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## N RESORPTION EFFICIENCY AND PROFICIENCY IN RESPONSE TO WINTER COLD IN THREE EVERGREEN SPECIES

### ABSTRACT

*Background and aims* Along temperature gradients, changes in leaf nutrient status have been reported by different authors, which suggest the existence of differences in nutrient availability and in the patterns of nutrient use. However, the effects of temperature on nutrient resorption efficiency within a species have rarely been studied. Our aim here is to analyze the effects of the differences in winter temperatures on the nitrogen resorption of the leaves of three evergreen tree species.

*Methods* Green and senescent leaves were sampled from mature specimens of the three species at 11 sites with contrasting winter temperatures. N resorption efficiencies were calculated from the differences between N contents in green and shed leaves collected from the same tree individuals. Minimum N concentrations in leaf litter were used as an estimation of resorption proficiency. Leaf mass per unit area (LMA) and concentrations of hemicellulose and cellulose were also determined in green leaves.

*Results* N contents in green leaves did not show any response to temperature gradients. By contrast, N contents in leaf litter increased with decreasing temperature. As a consequence, N resorption efficiency and proficiency declined with decreasing temperature. LMA and the concentrations of structural carbohydrates increased with declining temperature.

*Conclusions* The species studied have a lower potential for N resorption in environments with lower winter temperatures. The main reason for this lower efficiency seems to be the higher amounts of N immobilized in the greater amount of cell wall needed to cope with lower winter temperatures. The evergreen habit would thus be associated with higher costs at cooler sites, because the cold resistance traits imply reduced N resorption efficiency and

increased dependence on soil N.

**Keywords:** leaf structural reinforcement, N resorption efficiency, *Pinus*, proficiency, *Quercus*, winter temperature.

## INTRODUCTION

Nutrient resorption during leaf senescence is an important mechanism for plant nutrient conservation, and plays a key role in the function and dynamics of ecosystems (Aerts 1996; Aerts et al. 2007; Han et al. 2013). This reutilization of nutrients, which can be expressed either as resorption efficiency (% of the mature leaf nutrient pool that is resorbed) or resorption proficiency (the level to which nutrient concentration is reduced in senesced leaves, Killingbeck 1996), affects key processes such as the carbon cycle and resource use efficiency (Aerts and Chapin 2000; Gleason and Ares 2007) or the decomposition rates (Manzoni et al. 2008, 2010) and may decisively determine competitive relationships and fitness in terms of growth and reproduction (Aerts and Chapin 2000; Berg and McClaugherty 2008; May and Killingbeck 1992).

Owing to the importance of the resorption process, interspecific differences in resorption have been studied exhaustively, although they are still far from being understood. Many factors have been proposed to explain differences in resorption percentages, such as the relative size of sinks (Nambiar and Fife 1991; Silla and Escudero 2003), the phloem transport rate (Chapin and Kedrowski 1983; Chapin and Moilanen 1991), leaf abscission dynamics (Del Arco et al. 1991; Milla et al. 2005) and water availability (Del Arco et al. 1991; Pugnaire and Chapin 1992). Soil nutrient availability and the nutritional status of the leaves are among the determinants of nutrient resorption most studied, although the results are so divergent that they prevent any generalization. Thus, while some evidence suggests that nitrogen resorption can be determined by the availability of this nutrient in the soil, with more resorption in low-fertility soils (Enoki and Kawaguchi 1999; Holub and Tuma 2010; Lovelock

et al. 2007; Yuan et al. 2005), in many others cases no relationship has been observed (Aerts 1996; Diehl et al. 2003; Eckstein et al. 1999; Vergutz et al. 2012). Similarly, while some studies failed to find any relationship between resorption efficiency and nutrient concentrations in green leaves (Aerts 1996; Huang et al. 2007; Niinemets and Tamm 2005), other studies have found resorption efficiency to be related to plant nutrient status (Fife et al. 2008; Kobe et al. 2005; Vergutz et al. 2012; Wright and Westoby 2003).

