

RESPONSES OF PHOTOSYNTHETIC PARAMETERS TO DIFFERENCES IN WINTER TEMPERATURES THROUGHOUT A TEMPERATURE GRADIENT IN TWO EVERGREEN TREE SPECIES

ABSTRACT

The temperature dependence of photosynthetic parameters has been a focus of interest during recent years owing to its profound implications in the new climate scenario. Many studies have addressed the short-term responses of photosynthetic parameters to temperature change. Less attention has been given to the intraspecific variability in the biochemical parameters of photosynthesis in response to differences in growth temperature. This study explores the effects of winter harshness on the leaf traits of two evergreen tree species (*Quercus ilex* and *Q. suber*). Leaf mass per unit area (LMA) and the concentrations of fiber, nitrogen (N), soluble protein, chlorophyll and ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) were determined in both species throughout a temperature gradient. Several photosynthetic parameters [maximum carboxylation rate (V_{cmax}), maximum light-driven electron flux (J_{max}), dark respiration rate (R_d), relative stomatal limitation to photosynthesis (RSL), and the apparent nitrogen fraction in Rubisco (P_R) and in the rate-limiting proteins of photosynthetic electron transport (P_B)] were assessed by measuring leaf response curves of net CO_2 assimilation versus intercellular CO_2 partial pressure. LMA and structural carbohydrate concentrations increased with the decrease in winter temperatures, whereas N concentrations did not show definite patterns. Chlorophyll, soluble proteins, Rubisco, V_{cmax} , J_{max} , P_R and P_B declined with the decrease in winter temperatures, whereas R_d at a set common temperature (25°C) was higher at colder sites. Our results suggest that an increase in LMA and in the concentration of structural carbohydrates in cold environments is associated with a reduced N allocation to the photosynthetic machinery, which leads to reduced photosynthetic capacity.

Key words: photosynthetic parameters, Rubisco, fibre concentration, nitrogen, evergreen

species, climatic gradient.

INTRODUCTION

The investigation of the leaf traits that determine the natural variability of photosynthetic capacity is one of the main objectives of the present ecological research, given the large impact of photosynthesis on carbon sequestration and its role in the mitigation of global warming (Friedlingstein et al. 2006, Booth et al. 2012). The biochemical model proposed by Farquhar et al. (1980) and later improved by von Caemmerer and Farquhar (1981), Sharkey (1985) and Harley and Sharkey (1991) has been the most commonly used for characterizing the photosynthetic performance of the different species and for simulating carbon assimilation at different times and spatial scales. Significant variation in the two key parameters of this model (i.e. maximum velocity of carboxylation, V_{cmax} , and maximum rate of electron transport, J_{max}) has been found both across different species (Wohlfahrt et al. 1999, Manter and Kerrigan 2004, Kattge and Knorr 2007, Niinemets et al. 2009a, Lin et al. 2013, Flexas et al. 2014), and within a single species in response to changes in environmental factors such as growth irradiance (Daas-Ghrib et al. 2011, Vaz et al. 2011, Zhang and Yin 2012), nutrient supply (Grassi et al. 2002, Warren et al. 2003, Daas-Ghrib et al. 2011), water availability (Misson et al. 2010, Vaz et al. 2010, Aranda et al. 2012) and canopy position (Montpied et al. 2009, Legner et al. 2014).

Among the different environmental factors, the temperature dependence of photosynthetic parameters has been a focus of interest during recent years because of its profound implications in the new climate scenario. Many studies have addressed the seasonal variability of temperature responses of photosynthetic parameters in different species (Medlyn et al. 2002, Han et al. 2004, Borjigidai et al. 2006, Han et al. 2008, Dillen et al. 2012, Lin et al. 2013). However, less attention has been given to the intraspecific variability in the biochemical parameters of photosynthesis in response to differences in leaf growth temperature. Various authors have analysed the effects of the differences in growth

temperature on the photosynthetic capacity of a single species, but most of these studies rely on short-term experiments with seedlings growing in temperature-controlled growth chambers (Harley et al. 1992, Bunce 2000, Ghouil et al. 2003, Yamori et al. 2005, 2006, Warren 2008, Pons 2012). In contrast, there are not many studies addressing the variability in the photosynthetic parameters in adult specimens of a single tree species growing in different temperature environments (Zhang et al 2007, Dusenge et al 2015).

It is known that differences in V_{cmax} and J_{max} are often related to differences in leaf morphology and chemical composition. For instance, strong positive relationships between V_{cmax} and leaf nitrogen content have been observed by many authors (Niinemets and Tenhunen 1997, Wilson et al. 2000, Frank et al. 2001, Yamori et al. 2011). Similarly, decreases in the photosynthetic parameters have been frequently found associated with decreases in the fraction of nitrogen invested in the photosynthetic functions as leaf mass per unit area (LMA) increases (Hikosaka et al. 1998, Poorter and Evans 1998, Onoda et al. 2004, Takashima et al. 2004, Coste et al. 2005). LMA and leaf thickness also affect the internal CO_2 diffusion, with a tendency for leaves with greater LMA and thickness to have lower internal conductance (Syvertsen et al. 1995, Kao and Chang 2001, Niinemets et al. 2005, Zhang et al. 2007, Flexas et al. 2008, Tomas et al. 2013) and a lower photosynthetic capacity. In several studies, changes in the leaf traits that determine the photosynthetic parameters have been reported throughout climatic gradients. Within a single species, LMA tends to be greater in colder environments (Ogaya and Peñuelas 2007, Atkin et al. 2008, Mediavilla et al. 2012). Similarly, several authors have reported increases in N contents in leaves produced in colder sites (Weih and Karlsson 2001, Jian et al. 2009), although in other studies the patterns in N content proved inconsistent (Hultine and Marshall 2000, Chen et al. 2013). Accordingly, changes in the photosynthetic capacity of a species could be expected in response to changes in leaf growth temperature.

In the present paper we analyse the variations in the photosynthetic capacity of mature specimens of two evergreen tree species (*Quercus ilex* ssp. *ballota* (Desf.) Samp and

Quercus suber L.) throughout a winter temperature gradient. Our objective was to assess the extent of the differences in V_{cmax} and J_{max} in specimens of the same species growing in the field under contrasting winter conditions, and to determine the factors controlling these possible differences. Previous studies with the same species revealed significant differences between sites in several leaf traits related to differences in the intensity of winter harshness. Thus, the leaves from one species growing in environments with cooler winters showed a greater leaf mass per unit area (LMA) and structural carbohydrates concentrations, but lower chlorophyll, soluble proteins and Rubisco, with no trends in the leaf nitrogen content between environments (Mediavilla et al. 2012, González-Zurdo et al. 2016). By contrast, these trends were not observed in deciduous species (González-Zurdo et al. 2016), which suggests that the differences between sites in winter conditions were the cause of the changes observed in leaf traits for the evergreen species. Most authors addressing the effects of temperature on photosynthetic capacity have reported significantly higher capacities in the cooler temperatures than in warmer temperatures (Medlyn et al. 2002, Yamori et al. 2005, Dusenke et al. 2015, Kaluthota et al. 2015). These observations are in line with the hypothesis that plants growing in cool environments make relatively larger N investment in photosynthetic enzymes, compared to plants growing in warmer environments, in order to compensate for the low enzyme activity and slower membrane processes at low temperatures (Berry and Björkman 1980, Hikosaka 1997, von Caemmerer 2000). However, our previous data suggest that in evergreen species the cold resistance traits imply reduced N allocation to the photosynthetic machinery, associated with a stronger structural reinforcement at colder sites (Mediavilla et al. 2012, González-Zurdo et al. 2016). These large differences in leaf structure and leaf N allocation between sites could be associated with significant differences in photosynthetic capacity, with lower V_{cmax} and J_{max} values at the colder sites. In addition, lower mesophyll conductance to CO_2 might be expected in the colder environments as a consequence of the higher LMA and thickness of the leaves produced under these conditions. Our hypothesis, then, is that evergreen trees should exhibit lower photosynthetic capacity at colder sites than at warmer sites, as a

consequence of the structural reinforcement of the leaves produced in colder environments.

