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Leaf anatomical properties in relation to differences in mesophyll conductance to CO_2 and photosynthesis in two related Mediterranean *Abies* species

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ABSTRACT

***Abies alba* and *Abies pinsapo* are closely related species with the same ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (*rbc*L) but contrasting hydraulic traits and mesophyll structure occurring in the Iberian Peninsula under contrasting conditions. As photosynthesis and hydraulic capacities often co-scale, we hypothesize that these species differ in mesophyll conductance to CO_2 (g_m). g_m and key anatomical traits were measured in both species. Drought-adapted population of *A. pinsapo* has higher photosynthesis than the more mesic population of *A. alba*, in agreement with its higher hydraulic capacity. However, *A. alba* exhibits the largest stomatal conductance (g_s), and so water use efficiency (WUE) is much higher in *A. pinsapo*. The differences in photosynthesis were explained by differences in g_m, indicating a correlation between hydraulic capacity and g_m. We report a case where g_m is the main factor limiting photosynthesis in one species (*A. alba*) when compared with the other one (*A. pinsapo*). The results also highlight the discrepancy between g_m estimates based on anatomical measurements and those based on gas exchange methods, probably due to the very large resistance exerted by cell walls and the stroma in both species. Thus, the cell wall and chloroplast properties in relation to CO_2 diffusion constitute a near-future research priority.**

Key-words: cell wall; chloroplast thickness; water use efficiency.

INTRODUCTION

Abies alba Mill. and *Abies pinsapo* Boiss. are included in a group of species called 'Mediterranean firs' (Aussenac 2002), which constitute a strongly supported monophyletic group (Scaltsoyiannes, Tsaktsira & Drouzas 1999; Xiang *et al*. 2009). In this way, Suyama, Yoshimaru & Tsumura (2000) found that the Mediterranean *Abies* species do not contain sequence divergence for the gene encoding the ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (*rbc*L), including the catalytic site. However, *A. alba* and *A. pinsapo* occur in the Iberian Peninsula under contrasting climatic conditions (Peguero-Pina *et al*. 2011). *A. alba* is always associated to humid and cold climates of the Pyrenees (Peguero-Pina *et al*. 2007), whereas *A. pinsapo* is restricted to the south-western Baetic mountain range under a Mediterranean-type climate with a dry summer season (Fernández-Cancio *et al*. 2007). Thus, in contrast to *A. alba*, *A. pinsapo* must cope with a relatively severe drought period, when the precipitation reaches a minimum and the evaporative demand reaches a maximum. Moreover, Peguero-Pina *et al*. (2011) reported higher water transport efficiency to the transpiring needles through the stem and the shoot in *A. pinsapo* than in *A. alba*, which could partly explain the niche segregation and the geographical separation of these two firs.

Taken together, all these observations could constitute somehow a paradox. On one hand, the catalytic properties of Rubisco, which are mainly determined by the structure of the *rbc*L (Galmés *et al*. 2005), should be the same for these species. For this reason, the maximum potential photosynthesis may be similar for both species, although certainly other differences such as in Rubisco content could yield differences in the potential photosynthesis. On the other hand, recent studies demonstrate strong interspecific correlations between hydraulic and photosynthetic traits (Brodribb *et al*. 2005; Sack & Holbrook 2006; Brodribb, Field & Jordan 2007; Brodribb 2009). Thus, based on the findings of Peguero-Pina *et al*. (2011), higher photosynthetic rates in *A. pinsapo* would be expected if these two species follow such general relationship.

Irrespective of both genetic and hydraulic traits considered above, net CO_2 assimilation is known to be influenced by CO_2 transfer resistance from ambient air to carboxylation sites in chloroplasts (Syvertsen *et al*. 1995; Patakas,

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Kofidis & Bosabalidis 2003). Specifically, the mesophyll conductance to CO_2 (g_m), which is often intrinsically co-regulated with the stomatal conductance (g_s) (Flexas *et al*. 2008), can also be influenced by changes in leaf anatomical characteristics, for example, the leaf thickness, the packing relative to the position of stomata, the chloroplast surface area facing intercellular air spaces (S_c), the thickness of the mesophyll cell walls (Hanba, Kogami & Terashima 2002; Evans *et al*. 2009; Kodama *et al*. 2011; Scafaro *et al*. 2011; Terashima *et al*. 2011; Tholen & Zhu 2011) and the thickness of chloroplasts (Tosens *et al*. 2012a). It should be noted that the mesophyll is a complex structure that varies greatly between species, especially in conifers with three-dimensional foliage cross-section (Niinemets 1999; Niinemets *et al*. 2007). In this regard, previous observations indicated that populations of *A. alba* and *A. pinsapo* showed clear differences in their mesophyll structure despite their genetic proximity. Thus, a more densely packed mesophyll per unit leaf area has also been demonstrated to result in greater assimilation rates (Niinemets *et al*. 2009) and might constitute another reason for greater photosynthetic capacity in *A. pinsapo*. In spite of this, the possible existence of differences in g_m between *A. alba* and *A. pinsapo* could justify by itself the apparent paradox exposed above.

