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Stomatal responses in deciduous oaks from southern Europe to the anthropogenic atmospheric CO_2 increase; refining the stomatal-based CO_2 proxy

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Abstract

Herbarium specimens of *Quercus robur* L. from 25 localities on the Iberian Peninsula covering the time period from 1901 to 2003 were studied to develop a suitable stomatal index (SI)-atmospheric CO_2 inference model that met the specific demands of the abundant fossil material from this geographical region. Significant SI changes were observed under the atmospheric CO_2 increase since the onset of industrialization, resembling a sigmoidal response. The highly comparable SI response rates of Iberian and Northwest European data sets confirmed that *Q. robur* has reached its response limit at 330 ppmv, and adaptation of *Quercus* to changing CO_2 concentration is in principle the same. A 3.5% SI difference between both data sets is likely to be a result of the differences in irradiation level at the high and low latitudes. An adjusted SI-CO_2 calibration data set for the Northern Iberian Peninsula is provided for the CO_2 range from 295 ppmv to 330 ppmv, where the best statistical fit for the CO_2 inference model is achieved when data are restricted to an altitudinal range of 0–1000 m a.s.l. Geographical extension of the model is attained by the incorporation of the month irradiance average (W/m^2) as an independent factor. Additional pore length increase was observed over the same CO_2 range. Usage of the Stomata-Pore Coefficient (proportion of pore length over the Stomatal length) was introduced in order to give a better description of the response of stomatal dimensions to the rising atmospheric CO_2 concentrations.
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1. Introduction

The inverse relationship between atmospheric CO_2 concentration and the stomatal index (SI) recorded in north–west European *Quercus* species, has been success-fully used as a proxy to reconstruct atmospheric CO_2 levels in the past (Van der Burgh et al., 1993; Kürschner, 1996; Kürschner et al., 1998; Van Hoof, 2004; Van Hoof et al., 2005a). In order to develop suitable CO_2 inference models, the adaptation of the stomatal frequency parameters in *Quercus petraea* (Mattuschka) Liebl. and *Quercus robur* L. to the anthropogenic atmospheric CO_2 increase of the past ∼ 150 years was quantified in herbarium material and leaf litter profiles from the United

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Kingdom, Germany and the Netherlands (Beerling and Chaloner, 1992; Kürschner, 1996; Van Hoof et al., 2005b). It has been demonstrated, however, that a wide variety of environmental factors have a distinct influence on the stomatal frequency parameters among which light intensity plays an important role (Poole et al., 1996; Kürschner et al., 1998; Wagner et al., 2000). Therefore, existing calibration models from northern Europe have to be tested and adjusted to site-specific conditions before they are applied in other geographical regions.

The availability of abundant well-preserved sub-fossil *Quercus robur* leaf assemblages recovered from road sections and sea-eroded cliffs in northern Spain provided the opportunity to extend the use of *Quercus* leaf fossils as bio-indicators for Holocene atmospheric CO_2 dynamics into the southern European realm. However, especially on the Iberian Peninsula a variety of additional factors have to be taken into account as the wide altitudinal range and the lower mid-latitude position at the Eurosiberian to Mediterranean climate transitional zone, introduce large differences in local climate and light conditions.

Quercus robur and *Q. petraea* are both Eurosiberian species of which the distribution on the Iberian Peninsula is reduced to a narrow band in northern Spain. A geographical, especially altitudinal, differentiation can be found between the two species, but when ecological requirements overlap, they occur together and hybridization may take place (Jalas and Suominen, 1976; Rivas-Martinez and Sáenz, 1991; Costa et al., 1997).

In the present study we evaluated the potential effects of site differences on the *Quercus robur* SI response to the anthropogenic atmospheric CO_2 increase, focussing on the effects of altitude and irradiance level on leaf micro-morphology. Iberian specimens from *Q. robur* and *Q. petraea* and their hybrids, from a 1600 m altitudinal range as well as contrasting geographical populations were sampled. Based on the results of the stomatal frequency analysis of the material, a CO_2 inference model was developed that met the specific demands associated with CO_2 reconstruction based on the abundant sub-fossil leaf material found in the Iberian Peninsula (García-Amorena, 1998). Comparison of the Iberian data set with previous models (Van Hoof et al., 2005b), allowed the extension of the model to other locations by including an irradiance correction factor.

