_m that best explained changes in photosynthesis with changes in C_i indicated by minimum variance in J (Harley et al. 1992; Singsaas et al. 2004; Warren 2006) (Figure 2b).

Stomatal and mesophyll limitations to photosynthesis

A/C_i response and values for g_s and g_m, were used to partition stomatal, mesophyll and biochemical limitations to photosynthesis. Values of stomatal conductance to CO_2 transfer were calculated by $g_s = A/(C_a-C_i)$ dividing values by 1.64 (Jones 1992). Relative stomatal limitations were calculated using the method of Farquhar and Sharkey (1982) as $L_s = 1 - A_{a\text{-}gs} / A_{i\text{-}gs}$ where $A_{a\text{-}gs}$ and $A_{i\text{-}gs}$ are the actual value of A and the value estimated when g_s is infinite, respectively. Similarly, following Bernacchi et al. (2002), relative limitation to photosynthesis imposed by g_m was calculated as: $L_m = 1 - A_{a\text{-}gm} / A_{i\text{-}gm}$ where $A_{a\text{-}gm}$ and $A_{i\text{-}gm}$ are the actual value of A and the value estimated when g_m is infinite, respectively. The CO_2 concentration in the chloroplasts, C_c, was calculated from $C_c = C_i - A/g_m$.

Foliage surface area and nutrient concentrations

Following the completion of A/C_i and A/Q curves, the leaf was carefully removed from the cuvette, scanned to determine leaf area by optical methods, dried at 70°C to constant mass and then both measurements used to calculate the foliage area to mass ratio, M. Foliage samples were finely ground, Dumas combusted and N concentrations determined by thermo conductivity (LECO, TruSpec CN, US) at the Laboratory of Soil and Foliar Analysis at Universidad Católica de Chile, Santiago, Chile. Nitrogen foliage concentrations are expressed on a hemi-surface area basis (N_a) and photosynthetic nitrogen-use efficiency, E_N, defined as A_{sat}/N_a.

Data analysis

All statistical analyses were undertaken at the plant level using The R System (R Core Team 2013). Variables were tested for normality and homogeneity of variance and transformations were made as necessary to meet the underlying statistical assumptions of the models used. All values are presented as means ± 1 standard error ($n = 15$) unless stated otherwise. A one way analysis of variance was used to compare diffusional and biochemical limitations to photosynthesis between sclerophyllous species. Tukey's least significant difference test was used to distinguish among individual means where applicable with a confidence level of $P < 0.05$. Differences in slopes and intercepts in the linear relationships between photosynthetic parameters and foliar nitrogen among sclerophyllous species were tested for significance by analysis of covariance.

Results and discussion
Overall photosynthetic performance

The Farquhar et al. (1980) model was fitted to the A/C_i curves while the Prioul and Chartier (1977) model to the A/Q curves measured for each leaf sample ($n = 45$). There was excellent correspondence between modelled and observed data independent of the sclerophyllous species (Figure 3).