Based on this assumption, we hypothesize that *A. pinsapo* would show higher values of g_m and, as a consequence, higher assimilation rates than *A. alba*, which agrees with the differences found in water transport ability between these species (Peguero-Pina *et al*. 2011). To discriminate among these hypothesis, we measured g_m, $V_{c,max}$ and key anatomical parameters responsible for g_m in one representative population of *A. alba* and one representative population of *A. pinsapo*. The measurements were done at the end of the spring, with moderate temperatures and in absence of water stress (Peguero-Pina *et al*. 2011). Therefore, the specific objectives of this work are: (1) to find a link between hydraulic and photosynthetic traits in these *Abies* species; (2) to show that this link is influenced by changes in g_m and differences in mesophyll structure; and (3) to clarify which of several anatomical parameters considered (e.g. intercellular air spaces, cell wall thickness, chloroplast distribution and thickness) is the most determinant factor to set the maximum value of g_m for these species.

MATERIALS AND METHODS

Study sites

We selected a stand located in the western Spanish Pyrenees (Gamueta, 42°52′ N, 0°49′ W, 1350 m a.s.l.) for *A. alba*, whereas *A. pinsapo* was measured in a naturalized reforested population which dates from 1913 (Pérez-Soba Diez del Corral 2010) at the southern 'Sistema Ibérico' range (Orcajo, 41°05′ N, 1°30′ W, 1150 m a.s.l.). Precipitation was higher in Gamueta (*A. alba*), whereas temperature and atmospheric vapour pressure deficit were higher in Orcajo (*A. pinsapo*) (see meteorological data in Peguero-Pina *et al*. 2011).

Gas exchange and chlorophyll fluorescence measurements

Needle gas exchange parameters were measured simultaneously with measurements of chlorophyll fluorescence using an open gas exchange system (CIRAS-2, PP-Systems, Amesbury, MA, USA) fitted with an automatic conifer cuvette (PLC-C, PP-Systems) with an FMS II portable pulse amplitude modulation fluorometer (Hansatech Instruments Ltd, Norfolk, UK). Six CO_2-response curves were obtained from different trees for each species. In light-adapted mature shoots, photosynthesis was stabilized at a cuvette CO_2 concentration (C_a) of 400 μmol mol^{-1}, and a photosynthetic photon flux density (PPFD) of 1500 μmol m^{-2} s^{-1}. Such light intensity was previously determined to be just above light saturation for both species (data not shown). Needle temperature was maintained at 25 °C during all measurements. Once steady state gas-exchange rates were reached (usually 30 min after clamping the leaf), a CO_2-response curve was determined. Gas exchange and chlorophyll fluorescence were first measured at a C_a of 400 μmol mol^{-1}, and then C_a was decreased stepwise down to 50 μmol mol^{-1}. Upon completion of measurements at low C_a, the shoot was returned to 400 μmol mol^{-1} to restore the original value of net CO_2 assimilation (A_N). Then, C_a was increased stepwise to 1800 μmol mol^{-1}. Leakage of CO_2 in and out of the cuvette was determined for the same range of CO_2 concentrations with a photosynthetically inactive shoot enclosed (obtained by heating the shoot until no variable chlorophyll fluorescence was observed), and used to correct measured leaf fluxes (Flexas *et al*. 2007a).

In addition to A_N and stomatal conductance (g_s), the incorporated fluorometer allowed determination of the photochemical efficiency of photosystem II (Φ_{PSII}) in light-adapted stage. This was determined by measuring steady-state fluorescence (F_S) and maximum fluorescence during a light-saturating pulse of ca. 8000 μmol m^{-2} s^{-1} (F'_M), and was calculated as $(F'_M - F_S)/F'_M$, following the procedures of Genty, Briantais & Baker (1989). The electron transport rate (J_{flu}) was then calculated according to Krall & Edwards (1992), multiplying Φ_{PSII} by PPFD and by α (a term which includes the product of leaf absorptance and the partitioning of absorbed quanta between photosystems I and II). α was previously determined for each species as the slope of the relationship between Φ_{PSII} and Φ_{CO2} (i.e. the quantum efficiency of CO_2 fixation) obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing <1% O_2 (Valentini *et al*. 1995). Five light curves per species were recorded to determine α.

