

## LOSSES OF LEAF AREA OWING TO HERBIVORY AND EARLY SENESCENCE IN THREE TREE SPECIES ALONG A WINTER TEMPERATURE GRADIENT

### ABSTRACT

In temperate climates, evergreen leaves have to survive throughout low temperature winter periods. Freezing and chilling injuries can lead to accelerated senescence of part of the leaf surface, which contributes to a reduction of the lifespan of the photosynthetic machinery and of leaf lifetime carbon gain. Low temperatures are also associated with changes in foliar chemistry and morphology that affect consumption by herbivores. Therefore, the severity of foliar area losses caused by accelerated senescence and herbivory can change along winter temperature gradients. The aim of this study is to analyse such responses in the leaves of three evergreen species (*Quercus ilex*, *Q. suber* and *Pinus pinaster*) along a climatic gradient. The leaves of all three species presented increased leaf mass per area (LMA) and higher concentrations of structural carbohydrates in cooler areas. Only the two oak species showed visible symptoms of damage caused by herbivory, this being less intense at the coldest sites. The leaves of all three species presented chlorotic and necrotic spots that increased in size with leaf age. The foliar surface affected by chlorosis and necrosis was larger at the sites with the coldest winters. Therefore, the effects of the winter cold on the lifespan of the photosynthetic machinery were contradictory: losses of leaf area due to accelerated senescence increased, but there was a decrease in losses caused by herbivory. The final consequences for carbon assimilation strongly depend on the exact timing of the appearance of the damage resulting from low temperature and grazing by herbivores.

**Key words:** abiotic stress, herbivory, leaf nitrogen, evergreen species, structural reinforcement, winter temperature.

## INTRODUCTION

The study of the changes in foliar traits along environmental gradients has become a useful tool to assess the effects of climate change on the productivity and distribution patterns of the different tree species (Mediavilla et al. 2012; Klein et al. 2013). The analysis of changes in leaf traits across different environments shows that there is a trend towards an increase in leaf mass per unit leaf area (LMA) in colder environments (Ogaya and Peñuelas 2007; Atkin et al. 2008). Similarly, several authors 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). These changes in LMA and, possibly, in nitrogen content along temperature gradients should affect productivity in different environments, given the effects of both traits on instantaneous photosynthesis per unit leaf mass (Reich et al. 1997; Mediavilla et al. 2001; Niinemets and Sack 2006).

Temperature gradients also affect other productivity-determining factors, for example, the effects of temperature on plant-herbivore interactions have been frequently reported (Veteli et al. 2002; Jamieson et al. 2012). Temperature effects on the growth of herbivore populations are often direct. Higher temperatures promote higher metabolic rates that lead to higher consumption, growth and development rates (Jamieson et al. 2012). Moreover, warmer late winter and early spring temperatures tend to enhance insect survival (Bale et al. 2002). Furthermore, differences in foliar nitrogen content affect herbivores' preferences and, therefore, higher leaf N concentrations lead to stronger attack rates (White 1984; Agrawal 2004). On the other hand, leaf mass per unit area is known to correlate positively with leaf rigidity and hardness (Kudo 1996), often being considered an adaptation aimed at reducing vulnerability to herbivore attacks and other stress factors (Wright and Cannon 2001; Shipley et al. 2006). Thus, LMA and nitrogen changes along climatic gradients might interact with the direct effects of temperature on herbivore activity, leading to differences in the levels of

herbivory. Herbivory is a major determinant of leaf mortality rates, and has been shown to affect plant fitness (Moles and Westoby 2000; Mothershead and Marquis 2000). Leaf area losses resulting from herbivory usually take place at the early stages of leaf development and can significantly reduce the photosynthetic surface for a large part of the leaf's lifespan (Coley 1983; Kudo 1996; Moles and Westoby 2000).

Apart from the damage caused by herbivory, abiotic stress produces different physiological reactions and structural changes resembling natural senescence. These processes occur before the age of programmed senescence and may thus be interpreted as accelerated cell senescence (Günthardt-Goerg and Vollenweider 2007). Among other abiotic stress factors, low temperatures are known to produce irreversible damage that accumulates as the leaf ages (Günthardt-Goerg and Vollenweider 2007). Accordingly, the intensity of leaf area losses should also vary along temperature gradients, provided that other stress factors remain similar. Frequently, abiotic stress, such as drought, frost or chilling, results in necrotic areas bordered by green tissue (Vollenweider and Günthardt-Goerg 2005), which implies that only part of the leaf area is affected and that the individual leaf may remain alive until it reaches its age of programmed senescence. Similarly, for species with a long leaf lifespan, a large amount of herbivory damage may occur before the leaves are shed (Poorter et al. 2004). Therefore, leaf area losses resulting from herbivory and anticipated senescence may lead to a significant reduction in the amount of photosynthetic machinery before the end of the leaf's lifespan. As a result, individual leaves may have a longer life span than any unit of leaf mass or nutrients (Mediavilla and Escudero 2003a). Obviously, the shorter duration of the photosynthetic machinery renders the estimates of leaf-level carbon balance inaccurate, when they are based on the lifespan of individual leaves (Mediavilla and Escudero 2003b; Kikuzawa and Lechowicz 2006). In evergreen species, leaf lifespan tends to increase with decreasing temperatures (Wright et al. 2005), probably reflecting the longer payback time for construction costs in such conditions, due to the shorter growing season. However, this trend could be compensated by anticipated losses of

leaf area under conditions of cold stress.

We are not aware of any studies devoted to the analysis of changes in the intensity of foliar damage due to abiotic factors along climatic gradients. Conversely, there are numerous studies on the damage caused by herbivory along different environmental gradients, albeit with contradictory results. Thus, for example, while there are cases where higher levels of herbivory were detected in warmer environments (Bale et al. 2002; Bjorkman et al. 2011), other authors' results show higher levels of consumption in colder environments (Coley and Aide 1991; Aizen and Williams 1995) or no trends along temperature gradients (Andrew and Hughes 2007; Adams et al. 2009). In most studies, the sampled species changed along the climatic gradients, which makes it difficult to identify the factors responsible for the trends in levels of herbivory. The task of analysing leaf damage caused by herbivory and other factors is very time-consuming, which could be one of the reasons why this aspect has not been integrated into previous production models. There is a clear need for studies that analyse differences in leaf area losses comparing different environments, so that we might understand the impacts of climate change on the potential distribution of tree species.

The purpose of this study is to analyse damage to the leaf area caused by different stress factors in three evergreen tree species differing in leaf life span (*Quercus ilex* ssp. *ballota* (Desf.) Samp, *Quercus suber* L. and *Pinus pinaster* Aiton) located at different sites along a temperature gradient and to determine the effects of differences in foliar traits on possible differences in damage intensity. An earlier study using the same species and locations showed that there were significant site-related differences in several leaf traits. Thus, the leaves of the analysed species exhibited higher LMA, thickness and structural carbohydrate content in colder areas, with no trends in nitrogen content (Mediavilla et al. 2012; González-Zurdo et al. 2015). These differences among sites suggest the need for greater structural reinforcement under harsher winter conditions, which would involve higher costs in the coldest areas, mainly due to the negative relationship between LMA and instantaneous photosynthesis (Reich et al. 1997; Niinemets and Sack 2006). These costs could be

compensated, however, if the greater reinforcement were to involve a decrease in herbivory-related leaf area losses in colder environments. Therefore, our aim is to test whether the greater leaf structural reinforcement in cold environments involves lower costs in terms of losses of photosynthetic area associated with herbivory. By contrast, we anticipate that losses of photosynthetic area caused by accelerated senescence would be higher in colder environments.