Light response curves (Figure 3a) were very similar among species up to an irradiance level of about 500 μmol photons m^{-2} s^{-1}, changing drastically thereafter with clearly distinct maximums of photosynthetic rates in the series: *Q. saponaria* > *C. alba* > *L. caustica* which reflects that the three species exhibited a similar initial pseudo-linear slope of the A/Q curve (the apparent maximum quantum efficiency: α) averaging 0.033 ± 0.007 mol CO_2 mol^{-1} quanta. The rate of photosynthesis at saturating irradiance (2000 μmol m^{-2} s^{-1}) and ambient CO_2 concentration (A_{sat}), on the other hand, was significantly
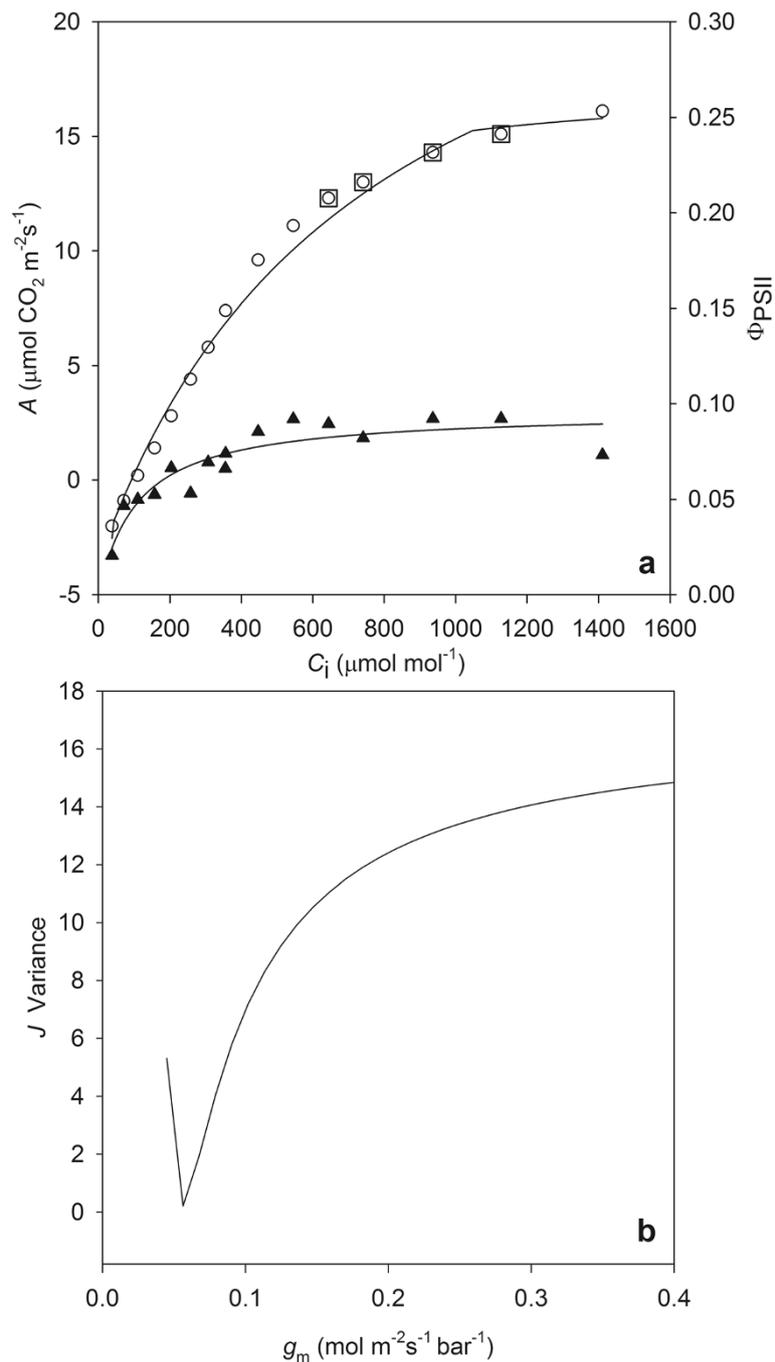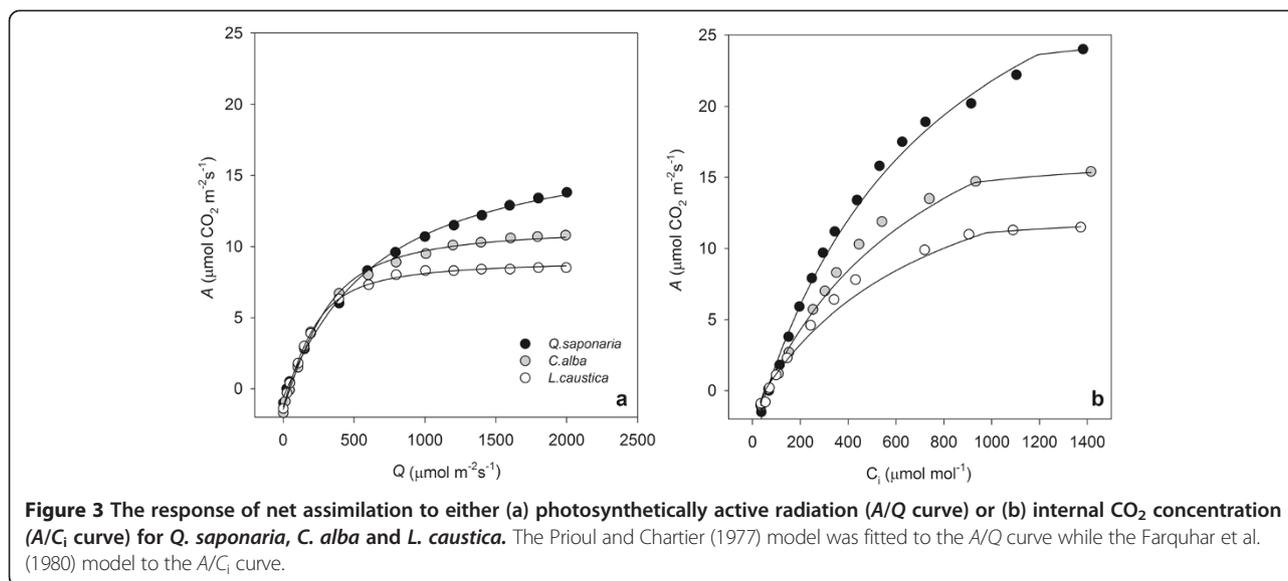
Figure 2 Graphic description of the constant *J* method to determine transfer conductance. (a) The rate of net photosynthesis (*A*; open circles) and photochemical efficiency of photosystem II (Φ_{PSII}; solid triangles) as a function of the intercellular CO_2 concentration (C_i) for a representative foliage sample. Solid lines represent a least-squares fit to the A/C_i and Φ_{PSII}/C_i response. Open squares are observed values used to estimate mesophyll conductance (g_m). These values are within the portion of the A/C_i response where Φ_{PSII} indicated that electron transport rate was constant. **(b)** The variance of estimated electron transport rates, *J*, for different values of g_m. Values of *J* were estimated for each of the four *A* values indicated as open squares in Figure 2a using the equation given by Harley et al. (1992). The g_m that minimized the variance of *J* estimates for this foliage sample was 0.054 mol m^{-2} s^{-1} bar^{-1}. This figure is equivalent to the one presented by Harley et al. (1992) and De Lucia et al. (2003) but with data drawn from this study.

greater in *Q. saponaria* (14.2 ± 0.8 μmol CO_2 m^{-2} s^{-1}) compared to *C. alba* (9.5 ± 0.4 μmol CO_2 m^{-2} s^{-1}) and *L. caustica* (7.8 ± 0.7 μmol m^{-2} s^{-1}) (Figure 3a; Table 1).