2. Material and methods

2.1. Leaf samples

Thirty-four Iberian *Q. robur* and *Q. petraea* specimens within a wide geographical range (Fig. 1) were collected
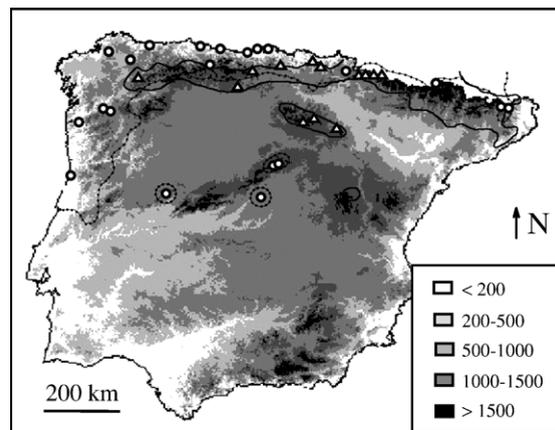

Fig. 1. Altitudinal map of the Iberian Peninsula. Altitudinal ranges are presented in metres. Dotted line: *Q. robur* distribution. Solid line: *Q. petraea* distribution. Circles: *Q. robur* collection sites. Triangles: *Q. petraea* collection sites.

from different herbaria supplemented with modern leaf material (Table 1) covering the last $\sim$ 100 years. Modern leaves were taken from above 3 m height on the south exposition of the canopy.

Sun/shade morphotypes were determined by periclinal wall undulation analysis (undulation index analysis—UI, see formula below) (Kürschner, 1997). Pilot measurements on shade leaves resulted in a UI > 1.5. All further measurements were restricted to sun leaf morphotypes in order to avoid the potential effect of canopy variations in humidity and CO_2 on the leaf morphology (Bazzaz and Williams, 1991; Kürschner, 1996; Poole et al., 1996; Lockheart et al., 1998).

Leaves were determined to species according to Flora Iberica (Castroviejo et al., 1990), and the identification of each sample was confirmed by the presence of stellated trichoms which characterize *Q. petraea* but are absent in *Q. robur* (Penas et al., 1994; Uzunova et al., 1997). Because the exact altitudinal positions of the sampled herbarium material were generally not recorded on herbarium sheets, samples were geo-referenced and maximum and minimum altitudes (Table 1) were obtained from the forest and topographic maps of the Iberian Peninsula (S.G.E., 1986 onwards; Ruiz de la Torre, 1990–1998).

2.2. Lower cuticle preparation and analysis

Squares of 5×5 mm were cut from the central area of the leaves, the most stable part in terms of stomatal and epidermal cell densities (Tichá, 1982; Poole et al., 1996). Fragments were boiled in water for 1 h to remove wax enclosures, and were bleached in 4% sodium-hypochlorite (NaHClO$_2$) for 12 h. After rinsing them several times,

Table 1
List of specimens

	C.D.	ID sample	Location (U.T.M.)			Stand altitudes	
			Zone	*X*	*Y*	−	+
Quercus petraea	1929	MA 160028	30N	638456	4756108	840	1390
	1929	MA 160027	30N	638456	4756108	840	1390
	1933	EMMA 917	30N	594406	4630333	850	1800
	1935	MA 255723	30N	539131	4651383	1300	1800
	1944	EMMA 902	30N	406206	4756958	850	1550
	1944	EMM 2546	30N	376915	4720907	930	1050
	1948	MA 51978	30N	638456	4756108	840	1390
	1948	MA 51978 (2)	30N	638456	4756108	840	1390
	1958	EMMA 909	29N	354667	4683860	1200	1600
	1983	MA 304492	30N	517506	4652133	1500	1500
	1989	MA 476590	30N	451981	4772808	1000	1300
Quercus robur	1901	MA 341149	30N	436206	4475633	600	690
	1924	MA 160014	30N	291331	4824608	10	130
	1928	MA 160020	29N	603889	4657914	630	1000
	1928	MA 160023	29N	585264	4788040	460	730
	1930	MA 160016	30N	408656	4806633	10	110
	1944	EMMA 869	30N	394705	4794614	140	650
	1948	EMMA 886	30N	607292	4758456	530	840
	1948	EMMA 887	31N	444142	4676067	790	1360
	1948	EMMA 883	31N	456499	4672619	440	1360
	1956	MA 205691	29N	667628	4809586	150	1160
	1956	MA 205692	29N	628937	4773880	480	730
	1960	EMMA 888	29N	742626	4483860	800	1000
	1966	U Min.Pt. 66	29N	537534	4629649	10	100
	1975	MA 276403	29N	535855	4511963	10	200
	1979	EMMA 874	29N	588848	4662713	550	1090
	1986	MA 330877	30N	438225	4804214	30	570
	1992	MA 511722	31N	314169	4745953	650	670
	1998	MA 652718	30N	314781	4770208	1170	1620
	2003	Rib.As.01.2003	30N	332545	4815094	10	100
Hybrids	1918	MA 50541	30N	456506	4547183	1090	1800
	1918	MA 50541 (2)	30N	456506	4547183	1090	1800
	1966	U GUI.PV.01	30N	551265	4769254	600	1100
	1992	MA 549567	30N	533606	4785183	690	1100