## **MATERIALS AND METHODS**

### *Species and study areas*

Three evergreen species were selected for the study: *Pinus pinaster* Aiton, *Quercus suber* L. and *Q. ilex* ssp. *ballota* (Desf.) Samp. The species were distributed over six sites located in the regions of Castilla-Leon and Extremadura (central-western Spain) between latitudes 41° 45' N and 39° 49' N and longitudes 6° 22' W and 2° 37' W (Table 1). Altitudes range between 466 and 1189 m a.s.l. Because of the differences in geographical location and altitude, two of the selected sites may be categorized as cold, with the lowest minimum temperatures and the highest number of frost days; two sites could be identified as warm and the remaining two as having intermediate winter conditions. The sites consisted of flat areas with sparse populations (between 50 and 100 specimens ha<sup>-1</sup>) of mature (over 100 years old) individuals. Each site was selected so as to include as many study species as possible and to cover a wide winter temperature gradient, at the same time making sure that the rest of climatic characteristics were as homogeneous as possible. Nevertheless, there is a tendency for rainfall levels to be higher in the hottest and southernmost sites, which helps 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

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	-0.90	-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
Precipitation (mm)						
Annual	986	672	495	460	567	646
Summer	54	51	60	54	107	111
Emberger's index	105	60	52	46	79	78
Ozone indicator AOT40 (µg m <sup>-3</sup> h)	23576	22192	21676	23318	20895	22928
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	Pp, Qi, Qs	Pp, Qi, Qs	Pp, Qi, Qs	Pp, Qi, Qs	Pp, Qi	Pp, Qi

Pp *Pinus pinaster*; Qi *Quercus ilex*, Qs *Quercus suber*

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). 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) programmed to obtain temperature data every 10 min and kept at each site for 6 years (October 2008 – October 2014). Data on ozone concentrations were obtained from The European Environment Agency (EEA) (The European air quality database and <http://www.eea.europa.eu/data-and-maps/data/airbase-the-european-air-quality-database-8>), using the information from the nearest control stations. In all cases the stations were situated at an altitude similar to the study sites. Ozone levels were expressed according to the index AOT40 (accumulated ozone over threshold  $80 \mu\text{g m}^{-3}$ ) along years 2012-2014 (Table 1). Soil samples were taken up to a depth of 20 cm (excluding the forest floor) from each stand. Determinations of soil granulometry, pH and N and P concentrations were carried out at the Soils Laboratory of the Institute of Natural Resources and Agrobiology of Salamanca, according to the methods described by Chapman and Pratt (1973) and Walkley and Black (1934).

#### *Sample collection and processing*

At each site, a branch from each of five mature specimens of each species selected randomly was taken at the top of the crown. The trees chosen were sun-exposed mature individuals whose trunk diameter at a height of 1.3 m ranged from 40 to 60 cm, mean heights being 8-10 m. All the samples were gathered in mid-October 2014.

The samples were taken immediately 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 sprouting in a given year were considered to belong to the same age class. All the leaves from each species and age were

visually analyzed and classified into different categories depending on whether they presented visible symptoms of damage or not. The damaged leaves were further classified according to damage type: leaves attacked by herbivores, leaves with symptoms of abiotic stress and leaves with both types of damage (herbivory and abiotic factors). There are many studies that offer plentiful graphic information that allows for identifying both biotic and abiotic stress symptoms in leaves (Vollenweider and Günthardt-Goerg 2005; Günthardt-Goerg and Vollenweider 2007). Samples from *Pinus pinaster* were only separated into two categories (healthy leaves and leaves presenting abiotic damage) because the herbivory levels detected for this species were practically negligible. After sorting, a count of the number of leaves in each category was made for each of the selected tree individuals of each species. A total of around 54,000 leaves were examined to detect damage and its type.

To check possible differences in the traits of the leaves of a same species according to the different environments and damage categories, 50 leaves of each species, leaf age class and category (healthy, with damage by herbivores and with abiotic damage) from each site (10 leaves of each category from each of the 5 selected specimens) were randomly selected. Leaf thickness was measured with a digital micrometer (Digimatic micrometer, Mitutoyo, Japan) as a mean of three 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). In the case of *P. pinaster*, we also measured needle length with the digital micrometer. The samples were then oven-dried at 70 °C to constant weight and the total dry mass was determined. From the data thus obtained, we calculated the leaf dry mass per area (LMA). Once all the data had been collected, a value for each specimen and leaf age class was calculated as the average of 10 leaves of each damage category. The 10 individual leaves taken from each specimen and leaf age class were ground together to obtain a sufficient amount of sample for the chemical analyses. Leaf N concentrations were determined with a



CE-Instruments NA-2100 auto analyser (ThermoQuest, Milan, Italy). After the N analyses, the remaining material was used to analyze the fibre content (hemicellulose, cellulose and lignin) with an Ankom Analyzer (A220, New York, USA), following the method of Goering and Van Soest (1970). The nitrogen and fibre contents of leaves were expressed per unit dry mass (as milligrams of nutrient or fibre per gram of leaf dry mass), and N also as per unit of leaf area, obtained as the N content per unit dry mass multiplied by LMA.

The foliar surface lost to herbivores or damaged by abiotic stress was also determined based on 10 leaves per tree individual up to a total of 50 from each species and age class, from each site, using the ImageJ programme (<http://rsb.info.nih.gov/ij/>; Abràmoff et al. 2004). The leaves were first subjected to high-resolution scanning (2300 x 3300 pixels). The ImageJ programme allowed for the analysis of the images obtained, measuring both the whole area of each leaf and those areas with a different colouration due to necrosis and chlorosis caused by abiotic stress damage. The area lost to herbivores was estimated by reconstructing leaf outlines and calculating the difference between the reconstructed leaf and the actual area. Thus, the percentage of surface loss caused by herbivory or other types of damage was estimated for each of the 50 leaves analysed in each case.

Additional branches were sampled on a 2-monthly basis to estimate leaf lifespan. Five mature specimens of each species were selected randomly at each site during each sampling session. A composite sampling of sun-exposed branches with leaves from different crown positions in each canopy was undertaken for each individual selected. The number of leaves or needles per shoot was counted for each age class and the data were used to construct static life tables, which made it possible to estimate the mean leaf life span for each species according to standard methods.