The light saturation point, Q_{sat}, which followed a similar pattern as A_{sat}, was consistently higher in *Q. saponaria* (1168 ± 57 μmol photons m^{-2} s^{-1}) compared to *C. alba*

Figure 3 The response of net assimilation to either (a) photosynthetically active radiation (A/Q curve) or (b) internal CO$_2$ concentration (A/C_i curve) for *Q. saponaria*, *C. alba* and *L. caustica*. The Prioul and Chartier (1977) model was fitted to the A/Q curve while the Farquhar et al. (1980) model to the A/C_i curve.

(964 ± 56 μmol photons m^{-2} s^{-1}) and *L. caustica* (987 ± 65 μmol photons m^{-2} s^{-1}). The rate of respiration in the absence of light at ambient CO$_2$ concentration (R_{dark}) was 0.77 ± 0.10 μmol CO$_2$ m^{-2} s^{-1} independent of the species. The light compensation point, or the irradiance value at which the rate of photosynthesis equals zero, did not differ across species, being 38 ± 4 μmol photons m^{-2} s^{-1}. The rate of transpiration at saturating irradiance (2000 μmol m^{-2} s^{-1}) and ambient CO$_2$ concentration (E) was 2831 ± 309 μmol H$_2$O m^{-2} s^{-1} while the instantaneous water use efficiency (E/A_{sat}) was 337 ± 41 mol H$_2$O mol^{-1} CO$_2$, unaffected by species (Table 1).

The response of photosynthetic rates (A) to internal CO$_2$ concentration (C_i) (Figure 3b), changed drastically among species for the whole range of C_i values in the series: *Q. saponaria* > *C. alba* > *L. caustica*. Consequently the initial slope of the A/C_i curves (dA/dC_i; often referred to as 'carboxylation efficiency') was greater in *Q. saponaria* (0.070) compared with *C. alba* (0.047) and *L. caustica* (0.038). A similar trend was observed for the rate of photosynthesis near saturating C_i (800 μmol mol^{-1}), A_{max}, being 12.03, 17.01 and 23.81 μmol CO$_2$ m^{-2} s^{-1} for *L. caustica*, *C. alba* and *Q. saponaria*, respectively (Table 1).

Mesophyll conductance

Mesophyll conductance (g_m) was similar between *L. caustica* (0.060 mol CO$_2$ m^{-2} s^{-1} bar^{-1}) and *C. alba* (0.065 mol CO$_2$ m^{-2} s^{-1} bar^{-1}), but collectively significantly lower than *Q. saponaria* (0.097 mol CO$_2$ m^{-2} s^{-1} bar^{-1}). Although unsignificantly, g_s tended to be greater for *Q. saponaria* (0.250 mol CO$_2$ m^{-2} s^{-1}) compared to *C. alba* (0.227 mol CO$_2$ m^{-2} s^{-1}) and *L. caustica* (0.221 mol CO$_2$ m^{-2} s^{-1}). The ratio g_m/g_s was on average 0.47 ± 0.08, independent of species (Table 1). Relative limitations posed by mesophyll

conductance to photosynthesis, L_m, (0.40 ± 0.02) were high compared to those imposed by stomata, L_s (0.07 ± 0.01) and not significantly influenced by species. The average CO$_2$ concentration in the intercellular air spaces (C_i) was 31.7 μmol mol^{-1} lower than in the atmosphere (C_a), while the average CO$_2$ concentration in the chloroplasts (C_c) was 130.6 μmol mol^{-1} lower than in C_i, independent of the species (Table 1).

The g_m values found in this study were generally lower than those reported for other plant functional groups. Flexas et al. (2008) argues that g_m is associated with leaf forms and plant functional groups, rather than evolutive trends: herbaceous plants exhibit generally the largest g_m values (~0.4 mol CO$_2$ m^{-2} s^{-1} bar^{-1}), perennial herbs and woody deciduous angiosperms display intermediate values (~0.2 mol CO$_2$ m^{-2} s^{-1} bar^{-1}), while woody evergreen plants exhibit g_m values slightly above and below 0.1 mol CO$_2$ m^{-2} s^{-1} bar^{-1} in angiosperms and gymnosperms, respectively. The mean value across all angiosperm sclerophyllous species studied was 0.073 mol CO$_2$ m^{-2} s^{-1} bar^{-1}, similar to those found by Niinemets et al. (2009b) for Australian sclerophyllous species (0.087 mol CO$_2$ m^{-2} s^{-1} bar^{-1}).

There seems to be a consistent pattern for sclerophyllous species to exhibit both low g_m and low g_m/g_s values (Gulias et al. 2002; Niinemets et al. 2009b; Tomas et al. 2013; Warren 2004). Consequently, differences between C_a and C_i are relatively small compared to the difference between C_i and C_c. This drawdown of CO$_2$ implies that relative limitations imposed by mesophyll conductance to photosynthesis are much greater than those posed by stomata. Warren (2004) found that mesophyll limitations (L_m ~ 0.19 to 0.38) were greater than stomatal limitations (L_s ~ 0.05 to 0.23) for *Eucalyptus globulus* which are similar to our study. Equivalent mesophyll conductance limitations were observed for other sclerophyllous

Table 1 Comparison of leaf and photosynthetic parameters for three common sclerophyll species in Central Chile