C.D.: collection date. U.T.M.: Universal Tranverse Mercator, datum Postdam.
Stand altitudes (−/+): Maximum and minimum altitudes (m) of the stand where the specimen was collected.

upper cuticles and mesophyll were removed; the lower cuticles were subsequently mounted on microscopic slides in glycerine jelly.

Stomatal and epidermal cells parameters were determined using a Leica Quantimet 500C/500+ Image Analysis System. Three to five leaves were analysed from each sample, and ten different and randomised areas of 0.0245 mm^2 within the alveoles were measured to obtain stomatal parameters. Measured epidermal properties include stomatal density (SD [mm^{-2}]: number of stomata per mm^2), epidermal cell density (ED [mm^{-2}]: number of non-stomatal epidermal cells per mm^2), stomatal length (SL [μm]: axial outer guard cell walls distance (Fig. 2)), pore length PL [μm]: axial inner guard cell walls distance (Fig. 2)), epidermal cell circumference (CC [μm]: non-stomatal epidermal cells average perim-

eter) and epidermal cell area (CA [μm^2]: non-stomatal epidermal cells average area). Stomatal index (SI [%]: proportion of total number of leaf surface cells (stomata and epidermal cells) that are stomata [formula a]); Salisbury, 1927) and undulation index (UI: non-stomatal epidermal cells undulation quantification [formula b]); Kürschner, 1997) were calculated as follows;

$$SI(\%) = 100 \frac{SD}{SD + ED} \qquad (a)$$

$$UI = \frac{CC(\mu m)}{CC(\mu m)} = \frac{CC}{2 \cdot \sqrt{\pi \cdot CA}} \qquad (b)$$

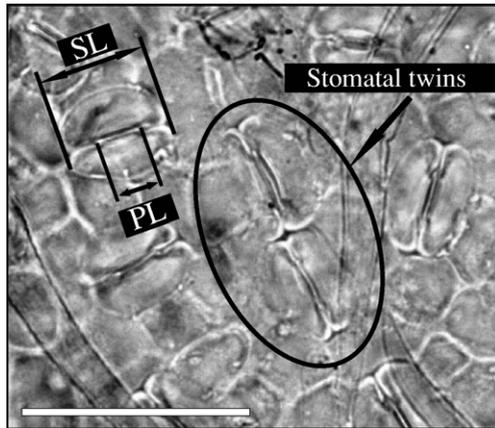where Co is the optimal circumference of the cell for its area.

Fig. 2. *Q. petraea* lower cuticle (sample MA 51978 (2)) with stomatal twins, SL (stomata length) and PL (pore length) indications. Scale bar = 50 μm.

Historical CO_2 (ppmv) values used for analysis, were derived from Mauna Loa monitoring (Keeling and Whorf, 2002) and were supplemented with shallow ice core data (Neftel et al., 1994). Statistical analysis of the data was performed with *Statgraphics Plus 5.0 programme*.

3. Results

3.1. Stomatal frequency analysis

Measured SI for *Q. robur*, *Q. petraea* and hybrid leaves, are presented in Fig. 3; A 5.7% SI decrease with atmospheric CO_2 is observed over the CO_2 range from 295 to 330 ppmv. At CO_2 values above 330 ppmv, SI response levels off around 19%.