### *Data analysis*

Once the leaves of each type had been counted up, we calculated the percentage of

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damaged leaf area for each tree. A final estimate of damage was obtained for each species and leaf age class from each site as a mean of the five trees selected in each case. One-way analysis of variance (ANOVA) was used to examine the differences among sites within each species. Linear regression analysis was used to explore the relationships within each species between levels of herbivory and leaf traits at the different sites. Regression analysis was also used to test the relationship between climate and level of damage at each site. Each site was given a single value for the average temperature and mean daily maximum and minimum temperatures, obtained as the average of the mean values measured each month over 4 years. A value for the maximum and minimum temperatures recorded was also obtained. In addition to temperature, the total annual and summer precipitation of each site and the Emberger's pluviothermic index (Emberger 1930) were used to analyse the effect of possible differences in water availability on the level of foliar damage recorded at each site. Emberger's index, commonly used in Mediterranean climates (Kunstler et al. 2007), was obtained through the following equation:

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

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). All statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

## RESULTS

### *Intraspecific variation in the traits of healthy leaves along the climatic gradient*

No significant differences were found between the two sites selected in each of the three climatic environments for any of the leaf traits analysed. Conversely, ANOVA results revealed differences among the three climatic environments for most of the leaf traits (Table

2). There was a tendency for leaves of a same species and age class produced at the warmest sites to be larger (larger area in the two *Quercus* and longer needles in *P. pinaster*), but thinner, with lower LMA and fibre content than in the coldest sites, with significant differences among sites in practically all of the cases (Table 2). The leaves from sites with intermediate climates also yielded intermediate values for thickness, LMA and fibre contents (Table 2). By contrast, between-sites differences in lignin concentrations were non-significant (not shown). Similarly, differences in nitrogen concentration per unit mass ( $N_{\text{mass}}$ ) were small and non-significant in most of the cases. The differences in N content per unit area ( $N_{\text{area}}$ ) tended to be more pronounced because of the increase in LMA at the coldest sites, although, in most cases, these differences did not reach significance either (Table 2). Leaf life span showed a non-significant trend to decrease with the increase in temperature in *P. pinaster*, but there were no trends for the other two species. The LMA, thickness and fibre content increased with the leaf longevity of the different species, whereas the opposite happened with nitrogen content per unit mass, which tended to be lower in leaves with longer life spans (Table 2).

#### *Intraspecific differences in the levels of foliar damage along the climatic gradient*

Most herbivorous damage was due to leaf area loss by leaf chewers and scars on leaf blades mainly by skeletonizers. The two species that presented significant damage caused by herbivory showed significant differences in level of damage in the different areas of the climatic gradient, the percentage of herbivore-attacked leaves and average area consumed per leaf always being significantly higher in the warmest sites (Table 3). In the case of *Q. ilex*, leaves also showed intermediate levels of consumption at the sites with intermediate climate characteristics (Table 3). The differences among environments could be observed in all leaf age classes. There were no differences in the intensity of herbivore damage among different age classes, the leaves of the different cohorts showing similar proportions of attacked leaves, as well as of consumed areas in both species (Table 3). Between the two

**Table 2.** Traits of the healthy leaves (SE in parentheses,  $n = 10$  trees per species) and mean leaf life span of the three species under different climatic conditions.

Species	Leaf age class	Sites temperatures	Leaf size	Leaf thickness ( $\mu\text{m}$ )	LMA ( $\text{g m}^{-2}$ )	H+C ( $\text{mg g}^{-1}$ )	Total fibre ( $\text{mg g}^{-1}$ )	Nmass ( $\text{mg g}^{-1}$ )	Narea ( $\text{g m}^{-2}$ )	LL (days)
<i>Q. suber</i>	1	Warm	5.23 (0.33) a	252 (7.29) b	166 (6.21) b	165 (2.48) b	315 (5.40) b	16.6 (0.33) a	2.74 (0.09) a	365 (0.5) a
		Intermediate	4.21 (0.24) b	274 (8.14) a	190 (5.96) a	233 (2.84) a	387 (4.54) a	15.8 (0.47) a	3.02 (0.15) a	382 (4.0) a
<i>Q. ilex</i>	1	Warm	3.02 (0.10) a	269 (3.92) c	202 (6.83) c	214 (3.73) b	350 (6.72) c	14.3 (0.42) a	2.91 (0.16) b	817 (62) a
		Intermediate	2.64 (0.10) ab	304 (7.05) b	236 (8.62) b	260 (2.54) a	403 (296) b	12.7 (0.60) b	2.99 (0.16) b	814 (41) a
		Cold	2.10 (0.09) b	353 (4.90) a	267 (12.9) a	284 (3.56) a	433 (3.83) a	14.2 (0.21) a	3.80 (0.16) a	820 (26) a
<i>P. pinaster</i>	2	Warm	2.96 (0.11) a	289 (4.18) c	215 (5.92) c	226 (3.38) c	371 (3.99) c	11.8 (0.47) b	2.54 (0.13) b	
		Intermediate	2.67 (0.12) ab	323 (16.4) b	247 (5.30) b	269 (3.91) b	421 (5.97) b	10.8 (0.25) c	2.66 (0.04) b	
		Cold	1.92 (0.14) b	362 (6.01) a	278 (8.97) a	308 (5.01) a	456 (7.53) a	12.8 (0.39) a	3.58 (0.20) a	
<i>P. pinaster</i>	1	Warm	133 (4.89) a	919 (20.0) c	355 (14.6) b	303 (5.36) c	456 (4.95) b	9.26 (0.42) a	3.29 (0.24) a	1099 (50) a
		Intermediate	117 (6.77) a	984 (35.2) b	369 (3.00) ab	336 (6.69) b	503 (8.20) a	10.1 (0.92) a	3.77 (0.42) a	1169 (70) a
		Cold	95 (2.56) b	1065 (16.0) a	393 (13.0) a	365 (3.04) a	514 (3.86) a	9.39 (0.70) a	3.68 (0.28) a	1290 (101) a
	2	Warm	139 (2.72) a	951 (12.7) b	367 (23.3) b	321 (5.33) b	474 (4.75) b	8.92 (0.46) a	3.26 (0.18) b	
		Intermediate	124 (6.21) ab	1076 (32.7) ab	391 (6.89) ab	351 (5.80) a	510 (10.2) a	9.58 (0.52) a	3.78 (0.66) ab	
		Cold	106 (4.46) b	1117 (38.3) a	414 (13.3) a	364 (8.60) a	520 (9.71) a	9.80 (0.43) a	4.05 (0.12) a	
<i>P. pinaster</i>	3	Warm	122 (7.67) a	978 (5.56) b	379 (3.89) b	312 (3.31) c	473 (3.75) c	7.60 (0.84) b	2.89 (0.34) b	
		Intermediate	106 (10.5) ab	1015 (31.9) b	410 (4.05) a	338 (2.92) b	506 (3.72) b	8.70 (0.69) a	3.57 (0.30) a	
		Cold	89 (6.20) b	1068 (8.19) a	423 (16.0) a	368 (2.12) a	538 (3.36) a	8.09 (0.60) ab	3.43 (0.32) ab	

For each leaf type, means with different letters indicate significant differences ( $P = 0.05$ ) between climatic categories.

