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ORIGINAL PAPER

Patterns of leaf morphology and leaf N content in relation to winter temperatures in three evergreen tree species

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Abstract The competitive equilibrium between deciduous and perennial species in a new scenario of climate change may depend closely on the productivity of leaves along the different seasons of the year and on the morphological and chemical adaptations required for leaf survival during the different seasons. The aim of the present work was to analyze such adaptations in the leaves of three evergreen species (*Quercus ilex, Q. suber* and *Pinus pinaster*) and their responses to between-site differences in the intensity of winter harshness. We explore the hypothesis that the harshness of winter would contribute to enhancing the leaf traits that allow them to persist under conditions of stress. The results revealed that as winter harshness increases a decrease in leaf size occurs in all three species, together with an increase in the content of nitrogen per unit leaf area and a greater leaf mass per unit area, which seems to be achieved only through increased thickness, with no associated changes in density. *P. pinaster* was the species with the most intense response to the harshening of winter conditions, undergoing a more marked thickening of its needles than the two *Quercus* species. Our findings thus suggest that lower winter temperatures involve an increase in the cost of leaf production of evergreen species, which must be taken into account in the estimation of the final cost and benefit balance of evergreens. Such cost increases would be more pronounced for those species that, like *P. pinaster*, show a stronger response to the winter cold.

Keywords Leaf density · Leaf mass per unit area · Leaf N content · Leaf thickness · Winter temperature gradient

Introduction

Research into the adaptations of plants to stress factors has long attracted the attention of scientists because plant adaptations are crucial in determining the functioning of ecosystems (Schulze and Mooney 1993; Wright et al. 2004). Artificial alteration of atmospheric chemistry further highlights this issue owing to the strong influence of plant production on the control of atmospheric CO_2 concentration. Thus, it is important to analyze foreseeable changes in the role of ecosystems in the carbon cycle. In this sense, the different strategies followed by the leaves of different tree species should receive special attention because the leaf traits of each species drive the carbon balance of ecosystems, both when the leaves are functional and after they have died (Gorham 1991).

One of the most important ecological consequences of climate change may be the replacement of current dominant species by others better adapted to the new conditions (Walther et al. 2002). However, more important than the mere replacement of some species by others is the possible change in leaf habits, in particular, the replacement of deciduous species by evergreen ones, or vice versa, that could occur if seasonal changes alter the carbon balance of such leaf habits in the opposite direction. In principle, milder winter conditions associated with global warming should favor evergreens at the expenses of deciduous species (Givnish 2002; Walther 2000), but the number of factors involved in the patterns of distribution of both leaf habits is very large (Givnish 2002), making it extremely difficult to predict future

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directions of change. Changes in leaf strategies may have profound effects on landscape, phenology, gas exchange, soil properties, etc. More importantly, the replacement of certain species by others with different leaf habits could affect the carbon cycle and the global response to climate change.

The competitive equilibrium between deciduous and evergreen tree species depends strongly on leaf productivity along the different seasons of the year and on the morphological and chemical adaptations necessary for leaf survival during the different seasons. The most transcendental changes as regards the deciduous-evergreen competitive balance in temperate climates will probably occur during the winter. During the warm seasons of the year, both groups of species maintain active leaves. Therefore, the possible climatic alterations that occur during the warm period would affect the leaves of both deciduous and evergreen species, although their responses could be different. However, the environmental changes occurring during colder periods of the year, in which deciduous species are devoid of leaves, would presumably lead to differences in the final leaf carbon balance and the competitive relations between both groups of species. Indeed, it is the climatic conditions prevailing in winter that are subject to a more pronounced and rapid change (IPCC 2007; Kreyling 2010). Despite this, however, there are very few studies that have addressed the implications of climate change during the winter for leaf adaptations. Among them, those carried out in temperate regions are particularly scarce (Kreyling 2010).

It is well known that the differences in photosynthesis and respiration between evergreen and deciduous species are associated with some leaf traits that help long-lived leaves to support the stressful conditions prevailing during the more unfavorable seasons of the year (Mediavilla et al. 2001; Takashima et al. 2004). Among the morphological traits that undergo the most pronounced change among leaf habits is leaf mass per unit area (LMA), which has a huge number of implications as regards leaf productivity and physiology (Wright et al. 2002). The variations in LMA and other morphological leaf traits have mainly been analyzed with respect to differences in nutrient and drought stress (Turner 1994a; Wright et al. 2002). It has also been observed that temperature may affect LMA directly (Atkin et al. 2008; Oliveira and Peñuelas 2000), although most of these analyses have been based on data obtained in young plants in controlled environments and this is not necessarily representative of how mature plants would respond to temperature in the field. There are some examples, such as that of Ogaya and Peñuelas (2007), who have shown in *Quercus ilex* that populations from colder sites tend to maintain a higher LMA than those from warmer areas. However, in general few studies have addressed the variations in leaf traits in different populations of the same tree species along environmental gradients (Jian et al. 2009; Uğurlu and Oldeland 2010). Accordingly, it is crucial to study these trends better in field conditions and in adult trees.

The general aim of the present work is to study the effect of differences in climate harshness during the colder seasons of the year on the leaf traits of three evergreen woody species widely distributed on the Iberian Peninsula (*Quercus ilex* L., *Q. suber* L. and *Pinus pinaster* Aiton). We attempt to contrast the hypothesis that the rigours of winter contributes to enhance the leaf traits that are responsible for leaf persistence. If the leaf traits of persistence were only associated with drought stress, there should be no large differences between coexisting deciduous and evergreen species in temperate zones, since during the hottest and driest months of the year both deciduous and evergreen species maintain active leaves. Instead, it must be winter conditions that govern the main morphological differences between deciduous and evergreen leaves. In any case, any variation in winter harshness could induce an acclimatization of leaf morphology and chemistry in evergreen species, which in turn would affect their photosynthetic capacity.

Materials and methods

Study area and sampling species

Three evergreen woody species were studied: *Pinus pinaster* Aiton, a gymnosperm with an average leaf life span about 52 months, and *Quercus suber* L. and *Q. ilex ssp ballota* (Desf.) Samp, with 15 and 24 months, respectively, of mean leaf life span (data taken from Mediavilla and Escudero 2003).