Estimation of g_m by gas exchange and chlorophyll fluorescence

Estimations of g_m for *A. alba* and *A. pinsapo* were performed using the method of Harley *et al*. (1992), as follows:

$$g_m = A_N/(C_i - (\Gamma^*(J_{flu} + 8(A_N + R_l))/(J_{flu} - 4(A_N + R_l)))) \quad (1)$$

where A_N (net CO_2 assimilation) and C_i (substomatal CO_2 concentration) were taken from gas- exchange measurements at saturating light, whereas Γ^* (the chloroplastic CO_2 photocompensation point in the absence of mitochondrial respiration) and R_l (the respiration in light) were estimated for each species using the Laisk (1977) method, following the methodology described in Flexas *et al.* (2007c). Although this method allows the calculation of g_m across the full range of CO_2 concentrations, we have fitted an average g_m value over a C_i range (150–400 μmol mol^{-1}) that best describes the entire curve (Flexas *et al.* 2007b). Calculated g_m values for each CO_2 concentration were used to convert A_N–C_i into A_N–C_c curves (where C_c is the chloroplastic CO_2 concentration) using the equation $C_c = C_i - (A_N / g_m)$. The maximum carboxylation and J_{flu} capacities ($V_{c,max}$ and J_{max}, respectively) were calculated from the A_N–C_c curves, using Rubisco kinetic constants and the temperature dependence of Rubisco kinetic parameters described on a C_c basis by Bernacchi *et al.* (2002). The Farquhar, von Caemmerer & Berry (1980) model was fitted to the data by applying iterative curve-fitting (minimum least-square difference) using Microsoft Excel Solver tool.

Estimation of g_m by a curve-fitting method

The estimation of g_m according to Ethier & Livingston (2004) is based on fitting $A_N - C_i$ curves with a non-rectangular hyperbola version of Farquhar's biochemical model of leaf photosynthesis (Farquhar *et al.* 1980). This is based on the hypothesis that g_m reduces the curvature of the Rubisco-limited portion of an $A_N - C_i$ response curve. The method has been successfully used in several studies, showing good agreement with other independent estimates of g_m (Niinemets *et al.* 2005; Warren & Dreyer 2006; Flexas *et al.* 2007b; Kodama *et al.* 2011). Values of the Michaelis–Menten constant for CO_2 (K_c), and oxygen (K_o) and Γ^* and their temperature responses used for these estimations, were obtained from the C_c-based *in vivo* values of Bernacchi *et al.* (2002). The C_i cut-off point was determined based on the method proposed by Ethier *et al.* (2006).

Estimation of g_m by anatomical measurements

After the gas-exchange measurements, cross-sections of 1 mm × 3 mm were cut from *A. alba* and *A. pinsapo* needles and fixed in 2% p-formaldehyde (2%) and glutaraldehyde (4%) in 0.1 M phosphate buffer solution (pH 7.2) for 24 h under vacuum at 4 °C and post-fixed for 2 h in 2% osmium tetroxide. Samples were dehydrated in: (1) a graded ethanol series and (2) propylene oxide and subsequently embedded in Embed-812 resin (EMS, Hatfield, PA, USA). A sliding microtome (Microm HM350S, Thermo Scientific, Walldorf, Germany) was used to obtain transverse sections of ca. 1 μm thick, which were stained by 1% toluidine blue in 1% sodium borate buffer (pH = 9.1) for light microscopy (Optika B-600TiFL, Optika Microscopes, Bergamo, Italy). Anatomical characteristics were studied from the resulting micrographs, which were analysed using Image-J software (http://rsb.info.nih.gov/nih-image/). Light microscopy images were used to determine: (1) the mesophyll thickness (I, μm); (2) the fraction of the mesophyll tissue occupied by the intercellular air spaces (f_{ias}) (Patakas *et al.* 2003); and (3) the chloroplast surface area facing intercellular air spaces per unit leaf area (S_c/S) (Syvertsen *et al.* 1995).

Another set of *A. alba* and *A. pinsapo* needles were observed with a low temperature scanning electron microscope (LTSEM, DSM 960 Zeiss, Jena, Germany, acceleration potential 15 kV, working distance 10 mm and probe current 5–10 nA). Fresh transverse sections taken after gas-exchange measurements were frozen in liquid N, gold sputtered and subsequently observed by this microscopic technique. The resulting images were used to measure cell wall thickness (ΔL_{cw}, μm), the average distance of the cell wall and the closest chloroplast (ΔL_{ct}, μm), and the diffusion path length in the stroma (ΔL_{st}, μm) of the mesophyll cells.

These anatomical parameters were used to estimate the mesophyll diffusion conductance according the one-dimensional within-leaf gas diffusional model of Niinemets & Reichstein (2003) as applied by Tosens *et al.* (2012a). The mesophyll diffusion conductance as a composite conductance for within-leaf gas, liquid and lipid components is given as:

$$g_m = \frac{1}{\frac{1}{g_{ias}} + \frac{RT_k}{H \cdot g_{liq+lip}}} \quad (2)$$

where g_{ias} is gas phase conductance inside the leaf from sub-stomatal cavities to outer surface of cell walls, $g_{liq+lip}$ is the conductance in liquid and lipid phases from outer surface of cell walls to chloroplasts, R is the gas constant (Pa m^3 K^{-1} mol^{-1}) and T_k is the absolute temperature (K), and H is the Henry's law constant (m^3 mol^{-1} K^{-1}). g_m is defined as a gas-phase conductance, and thus, $H/(RT_k)$ is the dimensionless form of Henry's law constant that is needed to convert the $g_{liq+lip}$ to corresponding gas-phase equivalent conductance (Niinemets & Reichstein 2003). The estimation of g_{ias} was obtained according to Niinemets & Reichstein (2003) as:

$$g_{ias} = (D_A f_{ias})/(\Delta L_{ias} \tau) \quad (3)$$

where D_A is the diffusivity of the CO_2 in the air (1.51×10^{-5} m^2 s^{-1} at 25 °C), f_{ias} is the fraction of intercellular air spaces, ΔL_{ias} (m) is the effective diffusion path length and τ is the diffusion path tortuosity (1.57 m m^{-1}, Syvertsen *et al.* 1995). We considered ΔL_{ias} as the half of the mesophyll thickness for *A. alba* and the average thickness of mesophyll ellipsoid up to the surface of the central cylinder for *A. pinsapo*, which shows a three-dimensional foliage cross-section with a big central cylinder, such as those species studied in Niinemets *et al.* (2007). On the other hand, $g_{liq+lip}$ was calculated as the sum of the inverse of serial conductances:

$$\frac{1}{g_{liq+lip}} = \left(\frac{1}{g_{cw}} + \frac{1}{g_{pl}} + \frac{1}{g_{ct}} + \frac{1}{g_{en}} + \frac{1}{g_{st}} \right) S/S_c \quad (4)$$

where S_c/S is the exposed chloroplast to leaf area ratio, and the partial conductances are: g_{cw} for cell wall, g_{pl} for plasmalemma, g_{ct} for cytosol, g_{env} for chloroplast envelope and g_{str} for chloroplast stroma. The different components of liquid phase diffusion pathway (g_i, where i stands either for cell wall, cytosol or stroma conductance) were calculated as:

$$g_i = \frac{r_{f,i} \cdot D_w \cdot p_i}{\Delta L_i} \quad (5)$$

where ΔL_i (m) is the diffusion path length in the corresponding component of the diffusion pathway, p_i ($m^3\ m^{-3}$) is its effective porosity, and D_w is the aqueous phase diffusion coefficient for CO_2 ($1.79 \times 10^{-9}\ m^2\ s^{-1}$ at 25 °C). The dimensionless factor $r_{f,i}$ accounts for the reduction of D_w compared with free diffusion in water (Weisiger 1998), and was taken 1.0 for cell walls (Rondeau-Mouro *et al*. 2008) and 0.3 for cytosol and stroma (Niinemets & Reichstein 2003). p_i of cytosol and stroma was set 1.0 (Nobel 1991). p_i of cell wall was set 0.055 for *A. alba* and 0.090 for *A. pinsapo*, according to the method of variable p_i approach (Tosens *et al*. 2012b). We used an estimate of 0.0035 m s^{-1} for both g_{pl} and g_{en} as previously suggested (Tosens *et al*. 2012a).

Analysis of quantitative limitations of A_N

To separate the controls on A_N resulting from limited stomatal conductance (l_s), mesophyll diffusion (l_m) and limited photosynthetic capacity (l_b), we used the quantitative limitation analysis of Grassi & Magnani (2005). The limitations of the different components, l_s, l_m and l_b ($l_s + l_m + l_b = 1$) were calculated as:

$$l_s = \frac{g_{tot}/g_s \cdot \partial A_N/\partial C_c}{g_{tot} + \partial A_N/\partial C_c} \quad (6)$$

$$l_m = \frac{g_{tot}/g_m \cdot \partial A_N/\partial C_c}{g_{tot} + \partial A_N/\partial C_c} \quad (7)$$

$$l_b = \frac{g_{tot}}{g_{tot} + \partial A_N/\partial C_c} \quad (8)$$

where g_s is the stomatal conductance to CO_2, g_m is the mesophyll diffusion conductance according to Harley *et al*. (1992, Eq. 1) and g_{tot} is the total conductance to CO_2 from ambient air to chloroplasts (sum of the inverse serial conductances g_s and g_m). $\partial A_N/\partial C_c$ was calculated as the slope of $A_N - C_c$ response curves over a C_c range of 50–100 μmol mol^{-1}. At least five curves per species were used, and average estimates of the limitations were calculated.

Statistical analysis

Data are expressed as means ± standard error. Student's *t*-tests were used to compare the photosynthetic parameters obtained for *A. alba* and *A. pinsapo*. All statistical analyses were carried out using SAS version 8.0 (SAS, Cary, NC, USA).