MATERIALS AND METHODS

Study species and sites

Two evergreen species were selected for the study: *Quercus suber* L. and *Q. ilex* ssp. *ballota* (Desf.) Samp. The two species were distributed on six sites located in the regions of Castilla & Leon and Extremadura (central-western Spain) between latitudes 41° 45' N and 39° 49' N and between longitudes 6° 22' W and 2° 37' W (Table 1). Owing to the differences in altitude and to the effects of continentality, there were great between-site differences in temperature, which were especially pronounced for the minimum winter temperatures and the number of frosts per year (Table 1). In contrast, the differences in summer temperatures were less intense. Accordingly, the annual temperature range was higher in colder sites mainly because of the effects of continentality (Ninyerola et al. 2000). According to differences in winter temperatures, two sites (A, B) may be classified as warm and two (E, F) as cold. Sites C and D had intermediate temperatures. The sites consisted of flat areas with sparse populations (between 50 and 100 specimens ha⁻¹) of mature (more than 100 years old) tree individuals. Each site was selected to cover a wide gradient in winter temperatures, although it was also ensured that the rest of climate characteristics were as homogeneous as possible. Nevertheless there was a tendency for rainfall levels to be higher in the hottest and southernmost sites, which helped to reduce the differences in the intensity of drought stress between cold and hot sites (Table 1). Owing to its lower tolerance to low temperatures, *Q. suber* was absent at the coldest sites. Rainfall data were obtained from the digital climatic atlas of the Iberian Peninsula (Ninyerola et al. 2005): a set of digital climatic maps of mean air temperature, precipitation and solar radiation elaborated with 200 m resolution by using data from climate stations and a combination of geographical variables (altitude, latitude, continentality, solar radiation and terrain curvature).

Table 1. Sites characteristics.

STANDS	A	B	C	D	E	F
Longitude (W)	5° 48'	6° 22'	5° 52'	5° 47'	2° 37'	2° 52'
Latitude (N)	40° 01'	39° 49'	41° 14'	41° 08'	41° 45'	41° 43'
Altitude (m.a.s.l.)	466	600	985	834	1189	1045
Climate characteristics						
Mean annual temperature (°C)	16.6	16.8	12.3	12.7	10.3	10.6
Mean spring temperature (°C)	19.3	19.0	15.0	14.9	13.0	13.2
Mean summer temperature (°C)	25.8	26.3	20.8	21.4	19.1	19.1
Mean autumn temperature (°C)	12.2	13.2	7.84	9.11	6.03	6.35
Mean winter temperature (°C)	9.07	9.81	5.55	5.30	3.14	3.53
Absolute minimum temperature (°C)	-4.15	-3.55	-8.18	-9.82	-13.7	-16.9
Mean daily minimum temperature of the coldest month (°C)	4.06	4.69	-0.47	-0.9	-2.93	-3.13
Number of days with frost per year	15	5	57	61	105	112
Mean duration of the period with frosts (days per year)	68	55	127	154	222	214
Annual rainfall (mm)	986	672	495	460	567	646
Daily mean radiation (W m⁻²)	185	184	186	183	177	179
Emberger's index	105	60	52	46	79	78
Soil						
Sand content (%)	77.2	73.9	84.6	74.3	75.1	84.0
Clay content (%)	11.7	12.3	6.40	12.2	14.4	8.30
Silt content (%)	11.1	13.8	9.00	13.5	10.5	7.70
Total soil N content (%)	0.143	0.104	0.072	0.021	0.115	0.048
P assimilable (ppm)	38	5	10	4	7	6
Organic matter (%)	4.47	4.10	2.60	0.37	5.12	1.92
pH	4.5	4.7	4.6	4.5	4.8	6.1
Species	Qi, Qs	Qi, Qs	Qi, Qs	Qi, Qs	Qi	Qi

Qi *Quercus ilex*, Qs *Quercus suber*

Temperature data were obtained for each site by means of data loggers (Hobo Pendant temperature/light datalogger, Part UA-002-08, Onset Computer Corporation, Pocasset, MA, USA). The data loggers were programmed to obtain temperature data every 10 minutes and were kept at each site for six years (2008–2014).

We used the Emberger's pluviothermic index (Emberger 1930) to analyse possible differences in water stress:

$$Q = \frac{1000 \times P}{(273 + \frac{T_X + T_N}{2}) \times (T_X - T_N)}$$

where P is the annual precipitation (mm), T_X is the average temperature of the hottest month (°C) and T_N is the average temperature of the coldest month (°C). This index is commonly used in Mediterranean climates (Kunstler et al. 2007).

Soil samples were taken up to a depth of 20 cm (excluding the forest floor) from each stand. The determination of soil granulometry, pH and N and P concentrations was carried out at the Soils Laboratory of the Institute of Natural Resources and Agrobiology in Salamanca according to the methods described by Chapmann and Pratt (1973) and Walkley and Black (1934).

Gas exchange measurements and biochemical modeling

Leaf gas exchange was measured with a portable steady-state photosynthetic system (CIRAS, PP-system, Hitchin, UK) equipped with a Parkinson leaf chamber (2.5 cm²). Measurements were conducted between 7:00 and 13:00 h local solar time during late spring and early summer, before the onset of summer drought, over a period of two years (2012-2013), on fully expanded sunlit leaves from the current year spring flush of both species. The A/C_i curves were produced under saturating light intensity (1800 μmol photons m⁻² s⁻¹), a temperature of 25°C and a relative humidity of around 60%. After allowing the gas exchange rates to stabilize for 15 minutes at 400 ppm CO₂ concentration (C_a), gas exchange rates were recorded over a range of intercellular CO₂ concentrations (C_i) resulting from changing the CO₂ supply in ten steps from 50 to 1800 ppm. The supply of CO₂ was reduced step-wise to the minimum value, then returned to 400 ppm again, and increased step-wise from that concentration to complete the A/C_i curve at the high C_a end. At each C_a, photosynthesis was allowed to stabilize for a minimum of 4 minutes and three successive

measurements were made at 30-s intervals, to ensure stability. A full A/C_i response curve usually took about 1.5 h to complete. In total, around 80 A/C_i curves were performed over the two growing seasons in all sites where *Q. ilex* was present and 60 curves were performed for *Q. suber*.

Photosynthetic parameters were estimated by fitting the model equations of Farquhar et al. (1980) and later modified by Sharkey (1985) and Harley and Sharkey (1991) to the measurements of leaf gas exchange by nonlinear least squares regression. For characterizing the photosynthetic capacity of the leaves, we chose light-saturated net photosynthesis rate (A_{\max} , measured at the CO_2 concentration close to $400 \mu\text{mol mol}^{-1}$ and $1800 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD), maximum carboxylation rate (V_{\max}) and maximum electron transport rate (J_{\max}) as parameters. In addition, day respiration rate (R_d) was calculated from the A/C_i curves. The Michaelis-Menten constants for CO_2 and O_2 , and the CO_2 compensation point in the absence of mitochondrial respiration were taken from Kosugi et al. (2003). We assumed an infinite CO_2 transfer conductance from the intercellular air spaces to the chloroplasts, i.e. the CO_2 partial pressure in the chloroplasts was assumed to be equal to C_i , as have numerous other researchers (Daas-Ghrib et al. 2011, van de Weg et al. 2012, Dillen et al. 2012, Lin et al. 2013, Dusenge et al. 2015). Estimation of internal conductance requires a considerable experimental effort using a combination of gas exchange measurements together with either carbon isotope fractionation or fluorescence techniques, which were not available in the present study. This assumption could induce underestimation of true V_{\max} values (Ethier and Livingston 2004), and therefore only apparent values of maximum carboxylation rates (V_{\max} , C_i) were displayed. Limitations of RuBP regeneration arising from the availability of inorganic phosphate for photophosphorylation (Sharkey 1985) were not considered in the present study.

The relative stomatal limitation to photosynthesis (RSL), an estimate of the proportion of the reduction in photosynthesis attributable to CO_2 diffusion between the atmosphere and the site of carboxylation, was calculated from A/C_i curves according to the method established

by Farquhar and Sharkey (1982), where $R = (1-A/A_0)100$ and A is the net photosynthetic rate when C_i equals ambient C_a . Under these conditions, A_0 is the rate of photosynthesis that would occur if there were no diffusive limitation to CO_2 transfer from the bulk atmosphere to the site of carboxylation. For this calculation, mesophyll conductance was also considered to be infinitely large. The intrinsic water use efficiency was calculated as the ratio of net CO_2 assimilation and stomatal conductance.

Leaf chemical and morphological analyses

Leaf morphology and nitrogen concentration were measured in the same leaves used for measuring photosynthesis. At the laboratory, leaf thickness was measured with a digital micrometer (Digimatic micrometer, Mitutoyo, Japan) and calculated as a mean of three measurements, taken at random positions on each leaf, but avoiding the main ribs on flat leaves. The total projected leaf areas were determined by an image analysis system (Delta-T Devices LTD, Cambridge, UK). The samples were then oven-dried at 70°C to constant weight and the total dry mass was determined. From the data obtained we calculated the leaf dry mass per area (LMA). Leaf N concentrations were determined with a CE-Instruments NA-2100 autoanalyser (ThermoQuest, Milan, Italy) and later expressed as per unit dry mass (N_{mass} , mg g⁻¹), and per unit of leaf area (N_{area} , g m⁻²), calculated as the concentration per unit dry mass multiplied by LMA.