The species were distributed on 12 plots located in the regions of Castilla-Leon and Extremadura (central-western Spain), between latitudes 41° 45′ N and 40° 01′ N and between longitudes 6° 07′ W and 2° 08′ W (Fig. 1). The sites consisted of flat areas with sparse populations (between 50 and 100 specimens/ha) of mature (more than 100 years old) individuals. Trunk diameter at 1.3-m height ranged from 20 to 60 cm and mean heights were 4–10 m. Each site was selected so as to include as many study species as possible and to cover a wide gradient in winter temperature, although taking care that the rest of the climate characteristics would be as homogeneous as possible. Nevertheless, there is a trend to have higher rainfall levels in the hottest and southernmost sites, which helps to reduce the differences in the intensity of drought stress among cold and hot sites (Table 1). Owing to its lower tolerance to low temperatures, *Q. suber* was absent at the coldest sites.

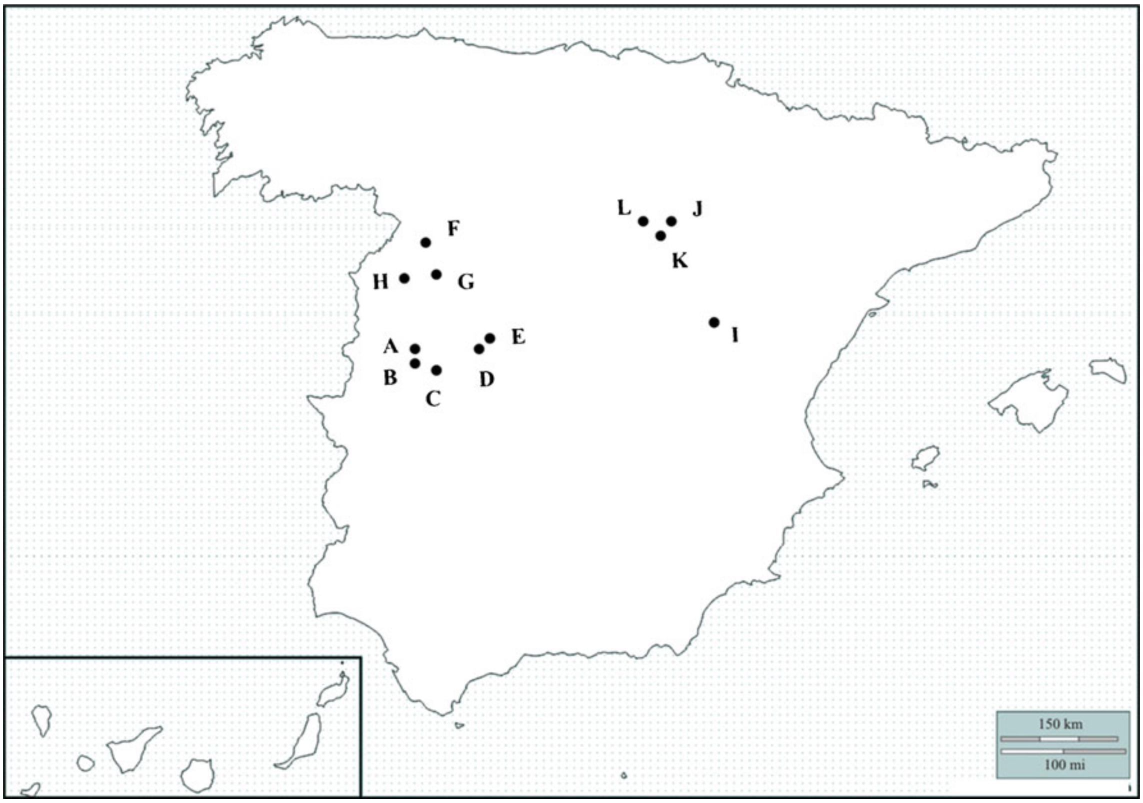

Fig. 1 Map of the study area showing location of study populations

Annual rainfall data from the station nearest to each plot were provided by the National Meteorological Institute of Spain. Temperature data for each site were obtained 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 they were kept at each site along two years (October 2008–October 2010). Two soil samples were taken from each plot, one at the surface (excluding the forest floor) and one at a depth of 50 cm. Determinations of granulometry, pH and the concentrations of the main mineral nutrients were carried out at the Soils Laboratory of the Institute of Natural Resources and Agrobiology in Salamanca according to the methods described by Bremner (1960), Chapmann and Pratt (1973) and Walkley and Black (1934).

Measurements of leaf morphology and chemistry

At each site, four or five mature specimens of each species were selected randomly on each sampling occasion. A composite sampling of sun-exposed branches with leaves from different crown positions in each canopy was undertaken for each individual selected. Samples were taken during three different periods of the year (autumn, winter and end of spring–beginning of summer) from October 2008 to October 2010, providing two-year data for each sampling date.

The samples were immediately taken to the laboratory and the branches were separated into annual segments (shoots) of different age classes. Only one flush of leaf growth was observed in all species. Accordingly, all the leaves born in one particular year were considered to belong to the same age class. All the shoots bearing leaves of a given age were identified as belonging to the same age class. Fifty individual leaf samples for each species and leaf age class were finally selected at each plot and sampling date for the morphological and chemical analyses. Temperature is known to exert a marked effect on the time of leaf emergence (Chmielewski and Rötzer 2001). Accordingly, the data corresponding to the spring period for the current-year leaf cohort were excluded in order to avoid possible differences among the plots due to the different state of development of the recently emerged leaves.

Leaf thickness was measured with a digital micrometer (Digimatic micrometer, Mitutoyo, Japan), as a mean of three leaf measurements taken at random positions on each leaf or needle, avoiding the main ribs on flat leaves. The total projected leaf and needle areas were determined by an image analysis system (Delta-T Devices LTD, Cambridge, UK). The leaf volume of flat leaves was calculated as the

Table 1 Site characteristics of the different experimental plots where leaf traits were measured