RESULTS

The photosynthetic parameters of *A. alba* and *A. pinsapo* needles are shown in Table 1. At 400 μmol CO_2 mol^{-1} air and saturating light, A_N was much higher in *A. pinsapo* (11.54 μmol CO_2 m^{-2} s^{-1}) than in *A. alba* (7.35 μmol CO_2 m^{-2} s^{-1}), whereas g_s was somewhat higher in *A. alba* (0.116 mol CO_2 m^{-2} s^{-1}) than in *A. pinsapo* (0.087 mol CO_2 m^{-2} s^{-1}), so that intrinsic water use efficiency (WUE) (A_N/g_s) was much larger in *A. pinsapo* than in *A. alba*. The analysis of the quantitative limitations of photosynthesis revealed that A_N in *A. alba* was mainly limited by the mesophyll (l_m = 51%), whereas l_s and l_b accounted for only 30 and 19%, respectively. By contrast, A_N in *A. pinsapo* was mainly limited by the stomatal conductance (l_s = 45%), whereas l_m and l_b accounted for only 24 and 30%, respectively. The differences in assimilation rate were partly associated with the more densely packed mesophyll and greater leaf dry mass per unit area in *A. pinsapo* (ca. 35 g cm^{-2}) when compared with *A. alba* (ca. 15 g cm^{-2}). In fact, when the photosynthetic rate was expressed per unit dry mass, no statistically significant differences ($P < 0.05$) were found between *A. alba* (30.62 ± 2.25 μmol g^{-1} s^{-1}) and *A. pinsapo* (28.73 ± 1.88 μmol g^{-1} s^{-1}). The relationship between A_N and C_i further demonstrated a higher initial slope and

Table 1. Mean values for the photosynthetic parameters analysed

	Abies alba	*Abies pinsapo*
A_N (μmol CO_2 m^{-2} s^{-1})	7.35 ± 0.43 a	11.54 ± 0.93 b
g_s (mol CO_2 m^{-2} s^{-1})	0.116 ± 0.010 a	0.087 ± 0.011 b
A_N/g_s (μmol mol^{-1})	63.4 ± 3.7 a	132.7 ± 13.7 b
g_m Harley (mol CO_2 m^{-2} s^{-1})	0.068 ± 0.004 a	0.162 ± 0.032 b
g_m Ethier (mol CO_2 m^{-2} s^{-1})	0.081 ± 0.011 a	0.140 ± 0.025 b
A_N/g_m Harley (μmol mol^{-1})	108.1 ± 5.2 a	71.2 ± 3.0 b
A_N/g_m Ethier (μmol mol^{-1})	90.7 ± 4.1 a	82.4 ± 3.5 b
C_i (μmol CO_2 mol^{-1} air)	195 ± 8 a	181 ± 16 a
C_c (μmol CO_2 mol^{-1} air)	77 ± 5 a	98 ± 7 b
V_{c,max_Cc} Harley (μmol m^{-2} s^{-1})	117 ± 6 a	162 ± 20 b
V_{c,max_Cc} Ethier (μmol m^{-2} s^{-1})	113 ± 14 a	179 ± 18 b
J_{max_Cc} Harley (μmol m^{-2} s^{-1})	138 ± 10 a	213 ± 17 b
J_{max_Cc} Ethier (μmol m^{-2} s^{-1})	158 ± 14 a	222 ± 10 b
J_{flu} (μmol m^{-2} s^{-1})	134 ± 16 a	177 ± 23 b
J_{max_Cc}:V_{c,max_Cc} Harley	1.18 ± 0.04 a	1.31 ± 0.06 b
J_{max_Cc}:V_{c,max_Cc} Ethier	1.40 ± 0.07 a	1.25 ± 0.05 b

Data are mean ± SE. Different letters indicate statistically significant differences ($P < 0.05$) between *Abies alba* and *Abies pinsapo*. A_N, net photosynthesis; g_s, stomatal conductance; g_m, mesophyll conductance to CO_2; C_i, sub-stomatal CO_2 concentration; C_c, chloroplastic CO_2 concentration; V_{c,max_Cc}, maximum velocity of carboxylation calculated from gas exchange on a C_c basis; J_{max_Cc}, maximum capacity for electron transport calculated from gas exchange on a C_c basis; J_{flu}, electron transport rate estimated by chlorophyll fluorescence.

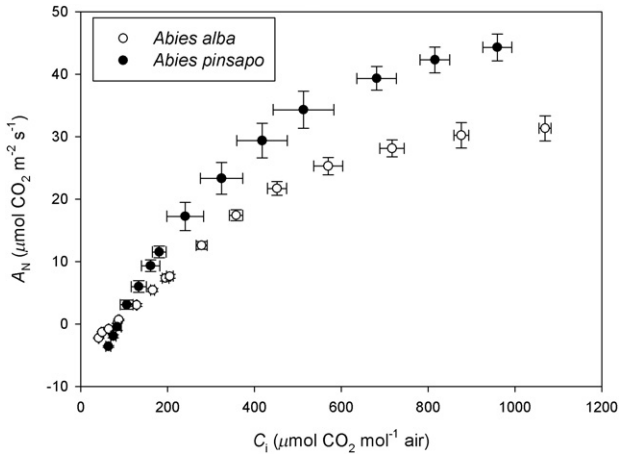

Figure 1. The relationship between A_N and C_i in *Abies alba* (open circles) and *Abies pinsapo* (closed circles) needles. Data are mean ± SE.