LMA and leaf nitrogen content per dry mass (N_{mass}) values were used together with V_{cmax} and J_{max} estimates to calculate the apparent nitrogen fraction in Rubisco (P_R), and in the rate-limiting proteins of photosynthetic electron transport (P_B), according to Niinemets and Tenhunen (1997):

$$P_R = V_{cmax} / (6.25 \times V_{cr} \times N_{mass} \times LMA)$$

$$P_B = J_{max} / (8.06 \times J_{mc} \times N_{mass} \times LMA)$$

where V_{cr} is the maximum rate of ribulose-1,5-bisphosphate carboxylation per unit Rubisco protein ($V_{cr} = 20.5 \mu\text{mol CO}_2 (\text{g Rubisco})^{-1} \text{s}^{-1}$ at 25°C), J_{mc} is the capacity for photosynthetic electron transport per unit cytochrome *f* ($J_{mc} = 156 \text{ mol e}^- (\text{mol cyt } f)^{-1} \text{s}^{-1}$ at 25°C), and scaling coefficients of 6.25 and 8.06 are based on the stoichiometry of nitrogen content of proteins and rate-limiting proteins (Niinemets and Tenhunen 1997).

The concentrations of chlorophyll, Rubisco, soluble protein and fibre (hemicellulose, cellulose and lignin) were determined in leaf samples collected in close proximity to the leaves used in the gas-exchange measurements. The samples used to determine the fibre concentration were harvested on the same dates as the gas-exchange measurements. Fibre concentrations were determined with an Ankom Analyzer (A220, New York, USA), following the method of Goering and Van Soest (1970). Since determining the concentration of Rubisco, chlorophyll and soluble protein is costly in terms of both time and money, we limited our analyses to samples taken on one single occasion (end of spring - beginning of the summer of 2013) from the same specimens sampled for the photosynthesis measurements. The samples were transported to the laboratory in a fridge. At the laboratory, the plant material was weighed and immediately plunged into liquid nitrogen and kept at -80°C until further analysis. Protein extraction and the determination of protein and chlorophyll concentrations were conducted using the Agrisera method (Sweden). Chlorophyll was measured according to Whatley and Arnon (1963) and total soluble protein was measured according to Bradford (1976). The dry mass of the leaves used for the analyses were also determined, and the chlorophyll (CF) and protein (PT) contents of leaves were expressed per unit dry mass. For Western blotting and Rubisco analysis we used the Agrisera method, with minor modifications (see Vicente et al. 2011). The relative amount of the Rubisco large subunit was calculated by densitometric scanning of PVDF membranes by image analysis using the Scion ImagePC software (Scion, MD, USA) and expressed in arbitrary units (AU).

Statistical analysis

The data concerning the two plots selected for each climatic category as a function of the intensity of their winter harshness (two warmer plots, two intermediate plots and two plots with the coldest winters) were pooled after checking that there were no significant differences in the mean values obtained for each of them in any of the variables (data not shown). Between-site differences in the parameters analysed were explored using one-way analysis of variance. All statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). The Levenberg-Marquardt estimation technique of SPSS was used for all non-linear regression analyses of A/Ci curves.

RESULTS*Leaf morphology and chemistry: intraspecific variability related to a winter temperature gradient*

In response to the temperature gradient, the two species studied showed significant between-site differences in several leaf traits. LMA, leaf thickness and the structural carbohydrates concentration (cellulose + hemicellulose) tended to be greater at the coldest sites, while the lignin concentration did not show differences between sites (Fig. 1). Also N_{area} tended to increase across the sites in correlation with the intensity of their winter harshness. However, the differences in N_{area} were merely the result of the increase in LMA associated with the decrease in temperatures, since N_{mass} did not vary significantly among sites in both species (Fig.1). The concentrations of chlorophyll and soluble proteins fell with the reduction in winter temperatures when they were expressed per unit of leaf mass, with significantly higher values in the leaves produced in warmer environments with respect to the colder sites (Fig. 2). The differences, however, tended to disappear when both constituents were expressed per unit leaf area, due to increased LMA in the coldest places.

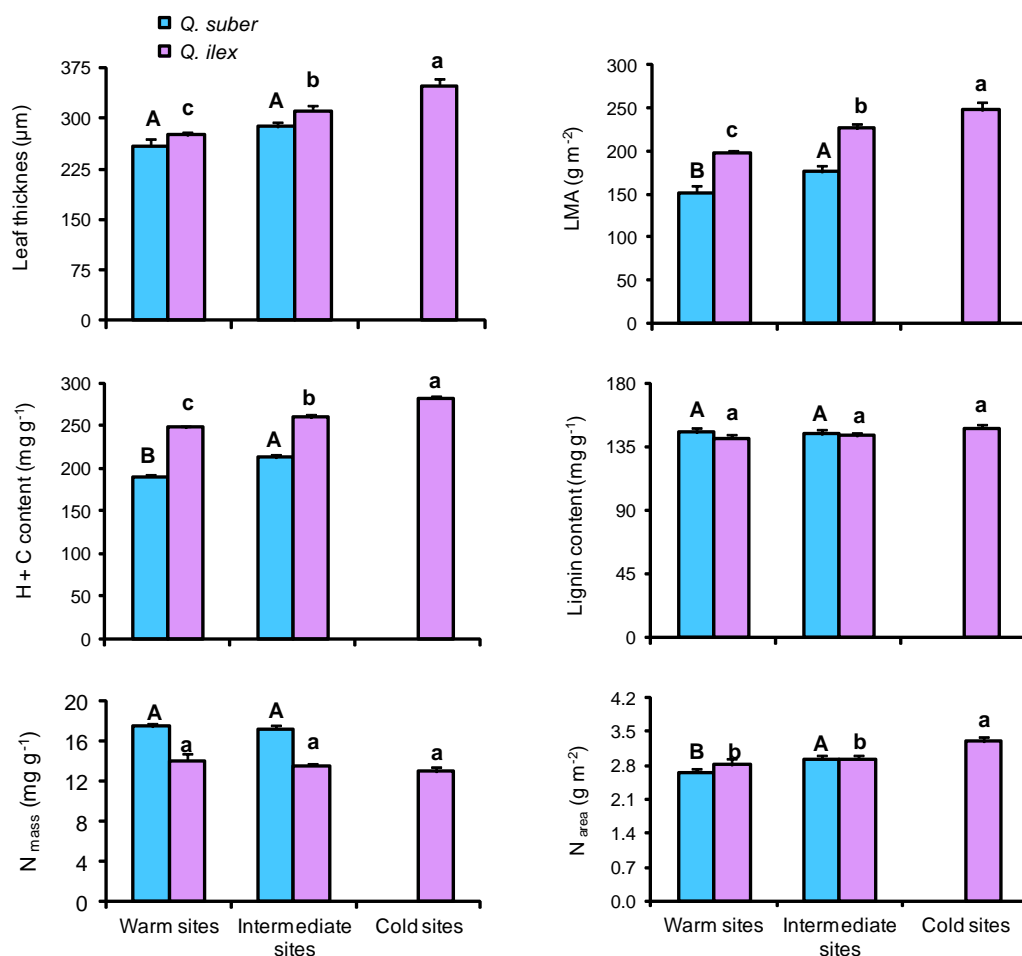


Figure 1. Mean (\pm SE, $n = 20$ -30) leaf traits at the different sites. The significant differences among sites are marked with different letters (Fisher LSD test, $P < 0.05$). LMA, leaf mass per unit area; H+C, hemicellulose + cellulose.

For *Q. ilex*, the sites with intermediate conditions were not always significantly differentiated from the warmer or colder sites, although they consistently showed intermediate values between both. In both species the relative amounts of Rubisco decreased with the increase in the intensity of winter harshness (Fig. 2).