Leaf size area ( $\text{cm}^2$ ) in oaks and needle length (mm) in pine, LMA leaf mass per unit area, H+C cellulose + hemicellulose concentration, Nmass N concentration per unit leaf mass, Narea N content per unit leaf area, LL mean leaf life span.

oaks, the species with the shortest-lived leaves, *Q. suber*, showed the highest percentage of damaged leaves and consumed area in each leaf (Table 3).

**Table 3.** Percentage of leaves attacked by herbivores and damaged by abiotic factors (SE in parentheses,  $n = 10$  trees per species).

Species	Leaf age class	Sites temperatures	No. of leaves examined	Leaves attacked by herbivores (%)	Area lost per leaf (%)	Leaves damaged by abiotic stress (%)	Area damaged per leaf (%)
<i>Q. suber</i>	1	Warm	3200	55.2 (0.99) a	15.69 (1.38) a	33.6 (0.84) b	9.64 (0.75) b
	1	Intermediate	4487	49.5 (1.59) b	10.80 (0.57) b	45.1 (2.13) a	17.3 (0.81) a
	2	Warm	100	56.5 (6.50) a	14.75 (1.49) a	44.0 (6.00) b	18.3 (1.61) b
	2	Intermediate	865	48.7 (3.09) b	11.39 (1.34) b	51.2 (3.09) a	25.0 (0.66) a
<i>Q. ilex</i>	1	Warm	6492	50.9 (0.80) a	7.48 (0.81) a	8.11 (0.67) a	6.17 (0.53) c
	1	Intermediate	4056	44.1 (0.63) ab	6.25 (0.64) ab	9.74 (0.29) a	11.0 (0.67) b
	1	Cold	5893	41.0 (4.10) b	4.87 (0.49) b	11.9 (2.27) a	18.9 (1.78) a
	2	Warm	2110	49.0 (0.41) a	7.34 (0.58) a	17.7 (0.79) b	9.35 (1.07) c
	2	Intermediate	2216	45.8 (0.82) b	6.34 (0.45) ab	20.2 (0.54) ab	16.4 (1.45) b
	2	Cold	1989	42.2 (1.13) c	5.06 (0.46) b	23.5 (3.13) a	22.5 (0.98) a
	3	Warm	210	48.2 (0.63) a	7.61 (0.65) a	23.5 (0.53) c	13.5 (3.33) c
	3	Intermediate	828	44.8 (0.84) b	6.61 (0.66) a	25.3 (0.58) b	22.0 (2.05) b
	3	Cold	214	42.4 (1.34) b	4.92 (0.32) b	30.5 (0.43) a	28.6 (1.97) a
<i>P. pinaster</i>	1	Warm	3079	-	-	18.84 (0.49) c	1.03 (0.12) b
	1	Intermediate	2849	-	-	21.97 (0.31) b	1.99 (0.06) a
	1	Cold	2552	-	-	24.92 (0.88) a	2.38 (0.18) a
	2	Warm	2513	-	-	25.39 (1.17) c	2.23 (0.21) b
	2	Intermediate	2532	-	-	31.99 (0.93) b	2.91 (0.27) ab
	2	Cold	2335	-	-	37.10 (0.79) a	3.63 (0.21) a
	3	Warm	1420	-	-	44.66 (1.26) c	3.11 (0.35) b
	3	Intermediate	1257	-	-	52.21 (1.36) b	3.92 (0.33) b
	3	Cold	1309	-	-	62.97 (2.67) a	4.91 (0.32) a
	4	Warm	212	-	-	78.28 (1.67) c	3.84 (0.42) b
	4	Intermediate	461	-	-	85.35 (1.43) b	4.48 (0.15) b
	4	Cold	968	-	-	91.03 (1.31) a	5.50 (0.33) a

Average loss of area (%) per individual leaf ( $n = 10$ ). Means with different letters indicate significant differences ( $P = 0.05$ ) between climatic categories.

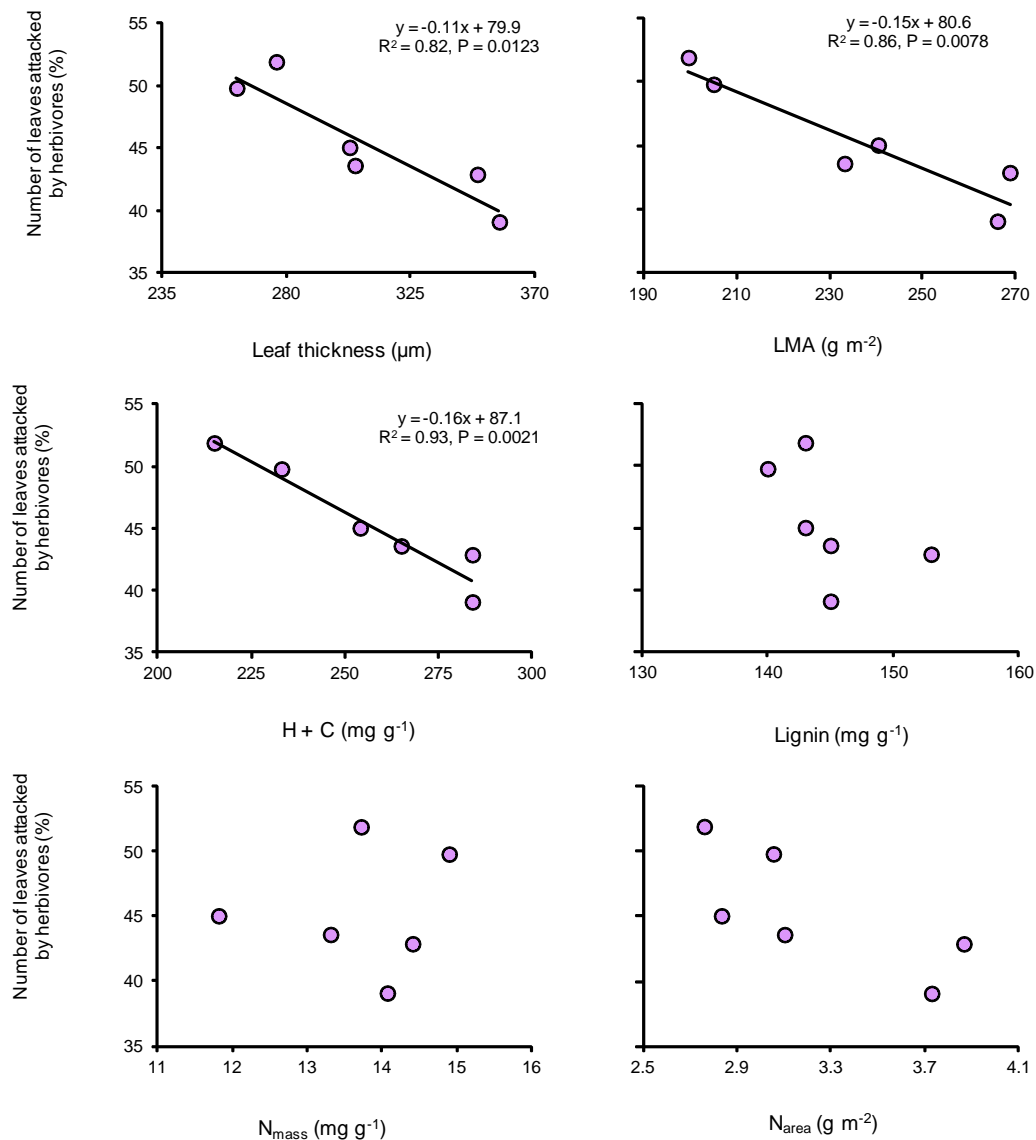
Trends of abiotic damage among sites proved opposite to those obtained for herbivory levels. For the two species represented in the three climatic environments (*P. pinaster* and *Q. ilex*), both the percentage of damaged leaves and the percentage of leaf area damaged, tended to be higher at the coldest sites than at the warmest ones, with intermediate values at sites with intermediate temperatures (Table 3). The same results were obtained for *Q. suber*, the intensity of damage caused by abiotic factors being much higher at the sites with lower temperatures (more damaged leaves and larger damaged area per leaf) as compared