Characteristics	Plots											
	A	B	C	D	E	F	G	H	I	J	K	L
Longitude (W)	5° 57′	5° 48′	5° 42′	5° 02′	5° 01′	5° 52′	5° 47′	6° 07′	2° 08′	2° 37′	2° 43′	2° 52′
Latitude (N)	40° 13′	40° 01′	40° 00′	40°11′	40° 16′	41° 14′	40° 08′	40° 55′	40° 59′	41° 45′	41° 32′	41° 43′
Altitude (m.a.s.l .)	619	466	261	449	748	985	834	832	1246	1189	982	1045
Mean temperature (°C)												
Annual	16.3	16.6	16.6	13.7	14.5	11.7	12.1	12.5	9.72	10.3	11.5	10.5
Spring	16.9	17.7	15.7	15.4	15.9	13.5	13.6	13.5	10.7	11.3	13.2	11.6
Summer	25.4	26.1	26.2	22.1	24.4	20.7	21.8	22.0	19.5	19.4	18.6	19.5
Autumn	12.0	11.9	11.5	9.79	9.64	7.31	8.44	8.05	5.98	4.69	6.47	4.88
Winter	9.20	9.24	8.55	7.52	6.35	5.15	5.20	5.79	2.55	2.72	3.67	3.28
Absolute minimum temperature	−2.15	−3.91	−5.50	−5.99	−6.35	−7.27	−9.82	−9.73	−11.3	−11.9	−15.0	−16.9
Mean daily minimum temperature of the coldest month	3.71	3.52	2.16	2.46	0.26	−0.34	−1.12	−1.98	−2.74	−2.86	−2.66	−3.09
Number of days with frost per year	7	11	14	17	26	55	63	40	100	86	110	112
Mean duration of the period with frosts (days per year)	30	80	94	94	126	153	184	201	191	203	246	203
Precipitation (mm)												
Annual	1320	977	1118	873	1078	521	368	380	392	485	652	478
Summer	82	42	57	61	43	42	51	45	52	66	71	53
Soil												
Sand content (%)	81.1	77.2	75.7	73.0	81.9	84.6	74.3	67.9	57.8	75.1	84.8	84.0
Clay content (%)	8.5	11.7	10.6	12.9	9.2	6.4	12.2	16.2	22.8	14.4	8.1	8.3
Silt content (%)	10.4	11.1	13.7	14.1	8.90	9.00	13.5	15.9	19.4	10.5	7.10	7.70
Total soil N content (%)	0.082	0.143	0.126	0.085	0.100	0.072	0.021	0.078	0.112	0.115	0.040	0.048
M.O. (%)	1.66	4.47	2.78	2.88	4.87	2.60	0.37	1.66	1.96	5.12	0.69	1.92
pH	5.7	4.5	5.1	5.8	5.4	4.6	4.5	4.4	7.6	4.8	7.0	6.1
Species	Qi, Qs	Qs	P, Qi, Qs	P, Qi, Qs	P, Qi	P, Qi, Qs	Qi, Qs	Qi, Qs	Qi	P	P, Qi	P, Qi

P = *Pinus pinaster*, Qi = *Quercus ilex*, Qs = *Quercus suber*

product of mean leaf thickness x leaf area. The transverse cross-sectional area of needles was measured by amplified scanning images, and needle volume was estimated as the product cross-sectional area x needle length. The samples were then oven-dried at 70°C to constant mass and the total dry mass was determined. From the data thus obtained, we calculated the leaf dry mass per area (LMA) and leaf tissue density (dry mass/volume, LTD). Leaf N concentrations were determined with a CE-Instruments NA-2100 autoanalyser (ThermoQuest, Milan, Italy), and expressed as N per unit leaf mass (N_{mass}, mg g^{-1}) and N per unit leaf area (N_{area}, g m^{-2}), these latter data obtained as the nutrient content per unit dry mass multiplied by LMA.

Data analysis

The relationships between leaf traits and the different temperature and precipitation measurements were described by means of linear regression analysis, using the average leaf trait values obtained for each leaf type at each site on each sampling occasion. To better explore between-site differences in leaf traits, we assigned a single value to each site for mean monthly temperature and mean monthly maximum and minimum daily temperatures, obtained as an average of the values measured for each month along the study period. In the same way, for each site we obtained a single value for the absolute maximum and minimum temperatures, the number of days with frost and the annual rainfall. The test for significantly different slopes in an analysis of covariance (ANCOVA) was used to determine when the slopes of the change in each leaf trait with temperature change were significantly different between the different species. All statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). For all statistical determinations, significance levels were established at $P<0.05$.

Results

Patterns of leaf morphology in relation to winter temperatures

The three species studied showed significant differences among sites in several leaf traits analyzed that were related to differences in winter severity. In all species LMA and leaf thickness were the two traits that showed the most pronounced response to changes in temperature, and especially to the harshening of winter climatic variables, since the minimum temperatures and the number of days with frost were the two variables that best accounted for the greater percentage of variation observed among sites in thickness and LMA (Table 2 and Fig. 2). In contrast, in many cases the maximum temperatures did not have significant effects on leaf morphology. Temperature also affected leaf size, the lowest temperatures giving rise to the smallest leaves and shorter needles (Table 2). Leaf tissue density (LTD) was the only trait that did not show any definite trend along the thermal gradient for any type of leaf (Table 2). Therefore, the reinforcement of the leaves with a greater LMA as the harshening of winter conditions progressed seems to occur only through leaf thickening, with no associated changes in the LTD. Although the warmest plots tended to have higher rainfall levels (Table 1), the differences in LMA among the populations of a given species were independent of the differences in precipitation among sites in an analysis of multiple regression with the number of days with frost as the other independent variable (Table 3). The only exception was the two-years-old leaf class of *Q. ilex*.

The slopes of the regression lines of leaf thickness against temperature (mean monthly daily temperature, mean monthly minimum daily temperature and minimum winter temperature) and the number of days with frost did not differ for the two *Quercus* species (data not shown), but were significantly higher in *Pinus* than in both *Quercus* species (Table 4). This suggests that *P. pinaster* reinforces its leaves against a decrease in temperature to a greater extent than *Quercus ilex* and *Q. suber*.

Patterns of N concentrations in relation to winter temperatures

The differences in leaf morphology in response to an increase in winter harshness were accompanied by differences in the leaf nitrogen content. N_{area} responded to the changes in temperature in all three species, increasing among plots with the intensity of their winter harshness (lower minimum temperatures and a higher number of days with frost) (Table 5). However, the maximum temperatures again either had no influence or the effect was less significant than that of winter harshness (Table 5). The differences in N_{area} were the result of the increase in LMA associated with the decrease in temperatures, since N_{mass} did not vary significantly among sites in most types of leaf (data not shown). No significant relationships were observed between N_{mass} and the different estimates used to describe winter harshness in any case (data not shown). The nitrogen concentration (N_{mass}) decreased with leaf age (data not shown). However, the same trends among sites were repeated in all the age classes (Table 5). In this case, the results of the ANCOVA revealed that there were no significant differences in the response slopes of N_{area} to the different estimates used to measure the intensity of winter harshness for any of the three species (data not shown).