greater maximum net assimilation rate at given C_i in *A. pinsapo* than in *A. alba* (Fig. 1). Chloroplast CO_2 concentrations (C_c) and leaf mesophyll conductance to CO_2 (g_m) were calculated from combined gas-exchange and chlorophyll fluorescence data (Table 1). g_m and C_c values, which were estimated using the Harley *et al.* (1992) method and the curve-fitting method by Ethier & Livingston (2004), were significantly higher in *A. pinsapo*, irrespective of the method used (Table 1). It should be noted that, although denser packing of mesophyll in *A. pinsapo* resulted in higher photosynthetic capacity per unit leaf area, it did not result in a greater drawdown of CO_2 from sub-stomatal cavities to chloroplasts (A_N/g_m) (Table 1). Parameterization of the Farquhar *et al.* (1980) model of photosynthesis yielded statistically significant differences ($P < 0.05$) between both species (Table 1). Thus, V_{c,max_Cc}, J_{max_Cc} and the ratio J_{max_Cc}:V_{c,max_Cc} were somewhat higher in *A. pinsapo* than in *A. alba*, irrespective of the method used.

Both species showed contrasted morphological and anatomical features at shoot, mesophyll and cell levels (Fig. 2, Table 2). Thus, the needles were amphistomatous in *A. pinsapo* and hypostomatous in *A. alba*. The thickness of the mesophyll and S_c/S was significantly higher in *A. pinsapo*, whereas f_{ias} was significantly higher in *A. alba* (Table 2). Moreover, ΔL_{cw} and ΔL_{st} were significantly higher in *A. alba*, whereas no differences were found in ΔL_{ct} These parameters were used to obtain an estimation of the different components of the CO_2 transfer resistances relative to total mesophyll resistance (see Materials and Methods section for details). It should be noted that r_{ias} was much higher in *A. pinsapo* (Table 3), which was caused by the low value of f_{ias} found in this species (Table 2). Anyway, the contribution of r_{ias} to the total resistance can be considered negligible for both species when compared with $r_{liq+lip}$ (Table 3). Regarding the liquid and lipid phases, the results demonstrated that *A. alba* showed higher values of resistances for all the components considered, specially r_{cw} and r_{str} (Table 3). As a consequence, the estimated value of mesophyll conductance in *A. alba* was much lower than that estimated for *A. pinsapo* (Table 3), which agreed with the lower g_m values obtained for *A. alba* by the simultaneous measurement of gas exchange and chlorophyll fluorescence (Table 1).

DISCUSSION

As hypothesized based on its larger hydraulic conductivity (Peguero-Pina *et al.* 2011), net photosynthesis was significantly higher in leaves of *A. pinsapo* than *A. alba*, thus fulfilling the generalized positive correlations observed between these two parameters (Brodribb *et al.* 2007; Brodribb 2009). Surprisingly, however, the pattern followed by A_N was opposite to that of stomatal conductance (g_s), but strongly related to that of mesophyll conductance (g_m), supporting recent findings of a close positive correlation between plant hydraulic capacity and g_m (Ferrio *et al.* 2011). Having larger g_m and lower g_s than *A. alba*, the ratio g_m/g_s was much larger in *A. pinsapo*, which was reflected in a much higher intrinsic WUE (A_N/g_s), as already observed in *Picea* (Duan *et al.* 2009), *Vitis* (Flexas *et al.* 2010) and ramellet tomato (Galmés *et al.* 2011). It is noteworthy, however, that in almost all cases, increased g_m/g_s and WUE was accompanied by *lower* A_N. The fact that these factors in *A. pinsapo* are accompanied by a *higher* A_N demonstrates that it may be possible to improve simultaneously photosynthesis and WUE, which is a key objective of agriculture for dry environments (Parry, Flexas & Medrano 2005; Flexas *et al.* 2010). On the other hand, possessing higher photosynthesis and WUE suggests that *A. pinsapo* is a species better adapted to the drought environments where it lives than *A. alba*.

Although $V_{c,max}$ was also somewhat higher in *A. pinsapo* than *A. alba* – most likely a consequence of higher amounts of Rubisco and/or a higher activation state, as Rubisco genes in these two species do not differ in their sequence (Suyama *et al.* 2000) – most of the observed differences in A_N between the two species were due to differences in g_m, which was about double in *A. pinsapo* than in *A. alba*. To the best of our knowledge, this is the first report showing a case where g_m alone sets most of the limitations to photosynthesis for a given species (*A. alba*), as g_m and g_s are often co-ordinated/co-regulated, hence exerting a similar degree of limitation (Flexas *et al.* 2008). This finding highlights the importance of g_m as a limiting factor for plant photosynthesis (Niinemets *et al.* 2009; Zhu, Long & Ort 2010). Despite its importance, the nature and determinants of g_m are not well understood, although leaf structural characteristics are believed to play an important role in determining g_m (Evans *et al.* 2009; Terashima *et al.* 2011). Among these, Tholen & Zhu (2011) recently identified in developing a 3-D model for g_m the resistances of the cell wall and chloroplast envelope as the most important factors constraining g_m. This is consistent with the observation in some species of good correlations between g_m and the surface of chloroplasts exposed to intercellular air spaces – S_c – (Evans *et al.* 1994, 2009) and cell wall thickness (Hassiotou *et al.* 2010; Scafaro