Intraspecific variability in photosynthetic parameters

The large between-environment differences in leaf structure and chemical composition were paralleled by significant variability in photosynthetic capacity as measured by apparent V_{cmax}

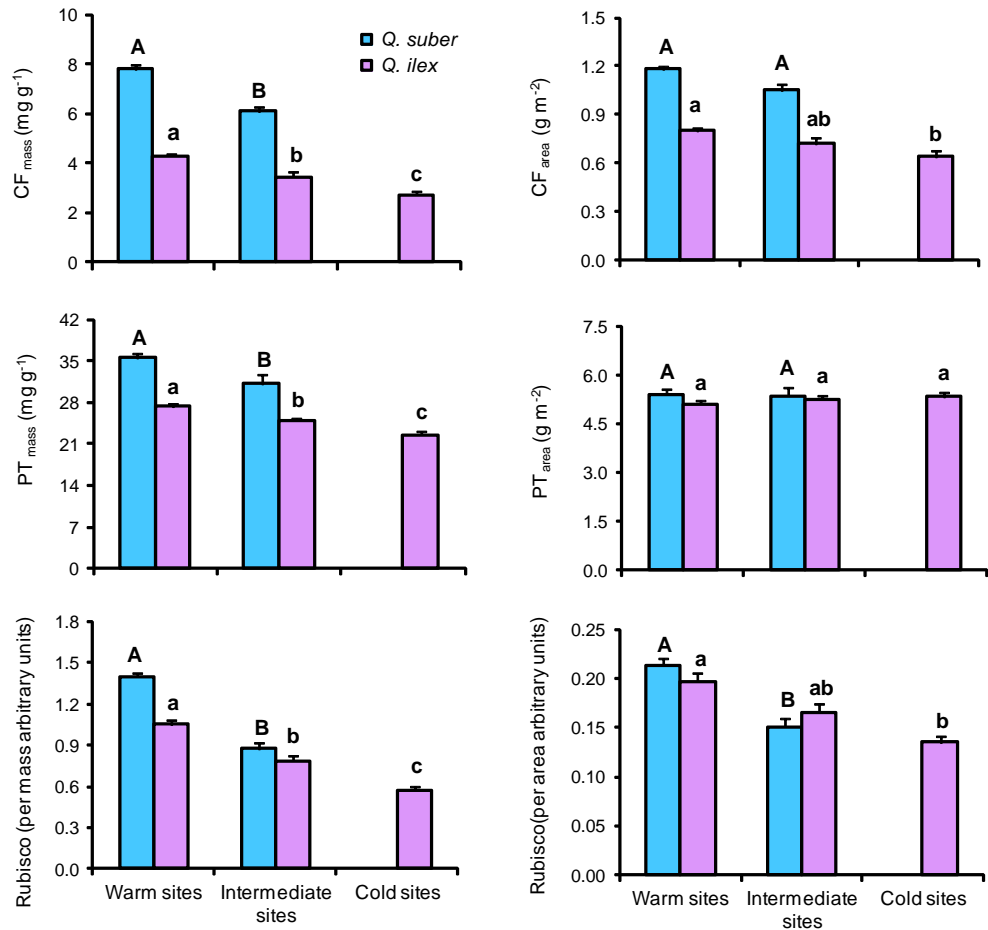


Figure 2. Mean (\pm SE, $n = 10$) concentration of chlorophyll (CF), soluble protein (PT) and Rubisco at the different sites. The significant differences among sites are marked with different letters (Fisher LSD test, $P < 0.05$).

and J_{\max} . The two parameters reached higher values at the warmer sites both when expressed per unit leaf area and per unit leaf mass (Fig. 3). By contrast, the day respiration rates (R_d) measured at 25°C were higher at the colder sites for both species (Fig. 3). No significant differences were observed for J_{\max}/V_{\max} between the different sites (Fig. 3).

Due to the differences in V_{\max} and J_{\max} , the light-saturated photosynthetic rates at an ambient CO_2 concentration around $400 \mu\text{mol mol}^{-1}$ were always higher at the warmer sites (Table 2). The between-site differences were especially marked when photosynthetic rates were expressed on a per leaf mass basis. The relative stomatal limitation to photosynthesis

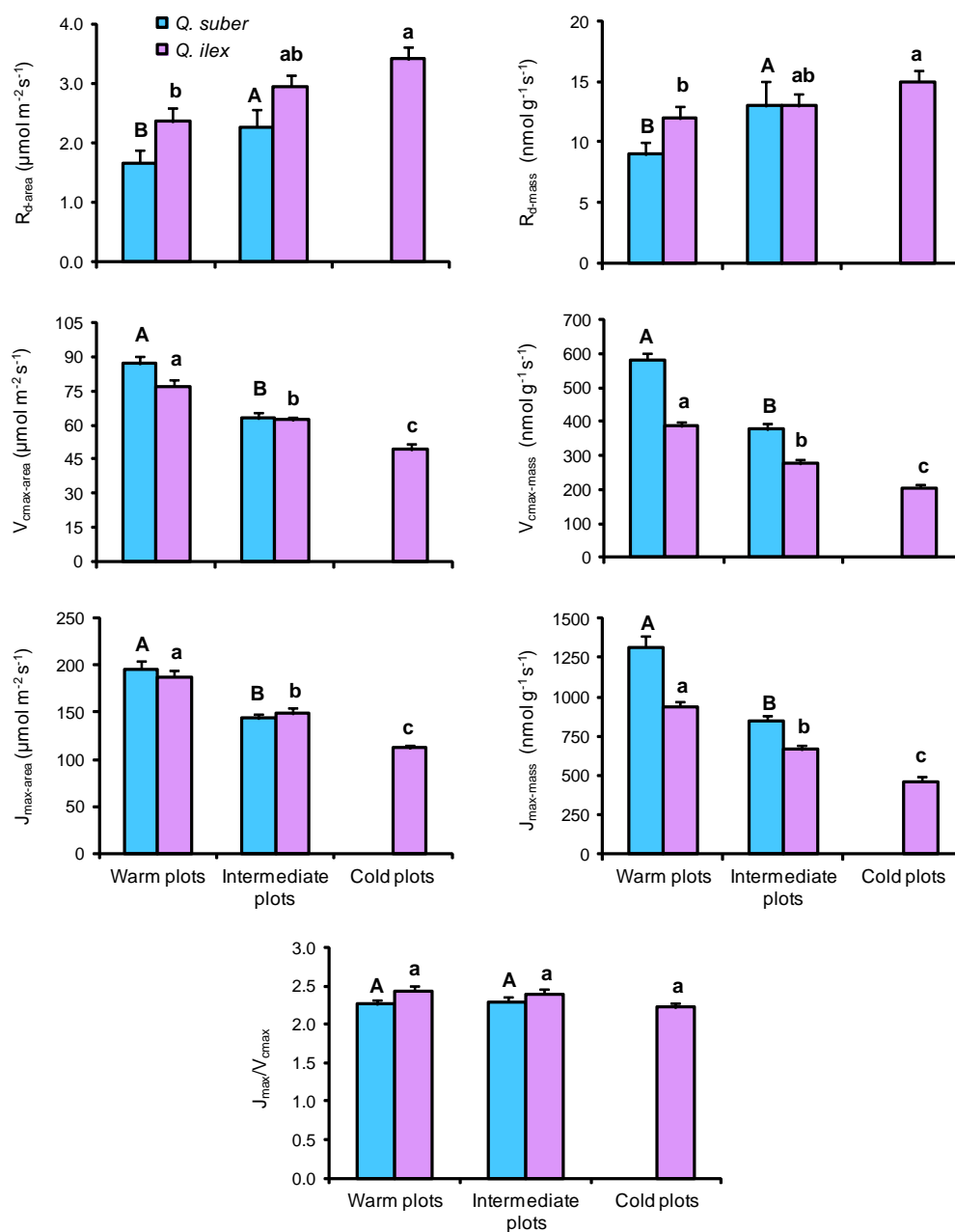


Figure 3. Mean (\pm SE, $n = 20-30$) day respiration rates and photosynthetic parameters at the different sites. The significant differences among sites are marked with different letters (Fisher LSD test, $P < 0.05$).

(RSL) was stronger in the warmer environments. Finally, the intrinsic water-use efficiency also reached significantly higher values at the warmer sites (Table 2). Under comparable temperature conditions, *Q. suber* exhibited higher values per unit leaf mass of V_{cmax} , J_{max} and light-saturated photosynthetic rate (Table 2 and Fig. 3).