Discussion

The three species studied here showed significant differences among sites in several of the leaf traits analyzed that were related to differences in the intensity of winter harshness among the sites. Lower temperatures during the colder months resulted in smaller leaves with a greater LMA in all species. Both traits figure among the leaf traits that have most often been seen to vary with temperature and/or precipitation (Jonas and Geber 1999; Li et al. 2006). The smaller size and the greater LMA normally shown by the leaves at the drier sites have been interpreted as a mechanism that allows leaves to increase their resistance to drought and improve their water use efficiency (Niinemets 2001; Turner 1994b). Less clear, however, are the results obtained concerning the differences in temperature. Some authors have reported that LMA increases with temperature (Chabot and Chabot 1977; Wright et al. 2004). In contrast, working with artificial heating chambers, Suzuki and Kudo (1997) failed to detect any significant differences in LMA and leaf size under the experimental conditions of the chamber with respect to control plants. Nevertheless, other studies have provided evidence that low temperatures induce decreases in leaf size and higher LMA values (Ogaya and Peñuelas 2007; Weih and Karlsson 2001), as was seen in the present work. In fact, the effects of winter temperatures on leaf traits were much more significant than the effects of maximum summer temperatures and total annual precipitation in the present study.

Leaf tissue density was the only trait that in no case showed any given trend along the thermal gradient for any of the leaf types. LMA depends on thickness and/or density (Niinemets 1999; Witkowski and Lamont 1991). However, it has repeatedly been shown that both traits may vary independently, such that LTD is sometimes the main determinant of the changes observed in LMA (Poorter et

Table 2 Line regression parameters for different morphological leaf traits depending on different climatic variables

Parameters	Mean leaf size				Leaf thickness (μm)				Leaf density (mg cm^{-3})				Leaf mass per unit area (g m^{-2})			
	Slope	y-intercept	R^2	*P*	Slope	y-intercept	R^2	*P*	Slope	y-intercept	R^2	*P*	Slope	y-intercept	R^2	*P*
Q.suber (C) (*n* = 28)																
mean m Tª (°C)	0.42	−0.42	0.27	0.0115	−7.43	339	0.48	0.0002	−3.79	805	0.08	NS	−6.72	269	0.43	0.0004
max Tª (°)	0.01	4.41	0.01	NS	−1.04	288	0.05	NS	−1.06	801	0.04	NS	−0.99	225	0.05	NS
min Tª (°C)	0.33	6.66	0.36	0.0013	−6.54	207	0.59	<0.0001	−2.33	750	0.12	NS	−6.06	150	0.61	<0.0001
F days	−0.02	6.11	0.41	0.0002	0.35	218	0.70	<0.0001	0.10	755	0.10	NS	0.30	162	0.66	<0.0001
Q.ilex (C) (*n* = 40)																
mean m Tª (°C)	0.16	0.56	0.52	<0.0001	−8.35	390	0.51	<0.0001	−5.29	859	0.08	NS	−7.81	324	0.51	<0.0001
max Tª (°)	0.06	0.23	0.20	0.0162	−2.09	378	0.11	NS	−1.67	865	0.03	NS	−2.52	334	0.19	0.0104
min Tª (°C)	0.07	3.10	0.46	<0.0001	−4.78	248	0.64	<0.0001	−2.66	774	0.07	NS	−4.51	192	0.72	<0.0001
F days	−0.01	2.93	0.51	<0.0001	0.30	256	0.75	<0.0001	0.18	777	0.09	NS	0.27	201	0.74	<0.0001
Q.ilex (C+1) (*n* = 60)																
mean m Tª (°C)	0.12	0.76	0.47	<0.0001	−7.76	392	0.54	<0.0001	−0.74	866	0.01	NS	−7.46	343	0.65	<0.0001
max Tª (°)	0.05	0.17	0.28	<0.0001	−2.86	415	0.25	0.0002	0.25	848	0.01	NS	−2.43	353	0.24	0.0003
min Tª (°C)	0.07	2.78	0.42	<0.0001	−4.22	265	0.61	<0.0001	−0.30	855	0.01	NS	−3.67	224	0.60	<0.0001
F days	−0.01	2.69	0.56	<0.0001	0.26	273	0.70	<0.0001	0.03	854	0.01	NS	0.23	229	0.67	<0.0001
P. pinaster (C) (*n* = 28)																
mean m Tª (°C)	6.87	53	0.32	0.0034	−20.2	1321	0.28	0.0096	−1.61	463	0.03	NS	−8.03	470	0.31	0.0058
max Tª (°)	3.22	0.99	0.13	NS	−4.80	1299	0.03	NS	−0.33	459	0.01	NS	−1.56	447	0.02	NS
min Tª (°C)	4.12	168	0.39	0.0003	−11.5	983	0.64	< 0.0001	−0.67	439	0.03	NS	−5.03	329	0.62	< 0.0001
F days	−0.25	162	0.45	< 0.0001	0.77	997	0.77	< 0.0001	−0.03	442	0.01	NS	0.31	338	0.73	< 0.0001
P. pinaster (C+1) (*n* = 42)																
mean m Tª (°C)	5.53	81	0.20	0.0030	−14.8	1350	0.32	0.0001	−2.81	497	0.03	NS	−7.21	502	0.45	< 0.0001
max Tª (°)	2.53	40	0.09	NS	−0.47	1211	0.01	NS	−0.70	494	0.01	NS	−0.49	447	0.01	NS
min Tª (°C)	3.82	177	0.42	< 0.0001	−9.47	1091	0.71	< 0.0001	0.04	467	0.0002	NS	−3.74	388	0.57	< 0.0001
F days	−0.22	167	0.39	< 0.0001	0.60	1110	0.74	< 0.0001	0.06	460	0.02	NS	0.25	394	0.73	< 0.0001
P. pinaster (C+2) (*n* = 42)																
mean m Tª (°C)	9.03	48	0.40	< 0.0001	−21.0	1463	0.56	< 0.0001	−1.17	479	0.01	NS	−6.83	521	0.55	< 0.0001
max Tª (°)	4.87	47	0.26	0.0010	−4.89	1435	0.07	NS	0.51	446	0.01	NS	−0.83	479	0.02	NS
min Tª (°C)	4.76	192	0.51	< 0.0001	−9.21	1150	0.62	< 0.0001	0.21	468	0.01	NS	−3.34	413	0.65	< 0.0001
F days	−0.29	182	0.53	< 0.0001	0.58	1166	0.68	< 0.0001	0.02	464	0.02	NS	0.21	420	0.71	< 0.0001