As altitudinal positions of the herbarium samples were too coarse, calculation of CO_2 partial pressures to account for the different altitudes could not be calculated (Table 1). However, reliability of the regression model was

increased by filtering the data set taking into account the maximum altitude of the sampled stands. A least squares simple regression analysis performed on the section of the SI to atmospheric CO_2 (ppmv) response that is linear (CO_2 from 295 to 330 ppmv) for the altitudinal ranges of 0–2000 m a.s.l. (Fig. 4A), 0–1500 m a.s.l.(Fig. 4B) and 0–1000 m a.s.l., did not show significant different linear models (Table 2). The higher explanation in SI variability by CO_2 was 57.10% in the 0 to 1000 m a.s.l. data. *Q. petraea* and hybrids were excluded from this data set,

Fig. 4. Linear stomatal index (SI %) response intervals versus atmospheric CO_2 (ppmv). Regression models (thick lines) and 95% confidence bands (thin lines). A: Least squares analysis for complete data set from 0 to 2000 m a.s.l. ($R^2 = 0.31$). B: Least squares analysis for data points between 0 to 1500 m a.s.l. Includes *Q. robur* data and three *Q. petraea* data. Hybrids data were removed ($R^2 = 0.46$). C: Weighted least squares analysis for data between 0 to 1000 m a.s.l. Three *Q. robur* data points from > 1000 m a.s.l. were removed together with *Q. petraea* and hybrids data (R^2 of 0.77).
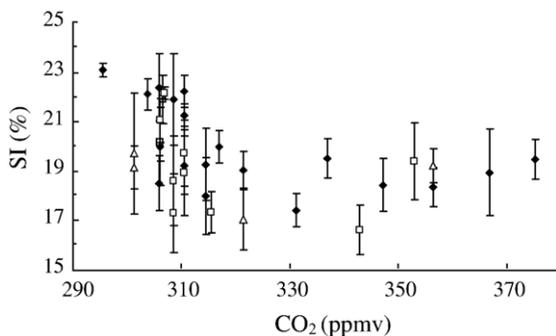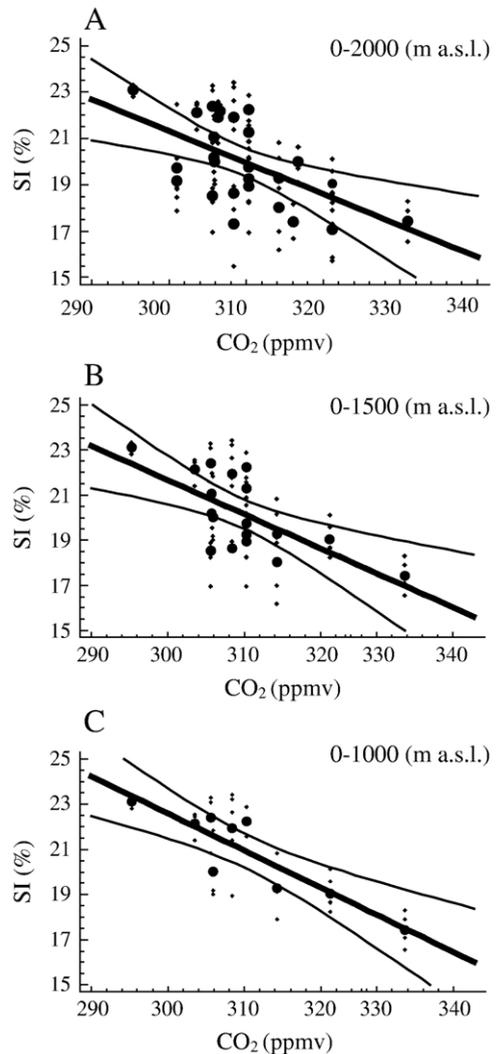

Fig. 3. Measured SI versus historical atmospheric CO_2 (ppmv). Rombs: *Q. robur*. Squares: *Q. petraea*. Triangles: hybrids. Error bars: ±1 SI standard deviation.

Table 2
* Least squares and ** weighted least squares regression analysis performed on the section of the *Q. robur* % SI response to atmospheric CO_2 (ppmv) that is linear, for the altitudinal ranges of 0–2000 m a.s.l., 0–1500 m a.s.l. and 0–1000 m a.s.l. (Statgraphics Plus 5.0 programme)