In addition to these controversial findings in the response to the factors most frequently studied, there are still many other factors whose effects have barely been addressed, such as temperature. Numerous studies have demonstrated changes in leaf morphology and chemical composition in response to air temperature, particularly as regards the foliar nutrient concentration (Han et al. 2011; Reich and Oleksyn 2004; Zhang et al. 2012). If it is true that temperature variations determine changes in the composition of the leaves, and if foliar nutrient status indeed finally affects resorption efficiency, one would then also expect to see effects of temperature on resorption percentages. In fact, however, very little is known about this issue, the existing information being limited to a few studies that have either been performed under controlled conditions (and also in this case with often conflicting results), or at a global scale through large environmental gradients. Declines in resorption efficiency with increasing temperature of the environment of origin have been observed, for example, in common-garden experiments conducted by some authors (Oleksyn et al. 2003; Oyarzabal et al. 2008), while others have found only a minor impact on nutrient resorption parameters (Aerts et al. 2007; Welker et al. 2005). Moreover, recent studies that have addressed geographic patterns of nutrient conservation strategies in relation to temperature are consistent in reporting a decrease in the N resorption efficiency in plants occupying warmer environments (Tang et al. 2013; Vergutz et al. 2012; Yuan and Chen 2009). However, these resorption patterns at a global scale include different plant communities and may be influenced by differences in the leaf traits of the different species and in environmental factors, such as the age of the soil substrate and associated soil

nutrient availability. Little is known, by contrast, about the effects of temperature on leaf resorption at smaller geographic scales and at the level of a same species. Given the importance of the resorption process for plants and ecosystems, more data are needed to clarify this issue, allowing us to incorporate nutrient resorption into models of plants' responses to climate change (Aerts et al. 2007; Chapin et al. 2011).

In the present work we analyze the effects of the differences in climate harshness during winter on the nitrogen resorption (efficiency and proficiency) of the leaves of three evergreen species widely distributed across the Iberian Peninsula (*Quercus ilex* ssp. *ballota* (Desf.) Samp, *Quercus suber* L. and *Pinus pinaster* Aiton). A previous study with the same species revealed significant differences between sites in several leaf traits related to differences in the intensity of winter harshness, with a similar response in all three species. Thus, the leaves from one species growing in environments with cooler winters had a greater leaf mass per unit area (LMA) than those produced at the sites with milder winters, with no trends in the leaf nitrogen content between environments (Mediavilla et al. 2012). Accordingly, our previous data suggest that winter climate harshness (in particular the intensity and frequency of frosts) contributes to increasing the sclerophyllic character of these evergreen species. Several authors (Chapin and Kedrowski 1983; Pugnaire and Chapin 1993) have proposed that resorption efficiency would depend on the availability of soluble forms of nitrogen, which facilitate remobilization. In particular, a large fraction of N is retained in cell walls (Reiter 1998) and this is only partially retranslocated (Yasumura and Ishida 2011). Thus, according to some authors, the potential for N resorption may vary with the amount of cell wall in the leaves, decreasing with a higher fraction of N associated with structural proteins occluded in the cell wall matrix (Hikosaka 2004; Niinemets and Tamm 2005). Possible differences in the leaf chemical composition associated with differences in the intensity of winter harshness could thus result in differences in resorption efficiencies. Our hypothesis is that if the resorption process varies with the availability of soluble forms of nitrogen for resorption, the amount of N retranslocated during senescence should be lower

in colder environments, where evergreen leaves tend to be more sclerophyllic than in warmer environments. Our aim therefore is to check whether in addition to the costs associated with a higher LMA at the coldest sites (Mediavilla et al. 2012), a lower resorption efficiency might also contribute to increasing the costs associated with a higher dependence on soil N in these environments. This information is essential to help understand the possible repercussions of climate change on the distribution of woody species and to improve predictions of vegetation responses to new climatic scenarios.

## **MATERIALS AND METHODS**

### *Study species and area*

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

The species were distributed on 11 sites located in the regions of Castilla-León and Extremadura (central-western Spain) between latitudes 41° 45' N and 40° 01' N and between longitudes 6° 22' W and 2° 08' W (Table 1). The sites consisted of flat areas with sparse populations (between 50 and 100 specimens ha<sup>-1</sup>) 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 broad gradient of winter temperatures, although taking care that the rest of the climate characteristics would be as homogeneous as possible. Nevertheless, there is a tendency for rainfall levels to be higher at 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. Annual rainfall data from the station nearest to each stand were provided by the

Table 1. Sites characteristics.