C current-year leaves, *NS* not significant

Leaf size = area (cm^2) for *Quercus* spp; needle length (cm) for *Pinus*

mean m Tª = mean monthly temperature; min Tª = minimum winter temperature; max Tª = maximum summer temperature; F days = total days with frost over study period

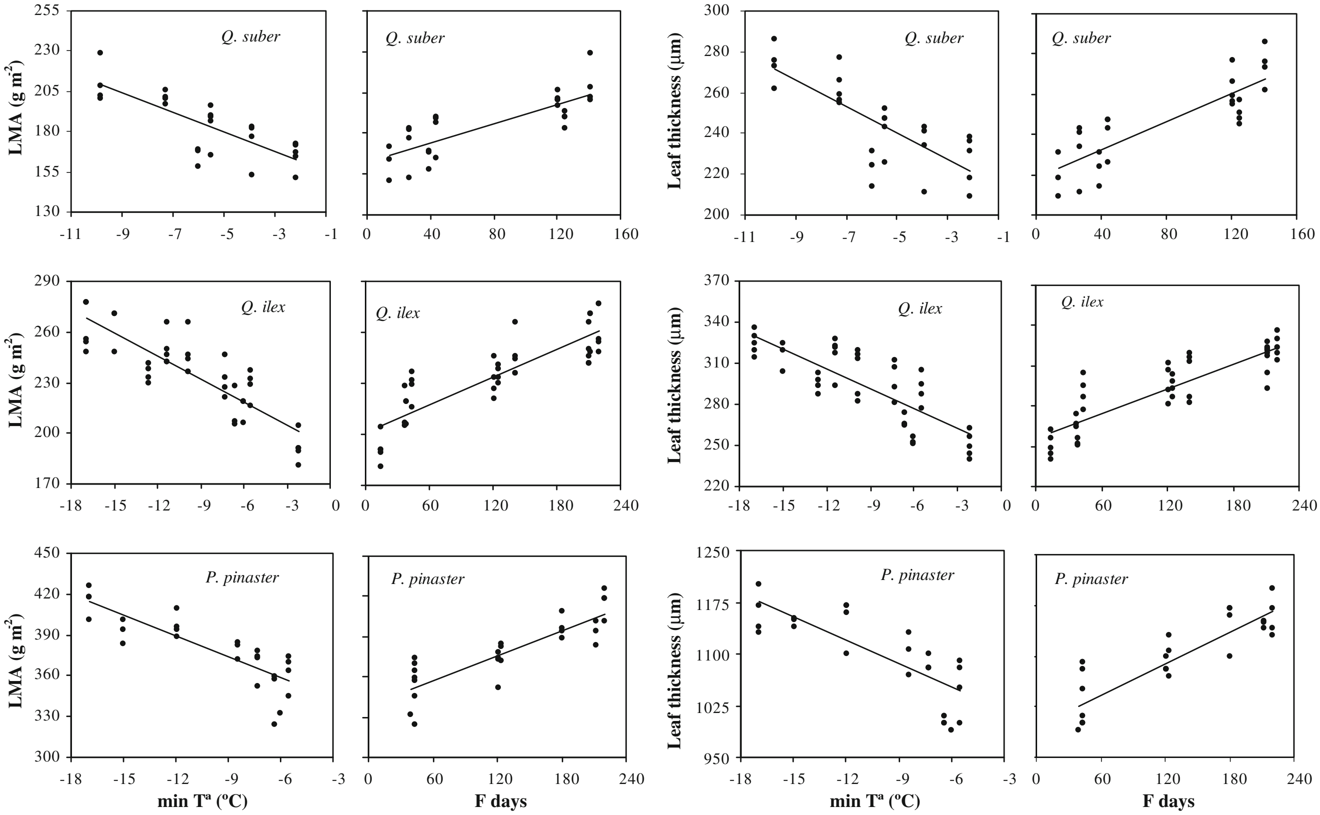

Fig. 2 Relationships between leaf morphological traits (leaf mass per unit area [LMA] and leaf thickness) and different estimates of winter harshness for the current-year leaves of three evergreen species. Min Tª = absolute minimum temperature (°C) registered along the study period. F days = total number of days with frost along the study period

Table 3 Multiple regression of the mean leaf mass per unit area of each leaf type (LMA, g·m^{-2}) against total days with frost over the study period (F days) and total annual precipitation (mm)

Leaf sample	Dependent variable	Independent variables	R^2	F	b	Beta coefficients	P
Q.suber (C)	LMA	F days	0.69	19.04	0.416	1.23	0.0150
		Annual rainfall			0.021	0.428	0.3580
		Intercept			28.5		0.0001
Q.ilex (C)	LMA	F days	0.75	55.42	0.245	0.778	0.0001
		Annual rainfall			−0.007	−0.102	0.4780
		Intercept			209		0.0001
Q.ilex (C+1)	LMA	F days	0.71	70.80	0.141	0.515	0.0001
		Annual rainfall			−0.023	−0.365	0.0070
		Intercept			257		0.0001
P. pinaster (C)	LMA	F days	0.73	29.75	0.339	0.91	0,0001
		Annual rainfall			0.070	0.08	0.6690
		Intercept			330		0.0001
P. pinaster (C+1)	LMA	F days	0.75	52.60	0.192	0.67	0.0001
		Annual rainfall			−0.017	−0.232	0.1070
		Intercept			413		0.0001
P. pinaster (C+2)	LMA	F days	0.71	40.50	0.229	0.932	0.0001
		Annual rainfall			0.007	0.107	0.5490
		Intercept			411		0.0001

C current-year leaves

Table 4 Analysis of covariance for leaf thickness of the current leaf cohort of *Pinus pinaster*, *Quercus suber* and *Q. ilex* . Factors in the model included species and different estimates of winter cold stress (mean monthly temperature; mean monthly minimum daily temperature; minimum winter temperature and total number of days with frost over study period) as the covariate