to the hottest sites. In contrast with herbivory, there were differences in damage caused by abiotic stress among the successive foliar cohorts, the proportion of damaged leaves and of the damaged part increasing with leaf age (Table 3).

Of the three species, the leaves of the one with the lowest leaf lifespan (*Q. suber*) once again showed higher intensities in the damage caused by stress factors when compared to leaves of the same age from the two other species (Table 3). Between *Q. ilex* and *P. pinaster*, *P. pinaster* showed a greater proportion of leaves with visible damage symptoms, although the percentage of damaged area per leaf in each of the environments was much lower than in *Q. ilex* (Table 3).

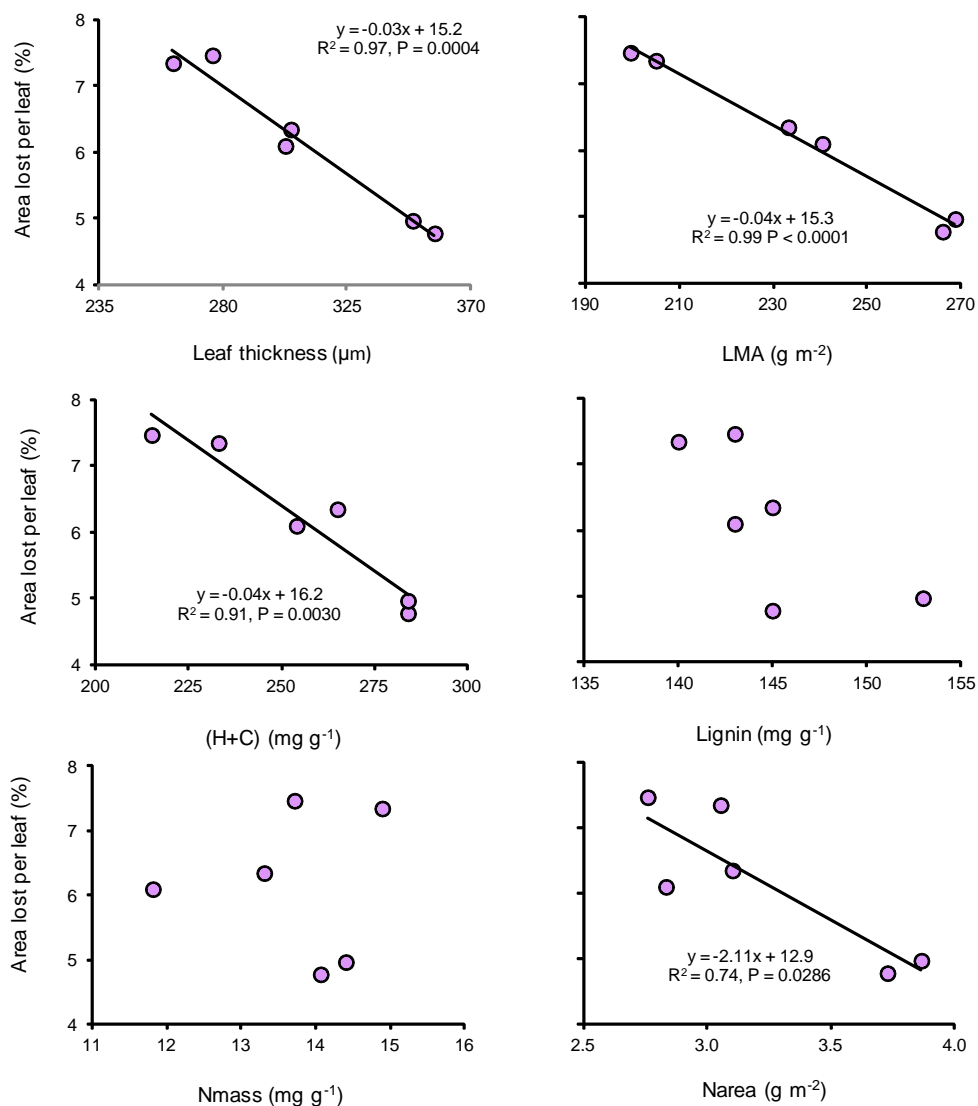
#### *Leaf traits and environmental factors that determine the differences in levels of foliar damage*

Regression analysis including data from the six sites studied was used to detect the foliar traits that determine the differences in the levels of herbivory among sites. Although we had herbivory data for the two oak species, the analysis was restricted to the leaves of *Q. ilex*, since *Q. suber* could only be found at four sites. Since the levels of herbivory were similar for the different leaf cohorts, the analyses were further limited to current-year leaves. There was a close relationship between all the traits related to the reinforcement of leaf structure and the levels of attack, the percentage of leaves consumed (Fig. 1) and the mean leaf area consumed (Fig. 2) becoming lower as LMA, thickness and structural carbohydrates increased along the climatic gradient. However, there was no significant relationship between levels of herbivory and concentrations of lignin and the nitrogen content per unit mass in the leaves from the different sites. There was a trend towards lower levels of herbivory as nitrogen content per unit leaf area increased, although it was only significant for the percentage of leaf area lost (Fig. 2).



**Figure 1.** Relationships between the percentage of leaves attacked by herbivores and different leaf traits of current-year leaves of *Quercus ilex* at different sites. *LMA* = leaf mass per unit area, *H+C* = cellulose + hemicellulose concentration,  $N_{mass}$  = N concentration per unit leaf mass,  $N_{area}$  = N content per unit leaf area.

The comparison between the traits of the healthy and the attacked leaves of each species at each site confirmed the influence of structural strengthening on herbivory levels (Table 4). There were no significant differences in nitrogen content per unit area between leaves with herbivore damage and intact leaves across sites. At the hottest sites, the current *Q. ilex* leaves that had been attacked by herbivores presented lower  $N_{mass}$  contents than did the



**Figure 2.** Relationships between the percentage of leaf area lost by herbivory and different leaf traits of current-year leaves of *Quercus ilex* at different sites. Abbreviations as in Fig. 1.

healthy ones. No differences were observed with regard to the individual area of intact and attacked leaves. Conversely, in many cases, there were significant differences in LMA between healthy and damaged leaves, both by herbivory and by abiotic stress factors. The healthy leaves tended to have greater LMA than do the damaged ones, regardless of the type of damage suffered (Table 4).