Table 2. Light-saturated photosynthetic rates per unit leaf area (A_{area}) and per unit leaf mass (A_{mass}), intrinsic water use efficiency (A/g) and relative stomatal limitation (RSL) at the different sites. Standard errors in parentheses ($n=20-30$). The significant differences among sites are marked with different letters (Fisher LSD test, $P < 0.05$)

Species	Sites	A_{area} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	A_{mass} ($\text{nmol g}^{-1} \text{s}^{-1}$)	A/g ($\mu\text{mol C mol}^{-1} \text{H}_2\text{O}$)	RSL (%)
<i>Q. suber</i>	Warm sites	11.22 (0.55) a	75 (4.05) a	96 (3.80) a	37 (2.72) a
	Intermediate sites	9.30 (0.31) b	54 (0.95) b	71 (4.04) b	30 (1.77) b
<i>Q. ilex</i>	Warm sites	9.00 (0.37) a	46.0 (1.81) a	109 (9.14) a	43 (1.80) a
	Intermediate sites	8.14 (0.31) ab	36.4 (1.42) b	90 (4.77) b	38 (2.23) a
	Cold sites	7.05 (0.56) b	29.2 (2.27) c	63 (4.53) c	31 (1.96) b

Relative investment of leaf nitrogen in photosynthesis

The estimated apparent nitrogen fraction in Rubisco (P_R) and in the rate-limiting proteins of photosynthetic electron transport (P_B) varied significantly among the different sites occupied by each species. Apparent leaf nitrogen investment in Rubisco (P_R) amounted to approximately 26% (*Q. suber*) and 21% (*Q. ilex*) at the sites with higher winter temperatures, but decreased to 17 and 12%, respectively, in the coldest locations for each species (Table 3). The higher fractional investment of foliar N in Rubisco in the warmer environments was also accompanied by a stronger investment in bioenergetics (P_B). Again, sites with intermediate temperatures exhibited intermediate values of P_B and P_R in *Q. ilex*

Table 3. Apparent nitrogen fraction in Rubisco (P_R) and in the rate-limiting proteins of photosynthetic electron transport (P_B) and photosynthetic nitrogen use efficiency (PNUE) at the different sites. Standard errors in parentheses ($n=20-30$). The significant differences among sites are marked with different letters (Fisher LSD test, $P < 0.05$)

Species	Sites	P_R (mg g^{-1})	P_B (mg g^{-1})	PNUE ($\mu\text{mol g}^{-1} \text{N s}^{-1}$)
<i>Q. suber</i>	Warm sites	257 (10.2) a	59.2 (2.70) a	4.36 (0.23) a
	Intermediate sites	173 (8.55) b	40.2 (1.51) b	3.21 (0.14) b
<i>Q. ilex</i>	Warm sites	208 (5.89) a	52.9 (1.74) a	3.40 (0.12) a
	Intermediate sites	160 (1.73) b	39.1 (1.27) b	2.78 (0.09) b
	Cold sites	122 (4.46) c	29.4 (1.43) c	2.12 (0.15) c

(Table 3). As a consequence, both species achieved significantly higher photosynthetic nitrogen-use efficiencies at the warmer sites (Table 3). Within each species, the decrease in P_R in response to the decrease in winter temperatures was of a similar order of magnitude to the decrease in P_B , and in both species P_B and P_R exhibited percentage reductions similar to those observed for V_{cmax} and J_{max} respectively. Under the same temperature conditions, *Q. ilex* maintained lower fractional investments of its leaf N in Rubisco and in the rate-limiting proteins of photosynthetic electron transport, as well as lower PNUE than *Q. suber* (Table 3).

DISCUSSION

Confirming previous reports, in both species the leaves that had developed in cooler winter environments had a greater thickness, LMA and a higher concentration of structural carbohydrates than those that had developed at sites with milder winters. These results suggest that a greater leaf structural reinforcement is required to cope with more severe winter conditions. The between-site differences in leaf traits have important repercussions on photosynthesis, with large differences in the parameters of photosynthesis across the temperature gradient in both species. Many authors have reported greater V_{cmax} and J_{max} values in the leaves of a species grown at cooler temperatures than for those grown at warmer temperatures (Bunce 2000, Fan et al 2011, Dusenge et al 2015). These responses to temperature would support the hypothesis that plants growing in cool environments make relatively larger N investments into photosynthetic enzymes compared to plants growing in warmer environments in order to compensate for the low enzyme activity at low temperatures (Dusenge et al. 2015).

In contrast, in the present study, we have shown that the two species had lower values of V_{cmax} and J_{max} and light-saturated photosynthetic rates at the sites with colder winter temperatures. Given the strong correlations often observed between N_{area} , V_{cmax} and J_{max} across different species, the differences in leaf N content are usually considered as the

main determinant of the variations in the photosynthetic parameters within a single species (Grassi et al. 2002, Medlyn et al. 2002, Warren et al. 2003, Whitehead et al. 2004, Misson et al. 2006, Zhang et al. 2007, Vaz et al. 2011, Zhang and Yin 2012). Several authors have reported higher N concentrations in the leaves produced under low temperatures (Reich and Oleksyn 2004), which also supports the hypothesis that higher investments in enzymes constitute an adaptation aimed at compensating for the shorter length of the favourable growth period at sites with lower temperatures. However, in the present study, the between-site differences in N_{area} were small. Accordingly, we did not observe any trend in increased N investments in colder sites.

Leaf structure, especially leaf mass per area (LMA), has been proposed by different authors as one of the determinants of photosynthetic rates, owing to the effects of LMA on the patterns of nitrogen allocation (Field and Mooney 1986, Evans 1989, Poorter et al. 2009). Greater allocation of leaf nitrogen to cell walls and reduced investment in Rubisco as LMA increased has been observed in different studies (Onoda et al. 2004, Takashima et al. 2004). In this study, the larger LMA at the colder sites was accompanied by lower concentrations of chlorophyll, soluble protein and Rubisco than in warmer environments. Despite the similar leaf N concentrations at the different sites, the fractional investments of foliar N in Rubisco (P_R) and in the rate-limiting proteins of photosynthetic electron transport (P_B) always reached significantly lower values at the colder sites. The greater thickness and LMA at colder sites is probably a consequence of the thickening of the cell walls, which explains the higher concentrations of structural carbohydrates, a characteristic of leaves growing in cold climates (Kubacka-Zebalska and Kacperska 1999, Stefanowska et al. 1999). It is known that cell walls accumulate a significant amount of nitrogen compounds: up to 10% of the cell content (Reiter 1998, Hikosaka and Shigeno 2009). Accordingly, it can be expected that a greater amount of available nitrogen would be allocated to cell walls in environments with harsher winters, leading to a reduction in the amount available for allocation to chlorophyll or photosynthetic proteins. This lower allocation is the most

plausible explanation for the lower values of photosynthetic capacity, V_{cmax} and J_{max} in the colder environments. In addition to the lower values of the photosynthetic parameters, leaf dark respiration rates at 25°C tended to be higher in the colder than in the warmer habitats, a trend previously reported by several authors (Mooney 1963, Oleksyn et al. 1998). These higher R_d values may reflect an inevitable increase in maximum respiratory capacity associated with repair functions under cold, bright conditions that may induce photoinhibition (Atkin et al. 2000). Our results have suggested that the within-leaf N allocation is a stronger determinant of the intraspecific variation in photosynthetic capacity than absolute leaf N content. Owing to the differences in the allocation of N to photosynthetic enzymes, leaf structural components and repair functions, the two species achieved higher N-use efficiency at the warmer sites.

The between-site differences in leaf structure should determine important differences in mesophyll conductance (g_m). Decreases in the internal diffusion conductance have been associated with increases in LMA and in the cell wall fraction in several species (Syvertsen et al. 1995, Kao and Chang 2001, Niinemets et al. 2005, 2006, Flexas et al. 2008, Tomas et al. 2013), which should contribute to limiting the supply of CO_2 to the chloroplasts, and to reducing the photosynthetic capacity. We made our calculations assuming infinite conductance of CO_2 transfer between the intercellular spaces and sites of carboxylation. Rates of photosynthesis are related to the concentration of CO_2 at the sites of carboxylation (C_c), not the more easily measured C_i , and it is possible that the draw-down from C_i to C_c varies among sites and this affects the results. Different authors have demonstrated that assuming an infinite mesophyll conductance leads to a large underestimation of V_{cmax} and to a lesser extent of J_{max} (Ethier and Livingston 2004, Manter and Kerrigan 2004, Flexas et al. 2008, Niinemets et al. 2009b). If leaf mesophyll conductance is lower in leaves with the high LMA and cell wall content typical of the colder environments, C_c will be much lower than C_i and will result in a more significant underestimation of V_{cmax} than that obtained in the warmer environments. The changes in mesophyll conductance could thus attenuate the

differences in the actual values of the photosynthetic parameters. However, the decrease in V_{cmax} and J_{max} with the decrease in winter temperatures observed in the present study was very strong (more than 30% in *Q. suber* and 35% in *Q. ilex*). It is hard to believe that this strong reduction in the photosynthetic parameters would be explained solely by calculation errors associated with the differences in g_m . Furthermore, the percent reduction in J_{max} was of similar magnitude to that of V_{cmax} , despite the fact that J_{max} is much less affected by g_m than V_{cmax} (Ethier and Livingston 2004, Flexas et al. 2008, Niinemets et al. 2009b).