Comparison	Source	df	Sum of squares	Mean squares	*F* value	*P* value
Quercus – Pinus	Species	1	478,232	478,232	276	< 0.0001
	mean m T^a (°C)	1	59,408	59,408	34.3	< 0.0001
	Species x mean m T^a	1	7,584	7,584	4.38	0.0399
	Residual	75	130,017	1,734		
Quercus – Pinus	Species	1	2,052,520	2,052,520	1315	< 0.0001
	mean m min T^a (°C)	1	72,078	72,078	46.2	< 0.0001
	Species x mean m min T^a	1	9,899	9,899	6.34	0.0139
	Residual	75	117,083	1,561		
Quercus – Pinus	Species	1	2,240,124	2,240,124	930	< 0.0001
	min T^a (°C)	1	67,660	67,660	43.2	< 0.0001
	Species x min T^a	1	9,826	9,826	5.47	0.0214
	Residual	52	102,716	1,575		
Quercus – Pinus	Species	1	2,315,595	2,315,595	1656	< 0.0001
	F days	1	86,090	86,090	61.6	< 0.0001
	Species x F days	1	10,120	10,120	7.24	0.0088
	Residual	75	104,839	1,398		

mean m T^a = mean monthly temperature; mean m min T^a = mean monthly minimum daily temperature; min T^a = minimum winter temperature; F days = total days with frost over study period

al. 2009), while on other occasions it is leaf thickness that is the most strongly correlated with LMA (Niinemets 1999). In fact, some authors have failed to obtain any correlation at all between thickness and LMA (Castro-Díez et al. 2000) or, as in the present case, between LTD and LMA (Coste et al. 2005). In the present study the strengthening of the leaf with a greater LMA as winter harshness increased only seemed to occur through a thickening of the leaves, with no associated changes in their density. In sum, therefore, in our species the cold triggered a decrease in leaf size and an increase in thickness.

The reduction in leaf dimensions leads to a better coupling of the leaf with the atmospheric environment, owing to cooling due to convective heat loss and a reduction in the resistance of the boundary layer to transpirational water losses (Fitter and Hay 2002; Nobel 2005). Traditionally it has been considered that the decrease in differences in temperature between the leaf and the surrounding air in smaller leaves is more important than the decrease in the resistance of the boundary layer in limiting the exchange of water vapour, which would result in fewer transpirational losses in smaller leaves (Nobel 2005). Accordingly, most authors have tended to assume that leaves should be smaller in environments susceptible to water stress (hotter and drier) (Lo Gullo and Salleo 1988; Turner 1986). Despite this, in recent studies the effects of the decrease in size have been interpreted in exactly the opposite way, suggesting that in cold climates lower leaf size and a reduced resistance of the boundary layer would involve a greater potential to lose water rapidly (Yates et al. 2010). Smaller dimensions and the consequent ability to transpire rapidly could be a trait clearly favorable for nutrient acquisition during the winter, especially in the case of species growing on sites subject to a nutrient deficit. In light of the evident connection between water flow and nutrients (Tinker and Nye 2000), any trait that will increase water flow would be potentially advantageous in such environments. In the present study soil fertility did not show any evident trend along the thermal gradient, although it has been reported that as temperatures decrease it is harder for the plants to absorb nutrients owing to the decrease in soil temperature (Jian et al. 2009; Weih and Karlsson 1999).

Regarding leaf thickness, different authors have indicated that the changes induced by the cold are due to a modification in the size and expansion patterns of mesophyll cells, and, in particular, to a thickening of the cell walls. Several works have shown that the increase in cell walls is a characteristic trait of leaves growing in cold conditions (Griffith and Brown 1982; Kubacka-Zebalska and Kacperska 1999). Thicker cell walls, according to some authors, would propitiate a change in the expansion of mesophyll cells from a longitudinal direction to a perpendicular one, contributing to the decrease in leaf area in colder climates (Stefanowska et al. 1999). The amount of cell wall seems to be related to the response capacity of

Table 5 Linear regression parameters for leaf N_{area} ($g \cdot m^{-2}$) depending on different climatic variables

Parameter			*Q. suber* (C) (*n* = 28)					
			Slope	y-intercept	R^2	*P*		
mean m T^a (°C)			−0.13	4.25	0.47	0.0003		
mean m max T^a (°C)			−0.07	4.03	0.23	0.0316		
mean m min T^a (°C)			−0.11	3.37	0.50	< 0.0001		
max T^a (°)			−0.03	3.64	0.11	NS		
min T^a (°C)			−0.10	2.03	0.45	0.0006		
F days			0.01	2.23	0.58	< 0.0001		
Parameter	*Q. ilex* (C) (*n* = 40)				*Q. ilex* (C + 1) (*n* = 60)			
	Slope	y-intercept	R^2	*P*	Slope	y-intercept	R^2	*P*
mean m T^a (°C)	−0.13	4.43	0.47	< 0.0001	−0.10	4.06	0.31	0.0006
mean m max T^a (°C)	−0.08	4.37	0.24	0.0243	−0.06	3.90	0.12	NS
mean m min T^a (°C)	−0.13	3.69	0.61	< 0.0001	−0.11	3.51	0.49	< 0.0001
max T^a (°)	−0.04	4.56	0.17	NS	−0.02	3.72	0.05	NS
min T^a (°C)	−0.08	2.27	0.60	< 0.0001	−0.07	2.24	0.47	< 0.0001
F days	0.01	2.44	0.62	< 0.0001	0.01	2.40	0.48	< 0.0001
Parameter	*P. pinaster* (C) (*n* = 28)				*P. pinaster* (C + 1) (*n* = 42)			
	Slope	y-intercept	R^2	*P*	Slope	y-intercept	R^2	*P*
mean m T^a (°C)	−0.15	5.06	0.34	0.0011	−0.15	5.79	0.23	0.0196
mean m max T^a (°C)	−0.12	5.43	0.17	0.0416	−0.14	6.62	0.12	NS
mean m min T^a (°C)	−0.08	2.57	0.45	< 0.0001	−0.12	4.69	0.32	< 0.0001
max T^a (°)	−0.03	4.55	0.02	NS	−0.04	5.55	0.03	NS
min T^a (°C)	−0.10	2.38	0.44	< 0.0001	−0.09	2.91	0.42	< 0.0001
F days	0.01	2.58	0.47	< 0.0001	0.01	3.24	0.44	< 0.0001

C current-year leaves, *NS* = not significant

mean m T^a = mean monthly temperature; mean m min T^a = mean monthly minimum daily temperature; mean m max T^a = mean monthly maximum daily temperature; min T^a = minimum winter temperature; max T^a = maximum summer temperature; F days = total days with frost over study period

plants to freezing, helping to increase cold tolerance through a reduction in the freezing rate (Rajashekar and Lafta 1996; Solecka and Kacperska 2003).