Variable	Units	Schlerophyll species			ANOVA statistics
		L. caustica	*C. alba*	*Q. saponaria*	
A_{sat}	(µmol m^{-2} s^{-1})	7.8 ± 0.7 a	9.5 ± 0.4 a	14.2 ± 0.8 b	***
α	(mol mol^{-1})	0.019 ± 0.002	0.024 ± 0.002	0.023 ± 0.002	ns
R_{dark}	(µmol m^{-2} s^{-1})	0.7 ± 0.1	1.0 ± 0.2	0.6 ± 0.1	ns
Q_{sat}	(µmol m^{-2} s^{-1})	987 ± 65 a	964 ± 56 a	1168 ± 57b	*
Q_{comp}	(µmol m^{-2} s^{-1})	36 ± 8	51 ± 9	27 ± 4	ns
E	(µmol H$_2$O m^{-2} s^{-1})	2133 ± 447	2913 ± 476	3492 ± 654	ns
1 / WUE	(mol H$_2$0 mol^{-1}CO$_2$)	376 ± 93	347 ± 57	285 ± 60	ns
SLA	(m^2 kg^{-1})	7 ± 0.4 a	10 ± 0.7 b	8.7 ± 0.5 ab	***
N_a	(mmol m^{-2})	120.2 ± 8.9 a	195.5 ± 21 b	165.8 ± 17.6 ab	**
E_n	(µmol mol^{-1} s^{-1})	69.8 ± 9.1 ab	56.4 ± 6.2 a	95.9 ± 8.8 b	**
A_{max}	(µmol mol^{-1} s^{-1})	12.03 ± 0.86 a	17.01 ± 0.81 b	23.81 ± 1.18 c	***
dA/dCi	(mol mol^{-1})	0.038 ± 0.003 a	0.047 ± 0.002 a	0.070 ± 0.004 b	***
g_m	(mol m^{-2} s^{-1} bar^{-1})	0.06 ± 0.005 a	0.065 ± 0.004 a	0.097 ± 0.007 b	***
g_s	(mol m^{-2} s^{-1})	0.221 ± 0.03	0.221 ± 0.025	0.25 ± 0.018	ns
g_m / g_s	-	0.54 ± 0.18	0.47 ± 0.14	0.43 ± 0.06	ns
L_s	-	0.07 ± 0.01	0.07 ± 0.02	0.08 ± 0.01	ns
L_m	-	0.35 ± 0.03	0.42 ± 0.02	0.43 ± 0.02	ns
C_a	(µmol mol^{-1})	368.3 ± 0.6	368.7 ± 1	368.7 ± 0.6	ns
C_i	(µmol mol^{-1})	337.7 ± 7	336.5 ± 6.9	333.6 ± 3.7	ns
C_c	(µmol mol^{-1})	220 ± 10.3	202.2 ± 8.2	192.8 ± 5.9	ns
V_{cmax} (C_i)	(µmol m^{-2} s^{-1})	26.8 ± 2.1 a	32.6 ± 1.1 a	49 ± 2.6 b	***
J_{max} (C_i)	(µmol m^{-2} s^{-1})	56.3 ± 4.1 a	79.6 ± 3.8 a	111.4 ± 5.6 b	***
J_{max} / V_{cmax} (C_i)	-	2.15 ± 0.1	2.44 ± 0.07	2.29 ± 0.07	ns
V_{cmax} (C_c)	(µmol m^{-2} s^{-1})	45.5 ± 5.1 a	64.4 ± 3.8 a	98.3 ± 5.7 b	***
J_{max} (C_c)	(µmol m^{-2} s^{-1})	60.4 ± 4.4 a	89.9 ± 4.2 b	126.4 ± 6.1 c	***
J_{max} / V_{cmax} (C_c)	-	1.41 ± 0.07	1.43 ± 0.06	1.31 ± 0.05	ns
R_d^*	(µmol m^{-2} s^{-1})	1.0 ± 0.1 a	1.7 ± 0.2 b	1.5 ± 0.1 b	***
C_i^*	(µmol mol^{-1})	33 ± 4.2 ab	23.2 ± 2.3 a	34 ± 2.4 b	*
$Γ^*$	(µmol mol^{-1})	55.5 ± 5.4	56.8 ± 4.1	59.2 ± 5.9	ns

Values are presented as means (± 1 SE, *n*=15) for the light-saturated rate of photosynthesis (A_{sat}), apparent maximum quantum efficiency (α), the rate of mitochondrial respiration at zero irradiance (R_{dark}), light saturation (Q_{sat}) and compensation point (Q_{comp}), evapotranspiration (E), the inverse of water use efficiency (1/WUE), specific leaf area (SLA), the nitrogen content on area basis (N_a), photosynthetic nitrogen-use efficiency (E_N), C_i-saturated rate of photosynthesis (A_{max}) and inicial slope of the A/C_i curve (dA/dC_i), mesophyll (g_m) and stomatal (g_s) conductance to CO$_2$ diffusion, and their ratio (g_m/g_s), relative stomatal, L_s, and mesophyll, L_m, limitations to photosynthesis, the external (C_a), intercellular (C_i) and chloroplastic (C_c) CO$_2$ concentration, maximal rate of Rubisco carboxylation (V_{cmax}), maximal rate of electron transport driving regeneration of RuBP (J_{max}), the ratio J_{max} / V_{cmax} on C_i and C_c basis, the rate of mitochondrial respiration in the light (R_d^*), the intercellular (C_i^*), and chloroplastic ($Γ^*$) CO$_2$ compensation concentration in the absence of mitochondrial respiration. Separation of means was determined by a Tukey test where applicable. Different letters between species indicate that means were significantly different at *P*<0.05. Significance of species on photosynthetic parameters are shown as: ns, non significant; *, significant at *P*<0.05; **, significant at *P*<0.01; ***, significant at *P*<0.001.

species (L_m ~ 0.20-0.50) by Niinemets et al. (2009b). This seems to be explained by leaf anatomical traits such as thickness, density and the leaf mass to area ratio (Flexas et al. 2012; Niinemets et al. 2009b; Tomas et al. 2013) and also by tissue and cell anatomical traits, such as chloroplast surface area exposed to the intercellular air spaces, cell wall thickness and palisade tissue path length, among others (Tomas et al. 2013; Flexas et al. 2012; Tosens et al. 2012; Terashima et al. 2011; Hassiotou et al. 2009).