**Table 4.** Mean traits of healthy and damaged leaves at the different sites (SE in parentheses,  $n = 10$  trees).

Leaf age class	Category of damage	Warm sites				Intermediate sites				Cold sites			
		LMA (gm <sup>-2</sup> )	N (mg g <sup>-1</sup> )	N (gm <sup>-2</sup> )	Leaf size	LMA (gm <sup>-2</sup> )	N (mg g <sup>-1</sup> )	N (gm <sup>-2</sup> )	Leaf size	LMA (gm <sup>-2</sup> )	N (mg g <sup>-1</sup> )	N (gm <sup>-2</sup> )	
1	Healthy	166 (6.21) a	16.6 (0.33) a	2.74 (0.09) a	4.21 (0.24) a	190 (5.96) a	15.8 (0.47) a	3.02 (0.15) a	-	-	-	-	-
	Abiotic stress	149 (3.53) ab	16.5 (0.41) a	2.46 (0.07) a	4.77 (0.16) a	168 (4.99) b	15.7 (0.30) a	2.64 (0.10) a	-	-	-	-	-
	Herbivory	146 (4.90) b	16.0 (0.34) a	2.34 (0.09) a	4.65 (0.15) a	164 (3.93) b	15.0 (0.48) a	2.46 (0.11) a	-	-	-	-	-
1	Healthy	202 (6.83) a	14.3 (0.42) a	2.91 (0.16) a	2.64 (0.10) a	236 (8.62) a	12.7 (0.60) a	2.99 (0.16) a	2.10 (0.09) a	267 (12.9) a	14.2 (0.21) ab	3.80 (0.16) a	
	Abiotic stress	203 (7.93) a	13.9 (0.49) ab	2.80 (0.12) a	2.69 (0.14) a	213 (4.92) b	12.2 (0.56) a	2.60 (0.15) a	2.09 (0.10) a	241 (7.06) b	13.9 (0.52) b	3.34 (0.18) a	
	Herbivory	183 (13.7) b	13.3 (0.40) b	2.42 (0.19) a	2.42 (0.14) a	209 (7.60) b	12.4 (0.29) a	2.58 (0.11) a	2.21 (0.13) a	242 (4.97) b	15.0 (0.61) a	3.63 (0.14) a	
2	Healthy	215 (5.92) a	11.8 (0.47) a	2.54 (0.13) a	2.67 (0.12) a	247 (5.30) a	10.8 (0.25) a	2.66 (0.04) a	1.92 (0.14) a	278 (8.97) a	12.8 (0.39) a	3.58 (0.20) a	
	Abiotic stress	205 (7.42) a	10.8 (0.48) b	2.22 (0.14) a	2.41 (0.15) a	237 (7.03) a	10.2 (0.33) a	2.41 (0.06) a	2.10 (0.13) a	256 (7.90) b	13.1 (0.25) a	3.35 (0.13) a	
	Herbivory	199 (3.35) a	11.6 (0.36) ab	2.31 (0.10) a	2.54 (0.16) a	237 (5.28) a	10.6 (0.29) a	2.52 (0.11) a	2.13 (0.24) a	257 (12.4) b	13.6 (0.39) a	3.51 (0.24) a	
1	Healthy	355 (14.6) a	9.26 (0.42) a	3.29 (0.24) a	117 (6.77) a	369 (3.00) a	10.2 (0.92) a	3.77 (0.42) a	95 (2.56) a	393 (13.0) a	9.39 (0.70) a	3.68 (0.28) a	
	Abiotic stress	317 (4.50) b	8.24 (0.52) b	2.61 (0.15) a	124 (6.24) a	339 (12.3) b	9.71 (0.85) a	3.31 (0.36) a	90 (2.04) a	354 (12.8) b	8.78 (0.66) a	3.11 (0.26) a	
	Healthy	367 (23.3) a	8.92 (0.46) a	3.26 (0.18) a	124 (6.21) a	391 (6.89) a	9.58 (0.52) a	3.78 (0.66) a	106 (4.46) a	414 (13.3) a	9.80 (0.43) a	4.05 (0.12) a	
2	Abiotic stress	348 (12.0) a	7.65 (0.26) b	2.66 (0.09) a	132 (3.16) a	366 (4.87) b	9.91 (0.72) a	3.63 (0.64) a	104 (3.89) a	383 (11.0) b	9.38 (0.96) a	3.56 (0.26) a	
	Healthy	379 (3.89) a	7.60 (0.84) a	2.89 (0.34) a	124 (6.21) a	410 (4.05) a	8.70 (0.69) a	3.57 (0.30) a	89 (6.20) a	423 (16.0) a	8.09 (0.60) a	3.43 (0.32) a	
	Abiotic stress	359 (4.46) a	6.55 (0.47) b	2.36 (0.18) a	102 (13.4) b	389 (6.24) a	8.39 (0.96) a	3.25 (0.38) a	74 (3.19) a	396 (10.6) b	8.18 (0.77) a	3.21 (0.22) a	

Means with different letters indicate significant differences ( $P = 0.05$ ) between categories of damage. Leaf size: area (cm<sup>2</sup>) in oaks and needle length (mm) in pine. LMA leaf mass per unit area.

The climatic factors responsible for the differences among sites in foliar damage caused by abiotic stress were determined by means of linear regression analysis between the percentage of leaves with damage symptoms and the percentage of damaged area per leaf against the different climatic variables recorded at each site (Table 5). Once again, *Q. suber* was excluded from the analysis because this species was only found in four plots. The

**Table 5.** Linear regression parameters for mean levels of damage by abiotic stress depending on different climatic variables at each site (C = current-year leaves).