Model		A) $SI = b_0 + b_1 \times CO_2$		B) $SI = b_0 + b_1 \times CO_2 + b_2 \times I$	
CO_2 range (ppmv)		295–330		295–330	
Altitudinal range (m)	0–1000**	0–1000*	0–1500*	0–2000*	0–1000**
b_0 (95.0% confidence)	71.09	72.45	69.54	64.35	−10.80
b_1 (95.0% confidence)	−0.16	−0.17	−0.16	−0.14	−0.12
b_2 (95.0% confidence)	–	–	–	–	0.29
R^2 (adjusted for df)	76.96%	57.10%	46.49%	31.44%	77.00%
F-Snedecor	27.73 ($P=0.00$)	14.31 ($P=0.00$)	17.51 ($P=0.00$)	12.47 ($P=0.00$)	62.99 ($P=0.00$)
Pearson's correlation coefficient	–	−0.78	−0.70	−0.58	–

df: degrees of freedom. P: probability value. A) Iberian data set. B) Dutch (Van Hoof et al., 2005b) and Iberian data set. I: month irradiance averages (W/m^2) over January 1975 to December 2003 (NOAA-CIRES, 2006).

as no herbarium material was available for this altitudinal range. A *weighted least squares* method applied on the 0–1000 m a.s.l. data set, by using the mean SI standard deviation as the weights for each observation, resulted in a better statistical fit ($R^2=0.77$), with a significant improvement of F-Snedecor accompanied by small changes in the model (Fig. 4C; Table 2). Subsequent statistical analysis of the four models secured the statistically significant relationship between the variables at the 99% confidence level, and confirmed previous assumptions of normality and independence of the residuals. This validated the statistical fit for a confidence higher than 90% (Draper and Smith, 1981; Saporta, 1990).

Epidermal cell density measurements on *Q. robur* for the 1000 m a.s.l. altitudinal range showed a pronounced and significant increase from mean 1210 mm^{-2} to 1447 mm^{-2}, ($b_1=4.31$; $R^2=0.06$) over the CO_2 range from 295 ppmv to 330 ppmv. In addition, a significant stomatal density decrease over the same CO_2 range was observed ($b_1=-1.29$; $R^2=0.08$).

3.2. Stomatal pore dimension analysis

Additional changes were further observed in PL of *Q. robur* for the altitudinal range of 0 to 1000 m (Fig. 5A). A pore length increase trend, from mean 8.69 µm at 295 ppmv CO_2, to 11.6 µm at 321 ppmv CO_2 became clearer when the stomatal dimensions, pore length and stomatal length defined as *Stomata–Pore Coefficient* (SC [%]=(100×(PL/SL)) were considered (Fig. 5B). SC correlation to changing CO_2 of $R^2=0.27$, need other environmental factors to be further evaluated.

4. Discussion

In the present study, significant changes were observed in SI, ED and SC, under the atmospheric CO_2 increase

since the onset of industrialization. Previous studies on SI showed that several plant species, including *Q. robur* and *Q. petraea*, are responding sensitively to atmospheric CO_2 changes (Woodward, 1987; Beerling and Chaloner, 1993; Kürschner, 1997; Wagner et al., 2004; Van Hoof et al., 2005b). However, the exact SI response range to CO_2 is highly species specific and in some cases, sensitive to latitudinal gradients and different geographical localities (Ferris and Taylor, 1994; Wang and Kellomäki, 1997; Hultine and Marshall, 2000; Wagner et al., 2000; Royer,
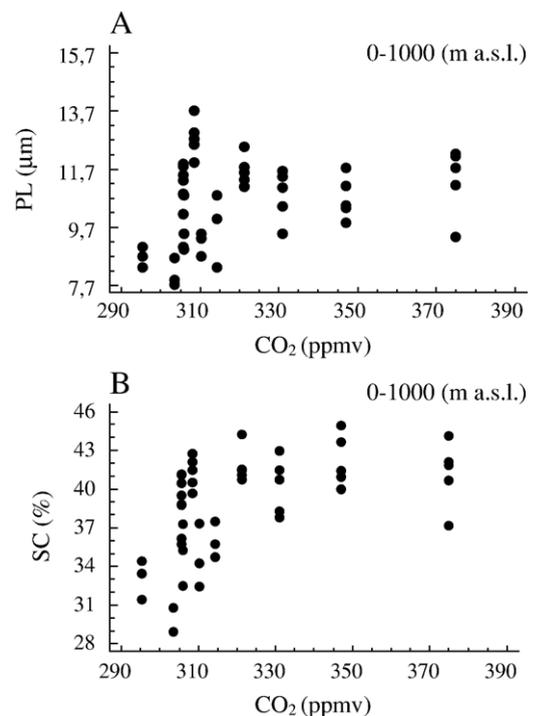

Fig. 5. A: *Q. robur* pore length (PL µm), and B: Stomata–Pore Coefficient (SC=100×PL/SL) response to CO_2, for the altitudinal range of 0 to 1000 m a.s.l.