C.albaThere are only a few studies on leaf anatomical traits for the sclerophyllous species considered in this study. *Q. saponaria* generally exhibits lower density palisade parenchyma and spongy mesophyll compared to *C. alba* and *L. caustica* (Gotor 2008); and additionally *L. caustica* exhibits substantially thicker cell walls compared to the other sclerophyllous species (Montenegro 1984). We also found *L.caustica* leaves to be thicker and denser, as shown by its lower foliage area to mass ratio

(M) (7.0 m^2 kg^{-1}), compared to *Q. saponaria* (8.7 m^2 kg^{-1}) and *C. alba* (10 m^2 kg^{-1}). Tomas et al. (2013) found that for sclerophyllous species g_m was strongly constrained by cell wall thickness (e.g. *L. caustica*). Such differences in leaf, tissue and cell anatomical traits may at least partially explain why values of g_m and photosynthetic rates are in the series: *Q. saponaria* > *C. alba* ≥ *L. caustica*.

In our study, values of A_{sat} were strongly and positively correlated with g_m ($A_{sat} = 1.92 + 100.49\ g_m$, $r^2 = 0.61$, P < 0.001). Intercepts ($F_{2,38} = 8.07$, $P < 0.01$) but not slopes ($F_{2,38} = 1.6$, $P = 0.21$) of this linear relationship were influenced by species (Figure 4). Values of g_s and g_m were uncorrelated ($r^2 = 0.03$, $P = 0.29$). An increase in the rate of photosynthesis with increasing g_m is consistent with previous findings from a wide range of species (Flexas et al. 2004; Grassi and Magnani 2005; Loreto et al. 1992; Niinemets et al. 2009b; Singsaas et al. 2004; Tomas et al. 2013; von Caemmerer and Evans 1991; Warren et al. 2003). Using the variable *J* method and carbon isotopes to estimate g_m for 15 angiosperm species, Loreto et al. (1992) estimated that the slope of the relationship between g_m (mol m^{-2} s^{-1} bar-1) and the rate of photosynthesis at saturating irradiance, A_{sat} (μmol m^{-2} s^{-1}) was 0.025 (i.e. $A_{sat} = 40\ g_m$). However, the magnitude of this slope in our study was 3.45 times greater. Grassi and Magnani (2005) estimated that the slope of the relationship
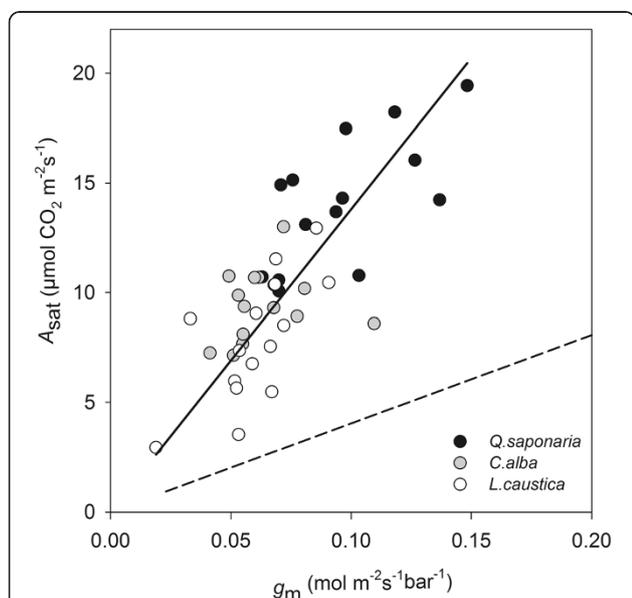

Figure 4 The relationship between Asat and g_m. A_{sat} stands for the rate of photosynthesis at saturating irradiance and ambient CO$_2$ concentration; and, g_m for mesophyll conductance. The A_{sat}/g_m linear relationship was highly significant ($A_{sat} = 2.4 + 109\ g_m$, $r^2 = 0.6$, P < 0.001; without intercept: $A_{sat} = 138\ g_m$). Intercepts ($F_{2,38} = 8.07$, P < 0.01) but not slopes ($F_{2,38} = 1.6$, P = 0.21) of the A_{sat}/g_m linear relationships differed between species. Dashed-lines are $A_{sat} = 40\ g_m$, $r^2 = 0.89$, determined for 15 angiosperms species by Loreto et al. (1992).

between g_m and A_{sat} was 0.0132 (i.e. $A_{sat} = 75.7\ g_m$) for oak trees, being our slope 1.8 times greater. This confirms that mesophyll conductance strongly limited the photosynthetic rates in the sclerophyllous species included in our study, compared to other plant groups; although they compare well with other sclerophyllous species.

The 'constant *J* method' used to estimate g_m is sensitive to errors in both the rate of mitochondrial respiration in the light, R_d^*, and the chloroplastic CO$_2$ compensation concentration in the absence of mitochondrial respiration, Γ^*. Values of Γ^* in our study were very similar across sclerophyllous species ($F_{2,41} = 0.26$, $P = 0.78$), with a mean value of 50.7 μmol mol^{-1} (Table 1). In contrast, R_d^*, was significantly lower ($F_{2,41} = 9.87$, $P < 0.001$) in *L. caustica* (1.0 μmol CO$_2$ m^{-2} s^{-1}) compared to *C. alba* (1.7 μmol CO$_2$ m^{-2} s^{-1}) and *Q. saponaria* (1.5 μmol CO$_2$ m^{-2} s^{-1}) (Table 1).