The morphological changes occurring in response to an intensification of winter harshness were also accompanied by changes in the nitrogen content per unit leaf area, with higher values at the colder plots, where the LMA was also greater. The inverse relationship between the mean air temperature and the leaf nitrogen concentration has been reported by many authors, both under field conditions (Morecroft and Woodward 1996; Weih and Karlsson 2001), and when working in environments subjected to artificial heating (Parsons et al. 1994; Suzuki and Kudo 1997). Higher N_{area} could arise as a passive consequence of the weaker dilution of nitrogen in the smaller area of leaves at cold sites, which could result in a smaller leaf area per unit soil surface area (Friend and Woodward 1990; Körner and Larcher 1988). However, a higher N content at lower temperatures could also reflect an adaptation aimed at increasing the metabolic activity and growth rates under such unfavorable conditions (Oleksyn et al. 1998; Woods et al. 2003). Low temperatures impose an important limitation to photosynthesis and a lowered efficiency in different physiological processes. A higher N content would allow the leaves, at least in part, to compensate that limitation at colder sites (Jian et al. 2009; Reich and Oleksyn 2004).

All these temperature-related changes have profound ecological implications. The evergreen habit demands that leaves must be present throughout the year, overcoming unfavorable seasons, among them winter. If, as is suggested by our observations, a greater LMA is necessary for plants to resist the challenge of harsher winter conditions, it could be deduced that in order to maintain their leaf longevity evergreen species must reinforce their leaves to a greater extent at the coldest sites. Said another way, the evergreen habit would imply higher costs at the coldest sites. A greater LMA requires a larger amount of plant material to achieve a given light interception area and hence leads to an increase in the costs of construction and maintenance of leaf area (Westoby et al. 2000). We also observed that the production of a given leaf area involves a greater amount of N at cold sites. However, the greatest costs associated with a larger LMA essentially derive from its negative relationship with instantaneous CO_2 assimilation (Mediavilla et al. 2001; Niinemets and Sack 2006). A greater allocation of biomass to structural versus photosynthetic components (Vitousek et al. 1990) together with greater internal competition and resistance to the transfer of CO_2 (Syvertsen et al. 1995) have been proposed as two of the factors responsible for the lower carbon assimilation rates in leaves with a greater LMA. In addition, lower allocation of N to photosynthetic versus non-photosynthetic leaf compounds in leaves with higher LMA values has also been reported (Niinemets 1999). For example, it is known that cell walls accumulate a significant amount of nitrogenous compounds, accounting for up to 10% of cell wall materials (Reiter 1998). According to our observations, the greater LMA of leaves growing at cold sites can be explained in terms of their thickness, which would be achieved by a thickening of the cell walls. If this were the case, a greater amount of available N should be assigned to cell walls in environments with harsher winters, leading to a reduction in the amount available for allocation to photosynthetic proteins and—necessarily—to a decrease in assimilation rates.

If it is indeed true that in cold climates the negative effects of high leaf longevity on photosynthesis per unit time are enhanced and the costs of the evergreen habit are greater, this would imply a disadvantage for evergreen species with respect to deciduous ones in such conditions. In principle, this disadvantage would be greater for those species that show a more pronounced response to the cold. Our findings indicate that *Pinus pinaster* was the species that most reinforced its leaves in response to winter harshness, undergoing a more marked leaf thickening than in the case of the two *Quercus* species. This suggests that in cold climates maintaining their leaves alive during the winter period would involve greater costs for *P. pinaster*.

It is difficult to extrapolate the responses observed here in a new scenario of climate change. In light of the present observations, any variation in winter harshness as a result of climate change could induce an acclimatization of leaf morphology and chemistry in evergreen species, which would in turn affect their photosynthetic capacity. In contrast, deciduous species, which only keep their leaves alive during part of the year, can respond to any climatic change merely by altering their phenology to better suit the temporal distribution of conditions favorable for their survival and production. Thus, the responses of deciduous and evergreen species to climate change should be drastically different, which probably will affect the competitive relations between both groups of species. The morphological responses to cold of the evergreen species studied by us were also different in intensity for the different species. Accordingly, these different responses may also alter the competitive relationships among the different evergreen species and hence the patterns of distribution and species composition of forest ecosystems. If climate change eventually leads to an increase in temperatures and a decrease in winter harshness (IPCC 2007), this could reduce the unfavorable effects associated with greater thickness and LMA shown by evergreen species under conditions of low temperatures. The reduction in unfavorable effects would be more marked for species that, like *P. pinaster*, display more intense responses to the winter cold. However, it has been also suggested that while mean wintertime temperature will significantly increase by the end of the 21st century, extreme cold events will not disappear (Kodra et al. 2011). Possible changes in leaf morphology in evergreen species as a response to increases in mean winter temperature could contribute to increases in leaf mortality during extreme cold events, making it more difficult to predict the ecological consequences of climate change. In any case, our data suggest that lower winter temperatures in colder sites would involve higher costs of construction and maintenance of leaves in evergreen species, which must be taken into account in the estimation of the final balance of costs and benefits of leaf adaptations in environments differing in temperature.

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References

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. Glob Change Biol 14:2709–2726

Bremner JM (1960) Determination of nitrogen in soil by the Kjeldahl method. J Agric Sci 55:11–31

Castro-Díez P, Puyravaud JP, Cornelissen JHC (2000) Leaf structure and anatomy as related to leaf mass per area variation in seedlings of a wide range of woody plant species and types. Oecologia 124:476–486

Chabot BF, Chabot JF (1977) Effects of light and temperature on leaf anatomy and photosynthesis in *Fragaria vesca*. Oecologia 26:363–377

Chapmann HD, Pratt PF (1973) Methods of analysis for soils, plants and water. University of California, Riverside

Chmielewski FM, Rötzer T (2001) Response of tree phenology to climate change across Europe. Agric For Meteorol 108:101–112

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

Fitter AH, Hay RKM (2002) Environmental physiology of plants, 3rd edn. Academic Press, London, p 423

Friend AD, Woodward FI (1990) Evolutionary and ecophysiological responses of mountain plants to the growing season environment. Adv Ecol Res 20:59–124

Givnish TJ (2002) Adaptive significance of evergreen vs. deciduous leaves: solving the triple paradox. Silva Fenn 36:703–743

Gorham E (1991) Northern peatlands: role in the carbon cycle and probable responses to climatic warming. Ecol Appl 1:182–195

Griffith M, Brown GN (1982) Cell wall deposits in winter rye *Secale cereale* L. "Puma" during cold acclimation. Bot Gaz 143:486–490