2001; Guy, 2003). Thus, SI standard curves based on herbarium material to infer atmospheric CO_2 from fossil leaves should ideally be generated from localities close to potential fossil sampling sites (Wagner et al., 1999; Royer, 2001).

A major problem related to the complex topography of northern Spain is for example the large altitudinal range for the sampling sites. By studying historical *Q. robur* leaf material from a wide variety of localities on the Iberian Peninsula, the evaluation of potential genetic variability or geographical error sources in the calibration data sets becomes possible. This procedure guarantees the development of specifically tuned CO_2 inference models and allows secure stomatal frequency based CO_2 estimates from the abundant sub-fossil *Q. robur* leaves in Holocene sections in northern Spain.

An Iberian stomata based CO_2 partial pressure regression model was hampered by the lack of exact altitudinal data and therefore of a low CO_2 partial pressures precision. However, as significant SI and SD correlation to decreasing CO_2 partial pressure with altitude were shown only above 1000 m a.s.l. for *Quercus kelloggii* Newb. (McElwain, 2004), data from stands which maximum altitude are above 1000 m a.s.l. were removed for the final calibration analysis (Fig. 4C). By applying this altitude filter, interference of environmental factors changing with altitude, e.g. light, temperature, atmospheric pressure (Körner and Mayr, 1981; Osborn and Taylor, 1990; Jones, 1992; Kürschner, 1997; Kürschner et al., 1998; Filella and Peñuelas, 1999; Kouwenberg et al., 2003), were avoided.

4.1. Stomatal index CO_2 signal

The analysed herbarium samples from the altitudinal range 0–1000 m showed a distinct SI response to changing CO_2 levels. This response resembled a sigmoidal response which has already been empirically modelled for herbarium studies, and demonstrated in several growth experiments for different taxa including *Betula*, *Q. petraea* and *Q. robur* (Kürschner et al., 1997; Van Hoof et al., 2005b).

The complete Iberian Peninsula data for the CO_2 range from 295 ppmv to 370 ppmv followed the sigmoidal pattern, although the response limit at low CO_2 concentration was not reached in the present study. This hampered the use of a sigmoidal model to fit the data. However, the SI response over the CO_2 range from 295 ppmv to 330 ppmv, showed a linear decrease from 23% SI to 18.5% SI, which was best modelled as $SI = 71.09 - 0.16 \times CO_2$ (Table 2). At CO_2 concentrations above 330 ppmv, SI remained constant.
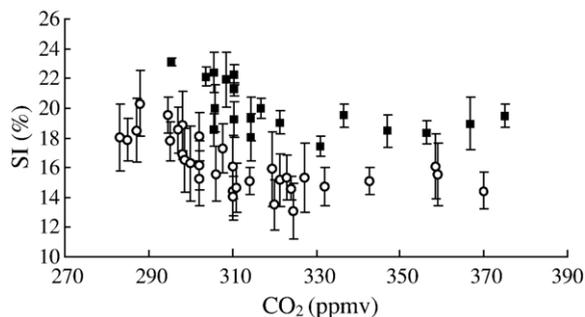

Fig. 6. *Q. robur* SI response to CO_2 changes for northern Europe (open circles), and for Iberian Peninsula (filled squares). Error bars: ±1 SI standard deviation.

Comparison of the Spanish *Q. robur* record and the Dutch record revealed a similar sigmoidal response trend (Fig. 6), where the response limit is reached between 320 ppmv and 330 ppmv at 15% SI for NW European material (Van Hoof et al., 2005b). The highly comparable decrease of stomatal sensitivity to changing CO_2 concentration at 330 ppmv in both data sets, supports earlier studies that suggest that several species have reached their SI response limit at present CO_2 values (Van de Water et al., 1994; Kürschner, 1997).

Between the CO_2 range of 295 ppmv and 330 ppmv, around 5% SI decrease was observed for both *Q. robur* data sets, with a − 0.16 slope for Spanish herbarium material versus − 0.14 for Dutch herbarium material supplemented with sub-fossil leaves (Van Hoof et al., 2005b). The calculated absolute SI values, however, were 3.5% consistently higher in the Iberian Peninsula material than in the northwest European *Q. robur* leaves (Fig. 6). Therefore, despite this difference in absolute SI levels, the highly similar response trends between Iberian and Dutch data sets enable the use of any SI calibration model to infer relative paleo-CO_2 changes.