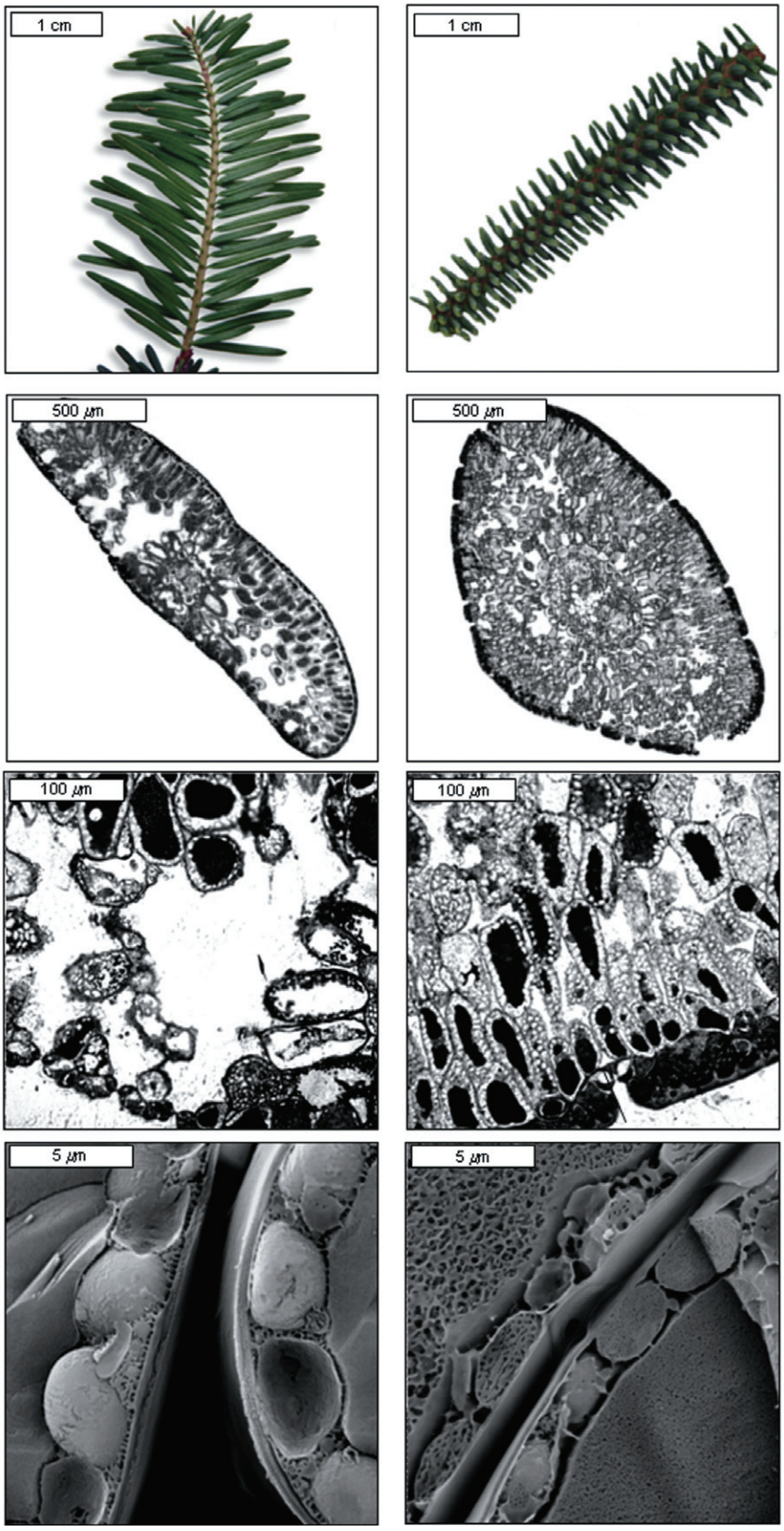

Figure 2. Shoot, transverse section of the mesophyll and mesophyll cells of *Abies alba* and *Abies pinsapo*.

Table 2. Needle type, mesophyll thickness (I, μm), fraction of the mesophyll tissue occupied by the intercellular air spaces (f_{ias}), cell wall thickness of the mesophyll (ΔL_{cw}, μm), the average distance of the gap between the cell wall and the closest chloroplast (ΔL_{ct}, μm), the diffusion path length in the stroma (ΔL_{st}, μm), and the chloroplast surface area facing intercellular air spaces (S_c/S, $m^2\ m^{-2}$) in *Abies alba* and *Abies pinsapo* needles

	Abies alba	*Abies pinsapo*
Needle type	Hypostomatous	Amphistomatous
I (μm)	436 ± 14 a	792 ± 16 b
f_{ias}	0.40 ± 0.05 a	0.10 ± 0.06 b
ΔL_{cw} (μm)	0.341 ± 0.014 a	0.248 ± 0.009 b
ΔL_{ct} (μm)	1.380 ± 0.025 a	1.115 ± 0.022 a
ΔL_{str} (μm)	1.850 ± 0.018 a	0.890 ± 0.016 b
S_c/S ($m^2\ m^{-2}$)	17.1 ± 1.4 a	32.3 ± 2.1 b