A recent paper by Gu and Sun (2014) suggests that the so-called Laisk method to estimate Γ^* and R_d^* seems to be invalid, and hence an alternative estimate for g_m in our study would reduce the uncertainty related to our results. We tested whether our results for g_m withhold when using values found in the literature for Γ^* and R_d^*. We used values for tobacco from Brooks and Farquhar (1985) (Set 1: $R_d = 0.8$ μmol m^{-2} s^{-1}, $\Gamma^* = 36.9$ μmol mol^{-1}) and for spinach from Bernacchi et al. (2001) (Set 2: $R_d = 1$ μmol m^{-2} s^{-1}, $\Gamma^* = 42.8$ μmol mol^{-1}), which have been commonly used to calculate g_m for evergreen species (Gallé et al. 2011; Hassiotou et al. 2009; Niinemets et al. 2009a; Niinemets et al. 2009b). We did not find significant differences within each species when comparing the three estimates of g_m i.e. using our estimates using the Laisk method, Set 1 and Set 2 (*Q. saponaria*: $P = 0.48$, *C. alba*: $P = 0.38$, *L. caustica*: $P = 0.12$). Tomas et al. (2013a, b) performed a similar analysis for deciduous, semideciduous and evergreen trees and herbs plants, obtaining similar values of g_m independent of the values chosen for Γ^* y R_d^*. This shows that our results hold independent of having used the Laisk method to estimate Γ^* y R_d^*.

Biochemical limitations to photosynthesis

Values of V_{cmax} (range 13–66, mean 36 μmol m^{-2} s^{-1}) and J_{max} (range 33–148, mean 82 μmol m^{-2} s^{-1}) in our study were within the range compiled for 109 C$_3$ plant species by Wullschleger (1993) (V_{cmax}: range 6–194, mean 64 μmol m^{-2} s^{-1}; J_{max}: range 17–372, mean 134 μmol m^{-2} s^{-1}). Wullschleger (1993) also provided specific values of V_{cmax} (range 11–119, mean 47 μmol m^{-2} s^{-1}) and J_{max} (range 29–237, mean 104 μmol m^{-2} s^{-1}) for temperate hardwoods and sclerophyllous species (V_{cmax}: range 35–71, mean 53 μmol m^{-2} s^{-1}; J_{max}: range 94–167, mean 122 μmol m^{-2} s^{-1}); which are similar to the ones found in our study. Pena-Rojas et al. (2004) observed values of 29 μmol CO$_2$ m^{-2} s^{-1} and 59 μmol electrons m^{-2} s^{-1} for V_{cmax} and J_{max}, respectively for *Quercus ilex*. Niinemets

et al. (2009b) observed values of 37 μmol CO_2 m^{-2} s^{-1} and 89 μmol electrons m^{-2} s^{-1} for V_{cmax} and J_{max}, respectively for different Australian sclerophyllous species. This emphasizes the fact that photosynthetic capacity in our study was more limited by g_m than by biochemical limitations, as our values of V_{cmax} and J_{max} were greater than those reported by Pena-Rojas et al. (2004) and Niinemets et al. (2009b) and comparable to averages for C_3 hardwoods.
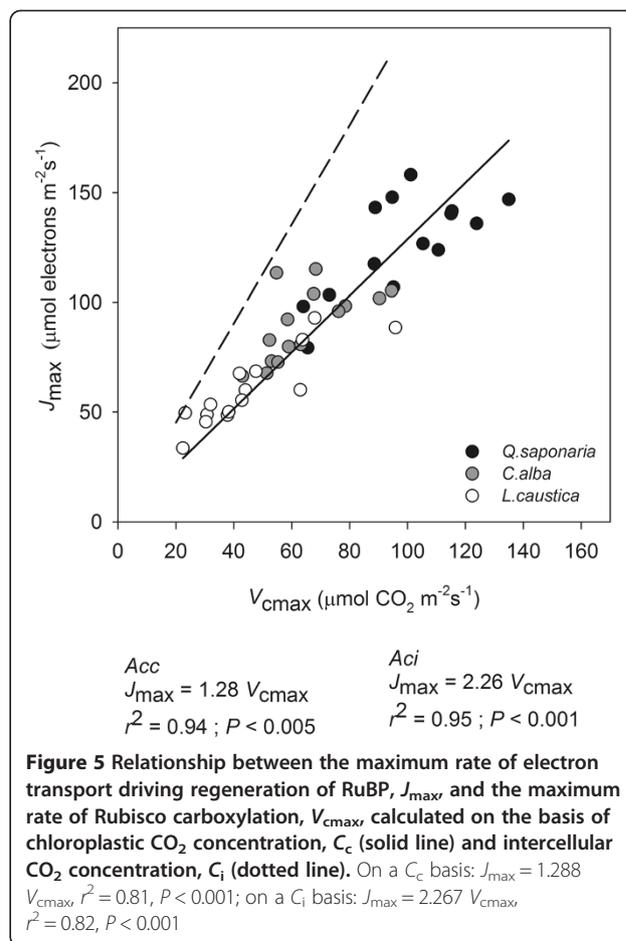
The maximal rate of Rubisco carboxylation (V_{cmax}) calculated on a C_i basis, were significantly greater in *Q. saponaria* (49.0 μmol CO_2 m^{-2} s^{-1}) compared to *C. alba* (32.6 μmol CO_2 m^{-2} s^{-1}) and *L. caustica* (26.8 μmol CO_2 m^{-2} s^{-1}) (Table 1). Corresponding values of V_{cmax} on a C_c basis were 98.3, 64.4 and 45.5 μmol CO_2 m^{-2} s^{-1}, respectively. Therefore, values of V_{cmax} calculated on a C_c basis were 101%, 97% and 70% greater than those on a C_i basis for *Q. saponaria*, *C. alba* and *L. caustica*, respectively.