Owing to the parallel variation in both photosynthetic parameters, we did not find significant between-site differences in the $J_{\text{max}}/V_{\text{cmax}}$ ratio. Other authors that have analyzed between-site differences in the photosynthetic parameters have reported decreases in the $J_{\text{max}}/V_{\text{cmax}}$ ratio at the warmer sites in several controlled experiments (Bernacchi et al. 2003, Yamori et al. 2005), as well as in other field studies with different species (Onoda et al. 2005, Kattge and Knorr 2007, Leuning 1997, Hikosaka et al. 1999, Bernacchi et al. 2001, Leuning 2002, Yamori et al. 2010). This would suggest that these species invest more nitrogen in RuBP regeneration processes (electron transport, ATP synthase, and Calvin cycle except for Rubisco) than in Rubisco as growth temperatures decrease. The higher apparent investment in J_{max} compared to V_{cmax} at the colder sites has been interpreted as an adaptation to the lower PAR levels at higher latitudes (Yamori et al. 2010, van de Weg et al. 2012). Obviously, this explanation is only valid when the temperature gradients are associated with large latitude gradients that imply differences in the available radiation levels. In our case, however, the differences in latitude of our sites are small, and, accordingly, the solar radiation levels were similar (Table 1). The constancy in the values of the $J_{\text{max}}/V_{\text{cmax}}$ ratio suggests that this ratio is insensitive to changes in temperature, which is consistent with the results obtained by other authors (Ferrar et al. 1989, Bunce 2000).

In conclusion, our results reveal that, owing to the leaf structural reinforcement typical of colder climates, the proportion of N allocated to the photosynthetic machinery in these environments is lower than in warmer locations. This lower allocation contributes to

decreasing the photosynthetic N-use efficiency at the colder sites with respect to the populations of the same species located in warmer areas. Although the relative stomatal limitation to photosynthesis (RSL) was lower in the colder environments, this lower limitation was not sufficient enough to compensate the apparent lower activity of the photosynthetic machinery, which results in lower rates of light-saturated photosynthesis at ambient CO₂ concentrations. It has been suggested that low photosynthetic rates of evergreen species in colder climates may be compensated by a longer leaf lifespan (Wright et al. 2005). However, for the two species included in the present study, differences in leaf lifespan were not significant across the temperature gradient (González-Zurdo et al. 2016). It could be argued that under water-limited conditions, as in Mediterranean environments, the lower photosynthetic capacity typical of colder environments is compensated by lower stomatal limitations during the drought-stressed part of the growth season. If drought stress was more intense at warmer sites, this would reverse the positive effects of higher temperatures and could shorten the growth season at the warmer sites when compared with the colder ones. However, in the present study, differences in summer temperatures were relatively low. At the same time, the warmer sites also received greater rainfall, which could reduce the differences in drought stress. In fact, the Emberger's pluviothermic index was uncorrelated with temperature (Table 1). Accordingly, under the conditions of the present study, the photosynthetic performance of evergreen leaves is significantly lower at colder sites and this disadvantage is not compensated for by other leaf traits.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES

- Aranda I, Rodríguez-Calcerrada J, Robson TM, Cano FJ, Alte L, Sánchez-Gómez D (2012)** Stomatal and non-stomatal limitations on leaf carbon assimilation in beech (*Fagus sylvatica* L.) seedlings under natural conditions. *For Syst* 21: 405–417.
- Atkin OK, Atkinson LJ, Fisher R, Campbell CD, Zaragoza-Castells J, Pitchford JW, Woodward FI, Hurry V (2008)** Using temperature-dependent changes in leaf scaling relationships to quantitatively account for thermal acclimation of respiration in a coupled global climate–vegetation model. *Global Change Biol* 14: 2709–2726.
- Atkin OK, Holly C, Ball MC (2000)** Acclimation of snow gum (*Eucalyptus pauciflora*) leaf respiration to seasonal and diurnal variations in temperature: the importance of changes in the capacity and temperature sensitivity of respiration. *Plant Cell Environ* 23: 15–26.
- Bernacchi CJ, Pimentel C, Long SP (2003)** In vivo temperature response functions of parameters required to model RuBP-limited photosynthesis. *Plant Cell Environ* 26: 1419–1430.
- Bernacchi CJ, Singaas EL, Pimentel C, Portis AR, Long SP (2001)** Improved temperature response functions for models of Rubisco-limited photosynthesis. *Plant Cell Environ* 24: 253–259.
- Berry J, Björkman O (1980)** Photosynthetic response and adaptation to temperature in higher plants. *Ann Rev Plant Phys* 31: 491–543.
- Booth BBB, Jones CD, Collins M, Totterdell IJ, Cox PM, Sitch S, Huntingford C, Betts RA, Harris GR, Lloyd J (2012)** High sensitivity of future global warming to land carbon cycle processes. *Environ Res Lett* 7: 024002.
- Borjigidai A, Hikosaka K, Hirose T, Hasegawa T, Okada M, Kobayashi K (2006)** Seasonal changes in temperature dependence of photosynthetic rate in rice under a free-air CO₂ enrichment. *Ann Bot* 97: 549–557.
- Bradford MM (1976)** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254.
- Bunce JA (2000)** Acclimation of photosynthesis to temperature in eight cool and warm climate herbaceous C₃ species: temperature dependence of parameters of a biochemical model. *Photosynth Res* 63: 59–67.
- von Caemmerer (2000)** Biochemical models of leaf photosynthesis. CSIRO, Collingwood.
- von Caemmerer S, Farquhar GD (1981)** Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* 153: 376–387.
- Chapmann HD, Pratt PF (1973)** Methods of analysis for soils, plants and water. University of California Press, Riverside, California USA.

-
- Chen Y, Han W, Tang L, Tang Z, Fang J (2013)** Leaf nitrogen and phosphorus concentrations of woody plants differ in responses to climate, soil and plant growth form. *Ecography* 36: 178–184.
- Coste S, Roggy JC, Imbert P, Born C, Bonal D, Dreyer E (2005)** Leaf photosynthetic traits of 14 tropical rain forest species in relation to leaf nitrogen concentration and shade tolerance. *Tree Physiol* 25: 1127–1137.
- Daas-Ghrib C, Montpied P, Ksontini M, Dreyer E (2011)** Functional relationships between leaf structure and photosynthetic traits as modulated by irradiance and nutrient availability in a sclerophyllous Mediterranean oak species. *Eur J For Res* 130: 503–512.
- Dillen SY, de Beeck MO, Hufkens K, Buonanduci M, Phillips N (2012)** Seasonal patterns of foliar reflectance in relation to photosynthetic capacity and color index in two co-occurring tree species, *Quercus rubra* and *Betula papyrifera*. *Agr Forest Meteorol* 160: 60–68.
- Dusenge ME, Wallin G, Gardesten J, Niyonzima F, Adolfsson L, Nsabimana D, Uddling J (2015)** Photosynthetic capacity of tropical montane tree species in relation to leaf nutrients, successional strategy and growth temperature. *Oecologia* 177: 1183–1194.
- Emberger L (1930)** La végétation de la région méditerranéenne. Essai d'une classification des groupements végétaux. *Revue Générale de Botanique* 43: 641–662 et 705–729.
- Ethier GJ, Livingston NJ (2004)** On the need to incorporate sensitivity to CO₂ transfer conductance into the Farquhar-von Caemmerer-Berry leaf photosynthesis model. *Plant Cell Environ* 27: 137–153.
- Evans JR (1989)** Photosynthesis and nitrogen relationship in leaves of C3 plants. *Oecologia* 78: 9–19.
- Fan Y, Zhong Z, Zhang X (2011)** Determination of photosynthetic parameters V_{cmax} and J_{max} for a C3 plant (spring hulless barley) at two altitudes on the Tibetan Plateau. *Agr Forest Meteorol* 151: 1481–1487.
- Farquhar GD, von Caemmerer S, Berry JA (1980)** A biochemical model of photosynthetic CO₂ assimilation in leaves of C3 species. *Planta* 149: 78–90.
- Farquhar GD, Sharkey TD (1982)** Stomatal conductance and photosynthesis. *Ann Rev Plant Physiol* 33: 317–345.
- Ferrar PJ, Slatyer RO, Vranjic JA (1989)** Photosynthetic temperature-acclimation in *Eucalyptus* species from diverse habitats, and a comparison with *Nerium oleander*. *Austr J Plant Physiol* 16: 199–217.
- Field C, Mooney HA (1986)** The photosynthesis-nitrogen relationship in wild plants. In: Givnish TJ (ed) *On the economy of form and function*. Cambridge University Press, Cambridge, pp 25–55.
- Flexas J, Diaz-Espejo A, Gago J, Gallé A, Galmés J, Gulías J, Medrano H (2014)** Photosynthetic limitations in Mediterranean plants: A review. *Environ Exp Bot* 103: 12–23.
- Flexas J, Ribas-Carbó M, Diaz-Espejo A, Galmés J, Medrano H (2008)** Mesophyll conductance to CO₂: current knowledge and future prospects. *Plant Cell Environ* 31: 602–621.
-