Data are mean ± SE. Different letters indicate statistically significant differences ($P < 0.05$) between *Abies alba* and *Abies pinsapo*.

et al. 2011). To assess which leaf structural trait was more limiting for g_m in the two *Abies* species, we performed a detailed microscopy analysis and estimated partial components of g_m – or its reciprocal, r_m – based on this analysis and assumed diffusion constants for CO_2 in each media, assuming no facilitation mechanism (Evans *et al*. 2009). The sum of cell wall and chloroplast resistances accounted for most of the total estimated resistance in both species (63% in *A. alba* and 53% in *A. pinsapo*). This finding reinforces recent results suggesting that both the cell wall and the stroma of the chloroplast exert the most important limitations to CO_2 diffusion inside leaves (Evans *et al*. 2009; Hassiotou *et al*. 2010; Scafaro *et al*. 2011; Terashima *et al*. 2011; Tholen & Zhu 2011; Tosens *et al*. 2012a). Overall, the sum of liquid and lipid phase resistance was much larger in *A. alba* than *A. pinsapo* due to the thicker cell wall and chloroplast of the former. As a consequence, the modelled g_m was higher in *A. pinsapo* than in *A. alba* (Table 3), which agreed with the higher g_m values measured in *A. pinsapo* (Table 1). Nevertheless, the mesophyll conductance modelled based on leaf anatomical properties was lower than that estimated using conventional methods in *A. alba* and *A. pinsapo*, respectively. This could be related to some biases of the model. For instance, the model has been applied over 2-D micrograph data, while g_m is regarded as an intrinsically 3-D property. Because of this, r_{ias} could be overestimated – simply because we estimate this resistance based on the shortest allowed ias path shown in the 2-D pictures, while on a 3-D scale there are possibly shorter paths for CO_2 diffusion in ias – but r_{cw} would not be affected by this bias – because cell wall must be crossed by CO_2 whatever direction it moves on. However, r_{cw} could be overestimated by other biases of the model, for instance if cell wall porosity and tortuosity significantly differ from the assumed values and/or between species. Alternatively, because modelled values were estimated assuming no facilitation process (i.e. based on pure CO_2 diffusivities for each media), the discrepancy between anatomical and gas-exchange estimates could indicate the existence of facilitating mechanisms in both species, but more developed in *A. pinsapo*. Aquaporins (co-oporins) and carbonic anhydrases have been proposed as facilitating mechanisms influencing g_m (Hanba *et al*. 2004; Flexas *et al*. 2006; Evans *et al*. 2009). These mechanisms may facilitate CO_2 diffusion in the stroma, which showed high modelled resistance values for both species. However, no facilitating mechanism for CO_2 diffusion in cell walls has been described. In view of its importance in setting maximum g_m, the cell wall and chloroplast properties in relation to CO_2 diffusion should be investigated in different species as a near-future research priority.

In summary, the present results demonstrate that – under the environmental conditions used in this study – drought-adapted population of *Abies pinsapo* has higher photosynthetic capacity than the more mesic population of *A. alba*, in agreement with its higher hydraulic capacity. However, it is the population of *A. alba* who presents the largest stomatal conductance, for which WUE is much higher in *A. pinsapo*, which may contribute to the habitat preferences of this species. This is one of the first reports showing that higher WUE in one species as compared with another is accompanied by higher photosynthetic capacity, and also the first to show g_m as the main factor limiting photosynthesis in one species (*A. alba*). It must be taken into account that these results may differ at varying temperatures naturally occurring in the field. The results also highlight the discrepancy between g_m estimates based on anatomical measurements and those based on conventional gas-exchange methods, suggesting that further efforts are needed to improve knowledge on CO_2 diffusion properties of different cellular components, particularly cell walls and chloroplasts, and/or to identify new facilitating mechanisms for CO_2 diffusion that could explain the observed differences.

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Table 3. CO_2 transfer resistances across the intercellular air space (r_{ias}, $s\ m^{-1}$), the cell wall (r_{cw}, $s\ m^{-1}$), the cytosol (r_{ct}, $s\ m^{-1}$), the chloroplast stroma (r_{str}, $s\ m^{-1}$), the liquid and lipid phases ($r_{liq+lip}$, $s\ m^{-1}$), and the CO_2 mesophyll conductance (g_m, $mol\ m^{-2}\ s^{-1}$) calculated from anatomical measurements in *Abies alba* and *Abies pinsapo* needles

	Abies alba	*Abies pinsapo*
r_{ias} ($s\ m^{-1}$)	56	151
r_{cw} ($s\ m^{-1}$)	1901	1382
r_{ct} ($s\ m^{-1}$)	2618	2115
r_{str} ($s\ m^{-1}$)	3509	1688
$r_{liq+lip}$ ($s\ m^{-1}$)	8599	5757
g_m ($mol\ m^{-2}\ s^{-1}$)	0.062	0.112

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