Similar to V_{cmax}, the maximal rate of electron transport driving regeneration of RuBP (J_{max}) calculated on a C_i basis, were significantly greater in *Q. saponaria* (111.4 μmol electrons m^{-2} s^{-1}) compared to *C. alba* (79.6 μmol electrons m^{-2} s^{-1}) and *L. caustica* (56.3 μmol electrons m^{-2} s^{-1}) (Table 1). Corresponding values of J_{max} on a C_c basis were 126.4, 89.9 and 60.4 μmol electrons m^{-2} s^{-1}, respectively. Therefore, values of J_{max} were 13%, 13% and 7% greater on a C_c than a C_i basis for *Q. saponaria*, *C. alba* and *L. caustica*, respectively.

Although there were significant differences in values of J_{max} and V_{cmax} among species, the J_{max}/V_{cmax} ratio was constant both on a C_i (2.29 ± 0.05) and C_c (1.38 ± 0.04) basis across species (Table 1). Similarly, the relationship between J_{max} and V_{cmax} was highly significant both on a C_i ($J_{max} = 2.267\ V_{cmax}$, $r^2 = 0.82$, $P < 0.001$) and C_c ($J_{max} = 1.288\ V_{cmax}$, $r^2 = 0.81$, $P < 0.001$) basis (Figure 5).

Most values of V_{cmax} and J_{max} reported in the literature are calculated from A/C_i response curves, rather than A/C_c curves, with the implicit assumption that mesophyll conductance is infinitely large. When this assumption is invalid, values of V_{cmax} and J_{max} are underestimated (Ethier and Livingston 2004; Ethier et al. 2006; Grassi and Magnani 2005; Harley et al. 1992; Long and Bernacchi 2003; Loreto et al. 1992; Manter and Kerrigan 2004; von Caemmerer 2000; Niinemets et al. 2009a). To prove this assumption Grassi and Magnani (2005) showed that the relationship between $V_{cmax,ci}$ and $V_{cmax,cc}$ values result in a slope of 1.62 ($r^2 = 0.94$), showing that the C_i calculation underestimated the real photosynthetic capacity of the leaf. This relation is similar to the one found in our study, which resulted in a slope of 1.28 ($r^2 = 0.76$).

There is mounting evidence that g_m changes with chloroplastic CO_2 concentration and irradiance, although some of that variation can be an artifact due to the mathematical methods employed (Gu and Sun 2014). Also

Figure 5 Relationship between the maximum rate of electron transport driving regeneration of RuBP, J_{max}, and the maximum rate of Rubisco carboxylation, V_{cmax}, calculated on the basis of chloroplastic CO_2 concentration, C_c (solid line) and intercellular CO_2 concentration, C_i (dotted line). On a C_c basis: $J_{max} = 1.288$ V_{cmax}, $r^2 = 0.81$, $P < 0.001$; on a C_i basis: $J_{max} = 2.267\ V_{cmax}$, $r^2 = 0.82$, $P < 0.001$

Tholen et al. (2012) suggests there are limitations in the precise estimate of g_m when taking several A values from varying CO_2 concentrations and rates of photorespiration to estimate g_m. Taking aside artefactual responses, it seems that g_m initially increases, then peaks to decline thereafter with increasing C_c; while g_m seems to increase with irradiance for the same level of C_c (Flexas et al. 2007). We are aware that converting A/C_i into A/C_c curves assuming a constant g_m can be invalid; but still useful for comparing our results with previous studies. For instance, the parameters and results found in our study were similar for the photosynthesis model stated by Farquhar et al. (1980) with model parameters in normal scenarios of $V_{cmax} \sim 50$ μmol CO_2 m^{-2} s^{-1}, $J_{max} \sim 125$ μmol electrons m^{-2} s^{-1}, and dark respiration rates ~ 1.25 μmol CO_2 m^{-2} s^{-1}. These values were approximate to an 'average' C_3 leaf based on C_c-derived estimates and in the range of the results for the three sclerophyllous species of our study; therefore emphasizing that our species were more limited by g_m that biochemical parameters. We are also aware that using 'standard' Rubisco kinetics from tobacco to calculate g_m, V_{cmax} and J_{max} may lead to biases (Walker et al. 2013) to parameterize photosynthesis in the sclerophyllous

species considered in this study. This emphasizes the need for future work to estimate Rubisco kinetics for sclerophyllous species in central Chile.

Foliar nitrogen and photosynthetic parameters

Photosynthetic rates are known to be closely related to foliar nitrogen concentrations (Field and Mooney 1986; Walcroft *et al.* 1997; Grassi *et al.* 2002; Ripullone *et al.* 2003). This is explained by the high proportion of total nitrogen partitioned to the carboxylating enzyme Rubisco (Sage and Pearcy 1987; Evans 1989; Warren and Adams 2002; Takashima *et al.* 2004) and also by the strong coupling effect among capacities V_{cmax} and J_{max} (von Caemmerer and Farquhar 1981; Wullschleger 1993; Sharkey 1985).