IPCC (2007) Climate change 2007: the physical science basis. Contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change. Cambridge, University Press, Cambridge

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

Jonas GS, Geber MA (1999) Variation among populations of *Clarkia unguiculata* (*Onagraceae*) along altitudinal and latitudinal gradients. Am J Bot 86:333–343

Kodra E, Steinhaeuser K, Ganguly AR (2011) Persisting cold extremes under 21st-century warming scenarios. Geophys Res Lett 38:1–5

Körner CH, Larcher W (1988) Plant life in cold climates. Symp Soc Exp Biol 42:25–57

Kreyling J (2010) Winter climate change: a critical factor for temperate vegetation performance. Ecology 91:1939–1948

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

Li C, Zhang X, Liu X, Luukkanen O, Berninger F (2006) Leaf morphological and physiological responses of *Quercus aquifolioides* along an altitudinal gradient. Silva Fenn 40:5–13

Lo Gullo MA, Salleo S (1988) Different strategies of drought resistance in three Mediterranean sclerophyllous trees growing in the same environmental conditions. New Phytol 108:267–276

Mediavilla S, Escudero A (2003) Photosynthetic capacity, integrated over the lifetime of a leaf, is predicted to be independent of leaf longevity in some tree species. New Phytol 159:203–211

Mediavilla S, Escudero A, Heilmeier H (2001) Internal leaf anatomy and photosynthetic resource-use efficiency: interspecific and intraspecific comparisons. Tree Physiol 21:251–259

Morecroft MD, Woodward FI (1996) Experiments on the causes of altitudinal differences in the leaf nutrient contents, size and δ13C of *Alchemilla alpine*. New Phytol 134:471–479

Niinemets U (1999) Components of leaf dry mass per area – thickness and density – alter leaf photosynthetic capacity in reverse directions in woody plants. New Phytol 144:35–47

Niinemets U (2001) Global-scale climatic controls of leaf dry mass per area, density, and thickness in trees and shrubs. Ecology 82:453–469

Niinemets Ü, Sack L (2006) Structural determinants of leaf light-harvesting capacity and photosynthetic potentials. Progress Bot 67:385–419

Nobel PS (2005) Physicochemical and environmental plant physiology. Academic Press, San Diego

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, Modrzynski J, Tjoelker MG, Zytkowiak 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

Oliveira G, Peñuelas J (2000) Comparative photochemical and phenomorphological responses to winter stress of an evergreen (*Quercus ilex* L) and a semi-deciduous (*Cistus albidus* L) Mediterranean woody species. Acta Oecol 21:97–107

Parsons AN, Welker JM, Wookey PA, Press MC, Callaghan TV, Lee JA (1994) Growth responses of four sub-Arctic dwarf shrubs to simulated environmental change. J Ecol 82:307–318

Poorter H, Niinemets Ü, Poorter L, Wright IJ, Villar R (2009) Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. New Phytol 182:565–588

Rajashekar CB, Lafta A (1996) Cell-wall changes and cell tension in response to cold acclimation and exogenous abscisic acid in leaves and cell cultures. Plant Physiol 111:605–612

Reich PB, Oleksyn J (2004) Global patterns of plant leaf N and P in relation to temperature and latitude. PNAS 101:11001–11006

Reiter WD (1998) The molecular analysis of cell wall components. Trends Plant Sci 3:27–32

Schulze ED, Mooney HA (1993) Biodiversity and ecosystem function. Springer, Berlin

Solecka D, Kacperska A (2003) Phenylpropanoid deficiency affects the course of plant acclimation to cold. Physiol Plantarum 119:253–262

Stefanowska M, Kuras M, Kubacka-Zebalska M, Kacperska A (1999) Low temperature affects pattern on leaf growth and structure of cell walls in winter oilseed rape (*Brassica napus L., var oleifera* L.). Ann Bot 84:313–319

Suzuki S, Kudo G (1997) Short-term effects of simulated environmental change on phenology, leaf traits, and shoot growth of alpine plants on a temperate mountain, northern Japan. Glob Change Biol 3:108–115

Syvertsen JP, Lloyd J, McConchie C, Kriedemann PE, Farquhar GD (1995) On the relationship between leaf anatomy and CO_2 diffusion through the mesophyll of hypostomatous 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

Tinker PB, Nye PH (2000) Solute movement in the rizosphere. Oxford University Press, New York

Turner NC (1986) Adaptation to water deficits: a changing perspective. Aust J Plant Physiol 13:175–190

Turner IM (1994a) Sclerophylly: primarily protective? Funct Ecol 8:669–675

Turner IM (1994b) A quantitative analysis of leaf form in woody plants from the world's major broadleaved forest types. J Biogeogr 21:413–419

Uğurlu E, Oldeland J (2010) Species response curves of oak species along climatic gradients in Turkey. Int J Biometeorol. doi:10.1007/s00484-010-0399-9

Vitousek PM, Field CB, Matson PA (1990) Variation in foliar δ13 C in Hawaiian *Metrosideros polymorpha*: a case of internal resistance? Oecologia 84:362–370

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

Walther GR (2000) Climatic forcing on the dispersal of exotic species. Phytocoenologia 30:409–430

Walther GR, Post E, Convey P, Menzel A, Parmesank C, Beebee T, Fromentin JM, Hoegh-Guldberg O, Bairlein F (2002) Ecological responses to recent climate change. Nature 146:389–395

Weih M, Karlsson PS (1999) The nitrogen economy of mountain birch seedlings: implications for winter survival. J Ecol 87:211–219

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

Westoby M, Warton D, Reich PB (2000) The time value of leaf area. Am Nat 155:649–656

Witkowski ETF, Lamont BB (1991) Leaf specific mass confounds leaf density and thickness. Oecologia 88:486–493

Woods HA, Makino W, Cotner JB, Hobbie SE, Harrison JF, Acharya K, Elser JJ (2003) Temperature and the chemical composition of poikilothermic organisms. Funct Ecol 17:237–245

Wright IJ, Westoby M, Reich PB (2002) Convergence towards higher leaf mass per area in dry and nutrient-poor habitats has different consequences for leaf life span. J Ecol 90:534–543

Wright IJ, Reich PB, Westoby M et al (2004) The worldwide leaf economics spectrum. Nature 428:821–827

Yates MJ, Verboom GA, Rebelo AG, Cramer MD (2010) Ecophysiological significance of leaf size variation in *Proteaceae* from the Cape Floristic Region. Funct Ecol 24:485–492