- Frank E, Le Roux X, Millard P, Dreyer E, Jaquen G, Saint-joanis B, Wendler R (2001)** Changes in total leaf nitrogen and partitioning of leaf nitrogen drive photosynthetic acclimation to light in fully developed walnut leaves. *Plant Cell Environ* 24: 1279–1288.
- Friedlingstein P, Cox P, Betts R, Bopp L, Von Bloh W, Brovkin V, Cadule P, Doney S, Eby M, Fung I et al (2006)** Climate carbon cycle feedback analysis, results from the C4MIP model intercomparison. *J Climate* 19: 3337–3353.
- Ghouil H, Montpied P, Epron D, Ksontini M, Hanchi B, Dreyer E (2003)** Thermal optima of photosynthetic functions and thermostability of photosynthesis in cork oak seedlings. *Tree Physiol* 21: 1031–1039.
- Goering HK, Van Soest PJ (1970)** Forage fibre analysis (apparatus, reagents, procedures and some applications). *Agric Handb* no. 379, pp 1–20. ARS-USDA, Washington DC.
- González-Zurdo P, Escudero A, Babiano J, García-Ciudad A, Mediavilla S (2016)** Costs of leaf reinforcement in response to winter cold in evergreen species. *Tree Physiol* doi:10.1093/treephys/tpv134
- Grassi G, Meir P, Cromer R, Tompkins D, Jarvis PG (2002)** Photosynthetic parameters in seedlings of *Eucalyptus grandis* as affected by rate of nitrogen supply. *Plant Cell Environ* 25: 1677–1688.
- Han Q, Kawasaki T, Nakano T, Chiba Y (2004)** Spatial and seasonal variability of temperature responses of biochemical photosynthesis parameters and leaf nitrogen content within a *Pinus densiflora* crown. *Tree Physiol* 24: 737–744.
- Han Q, Kawasaki T, Nakano T, Chiba Y (2008)** Leaf-age effects on seasonal variability in photosynthetic parameters and its relationships with leaf mass per area and leaf nitrogen concentration within crown. *Tree Physiol* 28: 551–558.
- Harley PC, Sharkey TD (1991)** An improved model of C3 photosynthesis at high CO₂-reversed O₂ sensitivity explained by lack of glycerate reentry into the chloroplast. *Photosynth Res* 27(3): 169–178.
- Harley PC, Thomas RB, Reynolds JF, Strain BR (1992)** Modeling photosynthesis of cotton grown in elevated CO₂. *Plant Cell Environ* 15: 271–282.
- Hikosaka K (1997)** Modeling optimal temperature acclimation of the photosynthetic apparatus in C3 plants with respect to nitrogen use. *Ann Bot* 80: 721–730.
- Hikosaka K, Hanba YT, Hirose T, Terashima I (1998)** Photosynthetic nitrogen-use efficiency in leaves of woody herbaceous species. *Funct Ecol* 12: 896–905.
- Hikosaka K, Murakami A, Hirose T (1999)** Balancing carboxylation and regeneration of ribulose-1,5-bisphosphate in leaf photosynthesis: temperature acclimation of an evergreen tree, *Quercus myrsinaefolia*. *Plant Cell Environ* 22: 841–849.
- Hikosaka K, Shigeno A (2009)** The role of Rubisco and cell walls in the interspecific variation in photosynthetic capacity. *Oecologia* 160: 443–451.

-
- Hultine KR, Marshall JD (2000)** Altitude trends in conifer leaf morphology and stable carbon isotope composition. *Oecologia* 123: 32–40.
- Jian Q, Keming M, Yuxin Z (2009)** Leaf-trait relationships of *Quercus liaotungensis* along an altitudinal gradient in Dongling Mountain, Beijing. *Ecol Res* 24: 1243–1250.
- Kao WY, Chang KW (2001)** Altitudinal trends in photosynthetic rate and leaf characteristics populations from central Taiwan. *Aust J Bot* 49: 509–514.
- Kaluthota S, Pearce DW, Evans LM, Letts MG, Whitham TG, Rood SB (2015)** Higher photosynthetic capacity from higher latitude: foliar characteristics and gas exchange of southern, central and northern populations of *Populus angustifolia*. *Tree Physiol* 35(9): 936–948.
- Kattge J, Knorr W (2007)** Temperature acclimation in a biochemical model of photosynthesis: a reanalysis of data from 36 species. *Plant Cell Environ* 30: 1176–1190.
- Kosugi Y, Shibata S, Kobashi S (2003)** Parameterization of the CO₂ and H₂O gas exchange of several temperate deciduous broad-leaved trees at the leaf scale considering seasonal changes. *Plant Cell Environ* 26: 285–301.
- Kubacka-Zebalska M, Kacperska A (1999)** Low temperature-induced modifications of cell wall content and polysaccharide composition in leaves of winter oilseed rape (*Brassica napus* L. var *oleifera* L.). *Plant Sci* 148: 59–67.
- Kunstler G, Thuiller W, Curt T, Bouchaud M, Jouvie R, Deruelle F, Lepart J (2007)** *Fagus sylvatica* L. recruitment across a fragmented Mediterranean Landscape, importance of long distance effective dispersal, abiotic conditions and biotic interactions. *Diversity Distrib* 13: 799–807.
- Legner N, Fleck S, Leuschner C (2014)** Within-canopy variation in photosynthetic capacity, SLA and foliar N in temperate broad-leaved trees with contrasting shade tolerance. *Trees* 28: 263–280.
- Leuning R (1997)** Scaling to a common temperature improves the correlation between photosynthesis parameters J_{\max} and V_{\max} . *J Exp Bot* 48: 345–347.
- Leuning R (2002)** Temperature dependence of two parameters in a photosynthesis model. *Plant Cell Environ* 25: 1205–1210.
- Lin YS, Medlyn BE, De Kauwe MG, Ellsworth DS (2013)** Biochemical photosynthetic responses to temperature: how do interspecific differences compare with seasonal shifts? *Tree Physiol* 33: 793–806.
- Manter DK, Kerrigan J (2004)** A/Ci curve analysis across a range of woody plant species. Influence of regression analysis parameters and mesophyll conductance. *J Exp Bot* 55: 2581–2588.
- Mediavilla S, Gallardo-López V, González-Zurdo P, Escudero A (2012)** Patterns of leaf morphology and leaf N content in relation to winter temperatures in three evergreen tree species. *Int J Biometeorol* 56: 915–926.
-

- Medlyn BE, Loustau D, Delzon S (2002)** Temperature response of parameters of a biochemically based model of photosynthesis. I. Seasonal changes in mature maritime pine (*Pinus pinaster* Ait.). *Plant Cell Environ* 25: 1155–1165.
- Misson L, Tu KP, Boniello RA, Goldstein AH (2006)** Seasonality of photosynthetic parameters in a multi-specific and vertically complex forest ecosystem in the Sierra Nevada of California. *Tree Physiol* 26: 729–741.
- Misson L, Limousin JM, Rodríguez R, Letts MG (2010)** Leaf physiological responses to extreme droughts in Mediterranean *Quercus ilex* forest. *Plant Cell Environ* 33: 1898–1910.
- Montpied P, Granier A, Dreyer E (2009)** Seasonal time-course of gradients of photosynthetic capacity and mesophyll conductance to CO₂ across a beech (*Fagus sylvatica* L.) canopy. *J Exp Bot* 60: 2407–2418.
- Mooney HA (1963)** Physiological ecology of coastal, subalpine, and alpine populations of *Polygonum bistortoides*. *Ecology* 44: 812–816.
- Niinemets U, Cescatti A, Rodeghiero M, Tosens T (2005)** Leaf internal diffusion conductance limits photosynthesis more strongly in older leaves of Mediterranean evergreen broad-leaved species. *Plant Cell Environ* 28: 1552–1566.
- Niinemets U, Cescatti A, Rodeghiero M, Tosens T (2006)** Complex adjustments of photosynthetic potentials and internal diffusion conductance to current and previous light availabilities and leaf age in Mediterranean evergreen species *Quercus ilex*. *Plant Cell Environ* 29: 1159–1178.
- Niinemets U, Diaz-Espejo A, Flexas J, Galmés J, Warren CH (2009b)** Importance of mesophyll diffusion conductance in estimation of plant photosynthesis in the field. *J Exp Bot* 60: 2271–2282.
- Niinemets U, Tenhunen JD (1997)** A model separating leaf structural and physiological effects on carbon gain along light gradients for the shade tolerant species *Acer saccharum*. *Plant Cell Environ* 20: 845–866.
- Niinemets U, Wright I, Evans J (2009a)** Leaf mesophyll diffusion conductance in 35 Australian sclerophylls covering a broad range of foliage structural and physiological variation. *J Exp Bot* 60: 2433–2449.
- Ninyerola M, Pons X, Roure JM (2000)** A methodological approach of climatological modelling of air temperature and precipitation through GIS techniques. *Int J Climatol* 20: 1823–1841.
- Ninyerola M, Pons X, Roure JM (2005)** Atlas Climático Digital de la Península Ibérica. Metodología y aplicaciones en bioclimatología y geobotánica. Universidad Autónoma de Barcelona, Bellaterra. http://opengis.uab.es/wms/iberia/espanol/es_model.htm. Accessed 26 January 2016.
- Ogaya R, Peñuelas J (2007)** Leaf mass per area ratio in *Quercus ilex* leaves under a wide range of climatic conditions. The importance of low temperatures. *Acta Oecol* 31: 168–173.
- Oleksyn J, Modrzyński J, Tjoelker MG, Zytowski R, Reich PB, Karolewski P (1998)** Growth and physiology of *Picea abies* populations from elevational transects: common garden evidence for altitudinal ecotypes and cold adaptation. *Funct Ecol* 12: 573–590.