A systematic offset between the two data sets implies principal differences that need further evaluation. As the material of both data sets were *Q. robur* leaves grown at comparable altitudes but different latitudes, environmental factors related to latitude is likely to explain SI changes. Contrasting light regimes have already been hypothesized to explain SI variations in several plants including *Q. petraea* and *Betula* sp. (Körner and Mayr, 1981; Tichá, 1982; Körner et al., 1986; Poole et al., 1996; Kürschner, 1997; Lockheart et al., 1998; Wagner et al., 2000; Kostina et al., 2001; Royer, 2001). Differences in radiation received have been shown to be a simple function of latitude for the same time of the day and of the year (Jones, 1992).

Considering latitudinal positions of the Iberian (≈ 43°N) and northwest Europe (≈ 52°N) material, the

3.5% SI difference in SI shown in Fig. 6 could be a result of the 5.68% W/m^2 annual irradiation increase at the northern Iberian Peninsula, compared to The Netherlands (calculated from month averages over January-1975 to December-2003; NOAA-CIRES, 2006). A weighted multiple linear regression model performed on the Dutch and <1000 m a.s.l. Iberian data over the CO_2 range from 295 to 330 ppmv resulted in a highly statistic significant relationship (R^2=0.77; F-Snedecor=63), between SI and the two independent variables (NOAA-CIRES month irradiance average (W/m^2) and CO_2 (ppmv)) (Table 2). This model permits the extension of the Q. robur stomatal-based CO_2 proxy to be used in low land regions where no calibration data set is available. The highly comparable SI response to CO_2 changes of Q. petraea (Fig. 3, Van Hoof et al., 2005b) suggests the applicability of this calibration model on Q. petraea fossil leaves found below 1000 m a.s.l., for the CO_2 range from 290 to 330 ppmv.

For further evaluation of the consistently higher SI values observed in the Spanish Q. robur leaves compared to those from northwest Europe, a number of additional anatomical parameters known to result from irradiance differences were analysed and compared. The results indicated smaller alveole areas, the presence of a dense layer of wax and more frequently occurring "stomatal twins" (Fig. 2) in Iberian Q. robur and Q. petraea material. Higher SI and the presence of more dense epicuticular waxes have already been reported as a low latitude and/or high luminosity adaptation (Osborn and Taylor, 1990; Kürschner, 1996). Early studies in 1904 by Zalenski (Tichá, 1982), showed that upper canopy leaves of nearly 50 plant species had more xeromorphic structures than lower leaves (e.g. smaller and more numerous stomata per unit leaf area, more dense leaf venation, smaller epidermical cells and more developed wax layer). Additional observations like higher Q. ilex L. specific leaf weight at higher locations (Filella and Peñuelas, 1999), and different sun and shade periclinal cell wall undulation in Q. petraea (Kürschner, 1997), also demonstrated that light environment during leaf development affects leaf morphology. These observations support the hypothesis that the presence of wax and the smaller alveole sizes are protection mechanisms from increasing UV-B at lower latitudes and also at high altitudes (Rozema et al., 1997).

4.2. Stomatal size responses

PL and SC, the indicative parameters for stomatal size, showed a clear increase for Q. robur under the CO_2 rise from 295 ppmv to 321 ppmv. Above 321 ppmv the response leveled off, thus showing the same response range as the measured SI values (Fig. 5). However, the increasing stomatal dimension response to increasing CO_2, that parallels SI reduction, needs further evaluation.

So far, changing leaf cell dimensions have been attributed to several environmental factors, e.g. irradiance, temperature and humidity or water availability, although some studies also attributed stomatal dimension changes to CO_2 concentrations (eg. Tichá, 1982; Körner et al., 1986; Bakker, 1991; Kostina et al., 2001; Royer, 2001). Evidence that the size development of leaf stomata is linked to changing CO_2 concentrations was provided by observations in birch stomata, where pore length increased by approximately 10% over the CO_2 range from 315 ppmv to 365 ppmv (Wagner et al., 1996). In this case however, the response limits for stomatal frequency parameters and pore dimension changes were not reached over the CO_2 interval studied. Leaf morphological analyzes of Myrtus communis L. and Juniperus communis L. grown under elevated CO_2 concentrations of 700 ppmv at natural CO_2 springs revealed significantly increasing pore dimensions (Tognetti et al., 2000).