At the plant level, observed N_a ranged almost sixfold from 54 to 339 mmol N m^{-2} being significantly greater in *C. alba* (195.5 mmol m^{-2}) compared to *Q. saponaria* (165.8 mmol m^{-2}) and *L. caustica* (120.2 mmol m^{-2}) (Table 1). Values of g_m were uncorrelated to N_a ($r^2 = 0.001$, $P = 0.40$); but as expected values of V_{cmax} and J_{max} on a C_i basis significantly increased with N_a ($F_{1,38} > 5.95$, $P < 0.019$). Intercepts ($F_{2,38} > 31.3$, $P < 0.001$) but not slopes ($F_{2,38} < 1.42$, $P > 0.25$) of the V_{cmax}/N_a and J_{max}/N_a linear relationships were significantly different between species ($V_{cmax} = a + 0.075\ N_a$, with $a = 17.728$ for *L. caustica*, $a = 32.489$ for *C. alba*, $a = 39.204$ for *Q. saponaria*, $r^2 = 0.61$, $P < 0.001$; $J_{max} = a + 0.149\ N_a$, with $a = 38.349$ for *L. caustica*, $a = 85.976$ for *C. alba*, $a = 101.296$ for *Q. saponaria*, $r^2 = 0.62$, $P < 0.001$). It is worth noting that *L.caustica* exhibited the lowest intercept followed by *C. alba* and *Q. saponaria* consistently for both the V_{cmax}/N_a and the J_{max}/N_a relationships which can be attributed to reasons other than foliage N (i.e. foliage N was accounted for in the slope of these relationships). We then may speculate that intercept differences among species can be attributed to distinct leaf N investment strategies. Consistently we found significant differences in Nitrogen use efficiency (E_n), being greatest in *Q. saponaria* (95.9 μmol CO$_2$ mol N s^{-1}), compared to *L. caustica* (69.8 μmol CO$_2$ mol N s^{-1}) and *C. alba* (56.4 μmol CO$_2$ mol N s^{-1}) (Table 1). Such differences in E_n, may suggest that *Q. saponaria*, that exhibits a greater photosynthetic capacity, may invest proportionally more N to Rubisco compared to *L. caustica* and *C. alba* that exhibit poorer photosynthetic performance.

Conclusions

In conclusion, values of stomatal conductance, g_s, were greater than those of mesophyll conductance, g_m, while their ratio (g_m/g_s) was not influenced by species, being on average 0.47. Therefore, the relative limitations imposed by g_m were high ($L_m \sim 0.40$, C_i-$C_c \sim 131$ μmol mol^{-1}) compared to those imposed by g_s ($L_s \sim 0.07$, C_a-$C_i \sim 32$ μmol mol^{-1}). Consequently photosynthetic rates in our study were mainly limited by

g_m as biochemical limitations V_{cmax} and J_{max} compare well to averages for C$_3$ plants. Photosynthetic performance was in the series: *Q. saponaria* > *C. alba* ≥ *L. caustica* which can be attributed first to mesophyll conductance limitations, probably mediated by leaf anatomical traits and then to species specific foliage N partitioning strategies.

Appendix

Abbreviations used throughout the text can be found in Table 2.

Table 2 Abbreviations

Symbol	Description	Units
A_{sat}	light-saturated rate of photosynthesis at ambient C_a	μmol m^{-2} s^{-1}
R_{dark}	respiration rate at zero irradiance	μmol m^{-2} s^{-1}
Q_{sat}	Light saturation point determined as the Q value for which assimilation is 90% of Asat	μmol m^{-2} s^{-1}
Q_{comp}	Light compensation point determined as the Q value for which the rate of photosynthesis exactly matches the rate of respiration	μmol m^{-2} s^{-1}
α	apparent maximum quantum efficiency	mol mol^{-1}
C_a	CO$_2$ concentration in the air outside the leaf	μmol mol^{-1}
C_i	CO$_2$ concentration in intercellular air spaces	μmol mol^{-1}
C_c	CO$_2$ concentration in the chloroplast	μmol mol^{-1}
Γ^*	chloroplastic CO$_2$ compensation point	μmol mol^{-1}
C_i^*	intercellular CO$_2$ compensation point in the absence of day respiration	μmol mol^{-1}
R_d^*	rate of mitochondrial respiration in the light	μmol m^{-2} s^{-1}
E_N	instantaneous photosynthetic nitrogen use efficiency	μmol mol^{-1} s^{-1}
L_s	relative stomatal (plus boundary layer) limitation to photosynthesis	%
L_m	relative mesophyll limitations to photosynthesis	%
g_s	stomatal (plus boundary layer) conductance to CO$_2$ diffusion	mol m^{-2} s^{-1}
g_m	Mesophyll transfer conductance to CO$_2$ diffusion	mol m^{-2} s^{-1} bar^{-1}
V_{cmax}	*in vivo* maximum rate of ribulose-1, 5-*bis*phosphate (RuBP) carboxylase-oxygenase (Rubisco) carboxylation	μmol CO$_2$ m^{-2} s^{-1}
J_{max}	electron transport driving regeneration of RuBP	μmol electrons m^{-2} s^{-1}
A_{max}	the rate of photosynthesis near saturating C_i (800 μmol mol^{-1})	μmol CO$_2$ m^{-2} s^{-1}
N_a	foliage nitrogen concentration on an area basis	mmol m^{-2}
M	Leaf area to mass ratio	m^2 kg^{-1}
VPD	Leaf-to-air vapour pressure deficit	kPa

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