	Number of leaves damaged (%)			Area damaged per leaf (%)		
	<i>y</i>	<i>R</i> <sup>2</sup>	<i>P</i> value	<i>y</i>	<i>R</i> <sup>2</sup>	<i>P</i> value
<i>Q. ilex</i> (C) ( <i>n</i> = 6)						
Mean monthly temperature (°C)	$y = -0.58x + 17.6$	0.81	0.0148	$y = -1.89x + 37.1$	0.88	0.0059
Maximum summer temperature (°C)	$y = -0.47x + 29.3$	0.23	NS	$y = -1.56x + 76.3$	0.25	NS
Minimum winter temperature (°C)	$y = -0.28x + 7.30$	0.74	0.0257	$y = -1.08x + 1.91$	0.96	< 0.0001
Number of days with frost per year	$y = 0.04x + 7.78$	0.77	0.0219	$y = 0.13x + 4.41$	0.97	< 0.0001
Total annual rainfall (mm)	$y = -0.01x + 12.1$	0.31	NS	$y = -0.01x + 18.0$	0.23	NS
Summer precipitation (mm)	$y = 0.07x + 7.11$	0.58	NS	$y = 0.22x + 2.29$	0.68	NS
Emberger's index	$y = -0.01x + 10.6$	0.01	NS	$y = 0.01x + 11.8$	0.01	NS
<i>Q. ilex</i> (C+1) ( <i>n</i> = 6)						
Mean monthly temperature (°C)	$y = -0.93x + 32.9$	0.8	0.0157	$y = -2.03x + 42.9$	0.96	< 0.0001
Maximum summer temperature (°C)	$y = -0.71x + 49.9$	0.2	NS	$y = -2.03x + 99.5$	0.41	NS
Minimum winter temperature (°C)	$y = -0.48x + 16.1$	0.71	0.0342	$y = -1.09x + 5.88$	0.93	0.0018
Number of days with frost per year	$y = 0.06x + 17.0$	0.82	0.0136	$y = 0.13x + 8.25$	0.98	< 0.0001
Total annual rainfall (mm)	$y = -0.01x + 23.8$	0.25	NS	$y = -0.01x + 23.8$	0.37	NS
Summer precipitation (mm)	$y = 0.11x + 15.8$	0.64	NS	$y = 0.20x + 7.17$	0.55	NS
Emberger's index	$y = -0.01x + 20.9$	0.01	NS	$y = -0.04x + 18.6$	0.02	NS
<i>P. pinaster</i> (C) ( <i>n</i> = 6)						
Mean monthly temperature (°C)	$y = -0.95x + 34.4$	0.94	0.0012	$y = -0.22x + 4.67$	0.99	< 0.0001
Maximum summer temperature (°C)	$y = -0.85x + 56.7$	0.32	NS	$y = -0.26x + 12.4$	0.59	NS
Minimum winter temperature (°C)	$y = -0.48x + 17.4$	0.82	0.0123	$y = -0.11x + 0.77$	0.87	0.007
Number of days with frost per year	$y = 0.06x + 18.3$	0.93	0.0019	$y = 0.03x + 0.99$	0.93	0.0017
Total annual rainfall (mm)	$y = -0.01x + 25.0$	0.26	NS	$y = -0.01x + 2.70$	0.44	NS
Summer precipitation (mm)	$y = 0.10x + 17.4$	0.64	NS	$y = 0.02x + 1.02$	0.39	NS
Emberger's index	$y = -0.01x + 22.2$	0.01	NS	$y = -0.01x + 2.25$	0.05	NS
<i>P. pinaster</i> (C+1) ( <i>n</i> = 6)						
Mean monthly temperature (°C)	$y = -1.83x + 55.6$	0.98	0.0002	$y = -0.21x + 5.77$	0.87	0.0061
Maximum summer temperature (°C)	$y = -1.84x + 107$	0.42	NS	$y = -0.19x + 10.7$	0.29	NS
Minimum winter temperature (°C)	$y = -0.96x + 22.5$	0.91	0.0032	$y = -0.11x + 1.90$	0.76	0.0234
Number of days with frost per year	$y = 0.12x + 24.5$	0.98	0.0002	$y = 0.01x + 2.10$	0.86	0.0072
Total annual rainfall (mm)	$y = -0.01x + 37.9$	0.32	NS	$y = -0.01x + 3.75$	0.31	NS
Summer precipitation (mm)	$y = 0.18x + 23.5$	0.56	NS	$y = 0.02x + 1.90$	0.59	NS
Emberger's index	$y = -0.02x + 32.9$	0.01	NS	$y = -0.01x + 3.12$	0.01	NS

results for the two younger leaf age classes of each species were included in this case because of the fact that foliar damage caused by abiotic stress increased with leaf age. Only differences in temperatures showed significant effects on damage levels. From the different temperature estimates, in most cases, the lowest temperatures and the number of frost days per year showed the highest percentages of explained variance (Table 5). Average temperatures also showed highly significant effects, mainly because of the strong effect of the lowest temperatures on the means. On the other hand, absolute maximum temperatures showed no significant effects on abiotic damage symptoms. No significant effects of the estimates of water availability in the different sites were observed in practically any of the cases (Table 5).

## **DISCUSSION**

The results obtained confirm those of previous studies that conclude that LMA is not exclusively a specific protection against drought and attack by herbivores (Niinemets 2001; Wright et al. 2002), but rather a response to a wide range of environmental stress factors, including low temperatures (Ogaya and Peñuelas 2007; Mediavilla et al. 2012; González-Zurdo et al. 2015). The tendency of the leaves of all three species was for LMA, contents of structural carbohydrates and thickness to be higher in colder than in warmer environments. In the two oak species, these differences in foliar traits among environments were accompanied by differences in levels of consumption by herbivores, with a lower percentage of attacked leaves and a smaller part of consumed area per leaf at the colder sites.

Differences in levels of herbivory at the different sites could be due to a direct effect of temperatures on the growth of herbivore populations (Bale et al. 2002; Jamieson et al. 2012). However, within a same site, the herbivore-attacked leaves presented lower LMA and thickness than the healthy ones did, which suggests a direct effect of these leaf traits on herbivores' preferences. At each site, herbivores seemed to first choose the species with lower leaf thickness and LMA and, within each species, the leaves with these same

characteristics. On the other hand, and again within each species, herbivores seem not to discriminate because of N concentrations.

Many leaf traits have been related to defence against herbivory. Foliar nitrogen content has been suggested as the main determinant for interspecific and intraspecific differences in levels of attack by several authors (White 1984; Agrawal 2004). Unlike the increase in the N content of leaves with the decrease in temperatures reported in some studies (Weih and Karlsson 2001; Jian et al. 2009), in the present study no trends in leaf N contents related to temperature changes were observed, which is, on the other hand, consistent with other studies (Hultine and Marshall 2000; Chen et al. 2013). Therefore, the between-sites differences in levels of herbivory observed in the species analysed showed no relationship to differences in foliar nitrogen contents.

Conversely, the level of herbivory significantly decreased with the increase in LMA, probably because of the positive effect of this trait on leaf rigidity (Kitajima and Poorter 2010; Onoda et al. 2011). These results suggest that, as defended by other authors (Poorter et al. 2004; Carmona et al. 2011; Cárdenas et al. 2014), leaf rigidity would be the best predictor of differences in the rate of herbivory. The higher LMA at the coldest sites was a result of a thickening caused by higher amounts of structural carbohydrates in the cell wall, with no changes in density (Mediavilla et al. 2012; González-Zurdo et al. 2015). Both thickness and cellulose and hemicellulose contents had a negative effect on the levels of herbivory at the different sites, while lignin content had no significant effects. Different authors (Coley 1983; Kitajima et al. 2012; Cárdenas et al. 2014) have proved the relevance of cellulose content as a predictor of herbivory-related damage. The lack of effects of lignin contents on levels of herbivory has also been observed in diverse studies (Kurokawa and Nakashizuka 2008; Kitajima et al. 2012). Conversely, our results are in contrast with those of other authors who state that the levels of herbivory depend on leaf density (Kitajima et al. 2012), while leaf thickness (Cárdenas et al. 2014) or hemicellulose content (Kitajima et al. 2012) show no significant effects. The reason for these discrepancies could be the fact that the conclusions

of other authors are based on interspecific comparisons, while in this study responses are analysed at the intraspecific level.