-
- Onoda Y, Hikosaka K, Hirose T (2004)** Allocation of nitrogen to cell walls decreases photosynthetic nitrogen-use efficiency. *Funct Ecol* 18: 419–425.
- Onoda Y, Hikosaka K, Hirose T (2005)** Seasonal change in the balance between capacities of RuBP carboxylation and RuBP regeneration affects CO₂ response of photosynthesis in *Polygonum cuspidatum*. *J Exp Bot* 56: 755–763.
- Pons T (2012)** Interaction of temperature and irradiance effects on photosynthetic acclimation in two accessions of *Arabidopsis thaliana*. *Photosynth Res* 113: 207–219.
- Poorter H, Evans JR (1998)** Photosynthetic nitrogen-use efficiency of species that differ inherently in specific leaf area. *Oecologia* 116: 26–37.
- Poorter H, Niinemets U, Poorter L, Wright J, Villar R (2009)** Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. *New Phytol* 18: 223–232.
- Reich PB, Oleksyn J (2004)** Global patterns of plant leaf N and P in relation to temperature and latitude. *Proc Natl Acad Sci USA* 101: 11001–11006.
- Reiter WD (1998)** The molecular analysis of cell wall components. *Trends Plant Sci* 3: 27–32.
- Sharkey TD (1985)** Photosynthesis in intact leaves of C₃ plants: physics, physiology and rate limitations. *Bot Rev* 78: 71–75.
- Stefanowska M, Kuras M, Kubacka-Zebalska M, Kacperska A (1999)** Low temperature affects pattern of leaf growth and structure of cell walls in winter Oilseed rape (*Brassica napus* L., var *oleifera* L). *Ann Bot* 84: 313–319.
- Syvertsen JP, Lloyd J, McConchie C, Kriedemann PE, Farquhar GD (1995)** On the relationship between leaf anatomy and CO₂ diffusion through the mesophyll of hypostomata leaves. *Plant Cell Environ* 18: 149–157.
- Takashima T, Hikosaka K, Hirose T (2004)** Photosynthesis or persistence: nitrogen allocation in leaves of evergreen and deciduous *Quercus* species. *Plant Cell Environ* 27: 1047–1054.
- Tomas M, Flexas J, Copolovici L, Galmes J, Hallik L, Medrano H, Ribas-Carbo M, Tosens T, Vislap V, Niinemets U (2013)** Importance of leaf anatomy in determining mesophyll diffusion conductance to CO₂ across species: quantitative limitations and scaling up by models. *J Exp Bot* 64: 2269–2281.
- Vaz M, Maroco J, Ribeiro N, Gazarini LC, Pereira JS, Chaves MM (2011)** Leaf-level responses to light in two co-occurring *Quercus* (*Quercus ilex* and *Quercus suber*): leaf structure, chemical composition and photosynthesis. *Agroforest Syst* 82: 173–181.
- Vaz M, Pereira JS, Gazarini LC, David TS, David JS, Rodrigues A, Maroco J, Chaves MM (2010)** Drought-induced photosynthetic inhibition and autumn recovery in two Mediterranean oak species (*Quercus ilex* and *Quercus suber*). *Tree Physiol* 30: 946–956.
-

- Vicente R, Morcuende R, Babiano J (2011)** Differences in Rubisco and Chlorophyll Content among Tissues and Growth Stages in Two tomato (*Lycopersicon esculentum* Mill.) varieties. *Agron Res* 9: 501–507.
- Walkley A, Black IA (1934)** An examination of the Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. *Soil Sci* 37: 29–38.
- Warren CR (2008)** Does growth temperature affect the temperature response of photosynthesis and internal conductance to CO₂? A test with *Eucalyptus regnans*. *Tree Physiol* 28: 11–19.
- Warren CR, Dreyer E, Adams MA (2003)** Photosynthesis-Rubisco relationships in foliage of *Pinus sylvestris* in response to nitrogen supply and the proposed role of Rubisco and amino acids as nitrogen stores. *Trees* 17: 359–366.
- van de Weg MJ, Meir P, Grace J, Ramos GD (2012)** Photosynthetic parameters, dark respiration and leaf traits in the canopy of a Peruvian tropical montane cloud forest. *Oecologia* 168: 23–34.
- Whatley FR, Arnon DI (1963)** Photosynthetic phosphorylation in plants. *Methods in Enzymology* VI, SP Colowick and NO Kaplan (eds). Academic Press, NY, pp 308–313.
- Weih M, Karlsson PS (2001)** Growth response of Mountain birch to air and soil temperature: is increasing leaf-nitrogen content an acclimation to lower air temperature? *New Phytol* 150: 147–155.
- Whitehead D, Walcroft A, Scott NA, Townsend JA, Trotter CM, Rogers GND (2004)** Characteristics of photosynthesis and stomatal conductance in the shrubland species Manuka (*Leptospermum scoparium*) and Kanuka (*Kunzea ericoides*) for the estimation of annual canopy carbon uptake. *Tree Physiol* 24: 795–804.
- Wilson KB, Baldocchi DD, Hanson PJ (2000)** Spatial and seasonal variability of photosynthetic parameters and their relationship to leaf nitrogen in a deciduous forest. *Tree Physiol* 20: 565–578.
- Wright IJ, Reich PB, Cornelissen JH, Falster DS, Groom PK, Hikosaka K, Lee W, Lusk CH, Niinemets Ü, Oleksyn J, Osada N, Poorter H, Warton DI, Westoby M (2005)** Modulation of leaf economic traits and trait relationships by climate. *Global Ecol Biogeogr* 14: 411–421.
- Wohlfahrt G, Bahn M, Haubner E, Horak I, Michaeler W, Rottmar K, et al (1999)** Inter-specific variation of the biochemical limitation to photosynthesis and related leaf traits of 30 species from mountain grassland differing in land use. *Plant Cell Environ* 22: 1281–1296.
- Yamori W, Nagai T, Makino A (2011)** The rate-limiting step for CO₂ assimilation at different temperatures is influenced by the leaf nitrogen content in several C₃ crop species. *Plant Cell Environ* 34: 764–777.
- Yamori W, Noguchi K, Hanba YT, Terashima I (2006)** Effects of internal conductance on the temperature dependence of the photosynthetic rate in spinach leaves from contrasting growth temperatures. *Plant Cell Environ* 29: 1069–1080.
- Yamori W, Noguchi K, Hikosaka K, Terashima I (2010)** Phenotypic plasticity in photosynthetic temperature

acclimation among crop species with different cold tolerances. *Plant Physiol* 152: 388–399.

Yamori W, Noguchi K, Terashima I (2005) Temperature acclimation of photosynthesis in spinach leaves: analyses of photosynthetic components and temperature dependencies of photosynthetic partial reactions. *Plant Cell Environ* 28: 536–547.

Zhang SB, Yin LX (2012) Plasticity in photosynthesis and functional leaf traits of *Meconopsis horridula* var. *racemosa* in response to growth irradiance. *Bot Stud* 53: 335–343.

Zhang SB, Zhou ZK, Hu H, Xu K (2007) Gas exchange and resource utilization in two alpine oaks at different altitudes in the Hengduan Mountains. *Can J For Res* 37: 1184–1193.