In this study distinct changes in stomatal dimensions under continuously increasing CO_2 concentrations are demonstrated for Q. robur for the first time. The significantly improved correlation of SC data (Fig. 5), showed that the use of SC rather than SL or PL minimizes the influence of environmental factors other than CO_2 (water availability, irradiance, etc.) that may cause stomatal size expansion. Whether this pattern is also present in the Dutch Q. robur material is difficult to assess, since stomatal dimension measurements were not available for this data set. Nevertheless, SC calculations from PL and SL determinations provided for Q. petraea, M. communis and J. communis (Kürschner, 1996; Tognetti et al., 2000), did not show significant differences at above present CO_2 values.

Stomatal resistance models showed that decreasing stomatal frequency leads to higher stomatal resistance, while enlargement of stomatal pores has the opposite effect (Parlange and Waggoner, 1970; Jones, 1992; Guy, 2003). Therefore, increased stomatal resistance caused by reduced stomatal frequency in response to CO_2 increase is compensated by simultaneously increased stomatal size in the Iberian Q. robur. An index that combine both parameters [Stomatal Pore Area Index (SPI)=product of SD and SL] was linearly correlated to leaf hydraulic conductance (Sack et al., 2003). Hence, observed stability of SPI over the CO_2 range from 290 to 330 ppmv suggested that subsequent leaf hydraulic conductance is also constant. The product of SP and SI, which better account for leaf transpiration surface (Guy, 2003), shows

the same relative stability. However, transpiration rates were demonstrated to increase with smaller but higher number of stomata for a given transpiration surface (von Denffer et al., 1990, p 328). It can, thus, be hypothesized from observed changes on the anatomy (SC) and distribution of stomata (SI) that plant benefits from reducing stomatal size and increasing stomatal frequencies under low CO_2 values by improving the timing and efficiency of stomatal opening processes. Such an argument is in good agreement with previous observations of inversely related stomatal aperture and the size of guard cells with changing CO_2 (eg. Miglietta and Raschi, 1993). Since only few studies including stomatal dimensions are available, and mostly provide data from experiments under elevated CO_2 concentrations, further testing of this hypothesis is required.

5. Concluding remarks

SI palaeo-CO_2 calibrations were demonstrated to be useful in a limited altitudinal range by comparison of the least squares regression statistical fitting from different altitudinal ranges. For a restricted altitudinal range, atmospheric CO_2 shifts explained up to 76.96% of the 5% SI decrease from 295 to 330 ppmv CO_2 in Iberian *Q. robur* material. Weighted least squares CO_2–SI calibration regression attained can be used to infer past atmospheric environment from under 1.000 m a.s.l. altitude.

A comparison with Northwest European data showed that Spanish *Q. robur* leaves typically have more epicuticular waxes, smaller alveole areas and 3.5% higher SI. These observations are likely to be a result of the ~ 6% W/m^2 difference in annual irradiation at the contrasting latitudes. Nevertheless, the good agreement between the SI response rates of the Iberian and the Dutch training set clearly shows that the adaptation of *Q. robur* to changing CO_2 concentration is in principle the same. The inclusion of the monthly irradiance average (W/m^2) in the model provided for the incorporation of the Dutch data set resulting in a highly improved model ($R^2 = 77$; F-Snedecor$=63$).

The observed changes in the historical *Q. robur* leaf material from northern Spain supports the potential of the abundant, well-preserved Iberian sub-fossil leaves to be used as a palaeo-atmospheric CO_2 proxy. However, the present data emphasizes the need of adjusted calibration data sets meeting the specific requirements of this geographical area. Thus, SI–palaeo-CO_2 calibration given models are useful in a limited altitudinal and latitudinal range to reconstruct past atmospheric CO_2 levels.

PL and SL variations over the CO_2 range of 295 ppmv to 321 ppmv, suggested a relationship with atmospheric CO_2 conditions. The significantly improved correlation of SC to CO_2 showed that the use of the stomata–pore ratio rather than absolute lengths may reduce the overprint of environmental factors other than CO_2. As a result of the increasing atmospheric CO_2, parallel plant response by reducing SI and also increasing the stomatal pore proportion were found. No further response to anthropogenic CO_2 increase is expected for Iberian *Q. robur*, as stomatal response limits were reached between 320 ppmv and 330 ppmv CO_2 at 18.5% SI, 11.64 μm PL and 41.8% SC.

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