Harsher climatic conditions during winter resulted in greater foliar damage by abiotic factors, with more leaves damaged by the effects of low temperatures and larger fractions of damaged area per leaf at the coldest sites for the three studied species. Accordingly, in contrast with its role in the protection against herbivores, the structural reinforcement in cold sites does not seem to contribute to protection against cold stress. Differences in rainfall and in Emberger's pluviothermic index had no effect on the level of damage of the leaves from the different sites. Other authors (Grossoni et al. 1998; Munné-Bosch and Peñuelas 2004) have found that drought stress also produces visible leaf damage symptoms. Mediterranean environments almost always suffer drought stress during the summer (Lionello et al. 2006). Therefore, the fact that rainfall levels have no effect on the intensity of abiotic damage suggests that, even though the rainfall levels recorded at each site were different, there were no differences in the intensity of drought stress during summer. In fact, Emberger's index varied little from site to site and showed no relation to the temperatures at each of them. Among the different environmental variables studied, minimum winter temperatures and the number of frost days per year had the strongest effects on the intensity of abiotic damage. On the other hand, the effects of maximum temperatures were non-significant.

Accelerated foliar senescence is usually related to oxidative stress (Günthardt-Goerg and Vollenweider 2007), so the most likely explanation for the higher levels of damage in cold climates is oxidative stress caused by excess light absorption in periods when temperatures represent a constraint for photosynthesis. In cold Mediterranean climates, during winter there are frequently periods of relatively high atmospheric pressure and clear skies when air temperature remains low during the whole day while leaves receive high levels of solar radiation. It is well-known that, if the energy absorbed from light that reaches the reaction centres exceeds the amount of energy that can be used, as is the case with low

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temperatures, photoinhibition may cause damage to the photosynthetic apparatus (Demming-Adams and Adams 1992; Lucinski and Jackowski 2006). This damage could be the main cause for the appearance of necrotic and chlorotic spotting in the leaves of the studied species. Depending on atmospheric conditions, UV irradiance may be expected to increase with altitude at a rate of between 3 and 7% km<sup>-1</sup> (Pfeifer et al. 2006). Accordingly, the differences in altitude between our sites could contribute to the responses observed in our species. In fact, increases in leaf thickness in response to UV-B radiation have been found in different experimental studies (Filella and Peñuelas 1999). The combination of low temperatures and high UV-B irradiances could exacerbate the appearance of visible symptoms of injury at higher altitudes. Damages by ozone have been reported at the Iberian Peninsula particularly in Mediterranean and other coastal areas (Saavedra et al 2012). However, to our knowledge, there are no reports of ozone damages in rural areas at the interior of the Iberian Peninsula for the three species included in the present study. Sclerophylly is considered as an adaptation that protects leaves against the effects of ozone, UV-B radiation and other pollutants (Bussotti and Ferretti 1998). Given the sclerophyllic character of the species studied, current ambient ozone levels in southern Europe are probably not high enough to induce visible injury in this species (Sanz and Calatayud 2016, <http://www.ozoneinjury.org/>). In any case, levels of ozone were very similar during the study years for the different sites (Table 1). Accordingly, differences in ozone effects cannot be responsible for the strong differences in visible symptoms observed between the different sites.

According to the results of the present study, in the environments with lower temperatures, evergreen tree species produced leaves with higher LMA and structural carbohydrates concentration. This structural reinforcement would have a negative impact on instantaneous C assimilation (Reich et al. 1997; Mediavilla et al. 2001; Niinemets and Sack 2006), which should be interpreted as a cost for the evergreen leaves produced at cold sites. This study shows that, in addition to this unfavourable effect on photosynthesis, damage caused by

abiotic factors was also higher at the sites with lower winter temperatures, which should contribute to a decrease in total CO<sub>2</sub> fixation across the entire lifespan of a leaf. On the contrary, at cold sites, losses resulting from herbivory were lower in both oak species. Moreover, lifespan of *Pinus pinaster* needles was slightly longer in colder than in warmer areas, although the trend was not significant. Therefore, the negative effects on leaf area persistence of the adaptation to cold might be partially counteracted by the positive ones. If these two types of effect are compared, in *Q. suber* the percentage of leaves attacked by herbivores was approximately 10% lower at the colder sites, which adds to 31% lower area consumed per leaf. Nevertheless, levels of damage by abiotic factors inversely increased in a proportion of 34% more damaged leaves and 79% more damaged area per leaf at the coldest sites. For current-year *Q. ilex* leaves, the decrease in levels of herbivory in the coldest plots in comparison with the warmest ones was of 19% less leaves attacked and 35% less average leaf area consumed. On the other hand, the intensity of damage caused by abiotic factors increased in around 47% more damaged leaves and 26% more average damaged area per leaf in the coldest environments compared to the warmest. Therefore, the reduction of anticipated losses of photosynthetic area caused by herbivory in cold environments would not, in principle, be enough to compensate the higher losses in photosynthetic area undergone in the coldest environments with respect to the warmest ones. Losses caused by herbivorous insects occur mainly during leaf formation, since herbivores show a preference for young leaves as opposed to old ones, which have lower concentrations of nutrients and higher rigidity (Coley and Aide 1991; Coley and Barone 1996). This means that the negative effects of area losses caused by herbivores take place over the leaf's whole lifespan. As opposed to this, damage caused by abiotic factors increases with leaf age, which allows the young leaves to maintain their photosynthetic area in healthy conditions during the most productive phases of their life cycles, because photosynthetic rates tend to decline with leaf age (Mediavilla and Escudero 2003b; Niinemets et al. 2005). Therefore, the timing of the appearance of both types of damage would mean that in equal amounts of damaged leaf area, the effect of herbivory on

productivity would be more negative than that of abiotic damage. However, losses caused by herbivory occur at very early phases of the leaf expansion period. If leaves are still small at the time of the attack, the volume of resource lost might be also relatively small. In fact, there were no significant differences in individual area between healthy leaves and those attacked by herbivores within a same species and site, which suggests that losses of the photosynthetic area due to this kind of attack are compensated during growth, so that the final leaf size is independent of herbivore attack. The differences between both types of leaf damage thus make it difficult to assess their final effects on leaf-level carbon balance. In any case, for both oak species, the increase in the intensity of abiotic damage at the coldest sites in relation to the warmest was stronger than the decrease in herbivory, which suggests that the negative impact of cold temperatures on the duration of the photosynthetic surface would be stronger than the positive effects. As far as *P. pinaster* is concerned, the percentage of leaves damaged by abiotic stress was very high, especially after their second year of life, although the percentage of damaged leaf area was small (around 4-5%). Therefore, the reduction in the photosynthetic area at cold sites was relatively small and lower than the lengthening of leaf lifespan in the coldest locations.

To our knowledge, this is the first study to provide data on the differences in anticipated losses of leaf area in populations of a same species located in different environments. Further study of other species is undoubtedly required to confirm our results. In any case, the anticipated loss of leaf area owing to herbivory and abiotic stress is a key factor that should be incorporated into models for estimating carbon sequestration capacity of the different tree species with the purpose of predicting future changes in ecosystem carbon balance.

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