SITES	A	B	C	D	E	F	G	H	I	J	K
Longitude (W)	5° 57'	5° 48'	6° 22'	5° 02'	5° 52'	5° 47'	6° 07'	2° 08'	2° 37'	2° 43'	2° 52'
Latitude (N)	40° 13'	40° 01'	39° 49'	40°11'	41° 14'	41° 08'	40° 55'	40° 59'	41° 45'	41° 32'	41° 43'
Altitude (m.a.s.l.)	619	466	600	449	985	834	832	1246	1189	982	1045
Mean temperature (°C)											
Annual	16.5	16.6	16.8	16.4	12.3	12.7	13.0	10.1	10.3	11.0	10.6
Spring	18.4	19.3	19.0	19.1	15.0	14.9	15.7	12.0	13.0	14.5	13.2
Summer	25.6	25.8	26.3	26.3	20.8	21.4	22.3	19.4	19.1	19.0	19.1
Autumn	12.6	12.2	13.2	11.5	7.84	9.11	8.32	6.09	6.03	6.51	6.35
Winter	9.55	9.07	9.81	9.2	5.55	5.30	5.86	3.04	3.14	3.99	3.53
Absolute minimum temperature	-2.15	-4.15	-3.55	-5.5	-8.18	-9.82	-7.50	-13.1	-13.7	-15.0	-16.9
Mean daily minimum temperature of the coldest month	4.5	4.06	4.69	3.19	-0.47	-0.90	-1.06	-1.96	-2.93	-3.28	-3.13
Number of days with frost per year	7	15	5	20	57	61	59	98	105	105	112
Mean duration of the period with frosts (days per year)	24	68	55	90	127	154	100	193	222	202	214
Annual precipitation (mm)	729	1055	598	615	385	328	380	383	496	473	506
Emberger's pluviothermic index	72	105	60	60	52	46	48	58	79	69	78
Soil											
Sand content (%)	81.1	77.2	73.9	75.7	84.6	74.3	67.9	57.8	75.1	84.8	84.0
Clay content (%)	8.5	11.7	12.3	10.6	6.4	12.2	16.2	22.8	14.4	8.1	8.3
Silt content (%)	10.4	11.1	13.8	13.7	9.00	13.5	15.9	19.4	10.5	7.10	7.7
Total soil N content (%)	0.082	0.143	0.104	0.126	0.072	0.021	0.078	0.112	0.115	0.040	0.048
P assimilable (ppm)	31	38	5	6	10	4	20	2	7	9	6
M.O. (%)	1.66	4.47	4.10	2.78	2.6	0.37	1.66	1.96	5.12	0.69	1.92
pH	5.7	4.5	4.7	5.1	4.6	4.5	4.4	7.6	4.8	7	6.1
Species	Qi, Qs	Qs	P, Qi, Qs	P, Qi, Qs	P, Qi, Qs	P, Qi, Qs	Qi, Qs	Qi	P, Qi	P, Qi	P, Qi

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

Spanish National Meteorological Institute. Air temperature data were obtained for each site by means of data loggers (Hobo Pendant temperature/light datalogger, Part UA-002-08, Onset Computer Corporation, Pocasset, MA, USA). The data loggers were programmed to obtain temperature data every 10 min and they were kept at each site for four years (October 2008 – October 2012). In spring, soil samples were taken down 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 in Salamanca according to the methods described by Chapmann and Pratt (1973) and Walkley and Black (1934).

### *Collection and processing of samples*

For each species, 5 individual trees were selected at random at each site. To obtain freshly fallen leaf litter, 10 leaves of each of the two oldest leaf cohorts present in the crown of each individual were held in place via a clip attached by a thread to the stem that allowed them to be retained on the plant after their abscission. 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. The tagged leaves were always situated at a mid-height in the sun-lit portion of the crown. The reduced size of the clips allowed the surface of the leaf covered by them to be sufficiently small for the damage to be almost negligible, as checked in previous assays. In any case, to minimize damage the clips were always emplaced in situ slightly before the start of the typical abscission period of each species. Although this method is obviously more time consuming than the “classic” litter-trap method, it does allow the errors associated with intra-canopy variability in leaf traits to be reduced, because the estimation of resorption is based on the comparison between green and senesced leaves collected from the same branches and with a similar size and mass per unit area. Once the clips had been set up, monthly samplings of the leaves shed were performed until all leaves selected had been collected. The sampling frequency was increased to once a week between late spring

and late summer to suitably record the periods of abscission typical of these evergreen species (Escudero and Del Arco 1987). Depending on the individual tree, most of the leaves were shed at ages between 1 and 2 years for *Q. suber*, 2-3 years for *Q. ilex* and between 3 and 6 years in *P. pinaster*. In spring of each year, before the abscission period, 25 green leaves of each of the two oldest leaf cohorts were collected from the same individuals and the same branches in which leaves were marked to collect leaf litter. The N content of these green leaves was then used to estimate N resorption efficiency. This sampling procedure was repeated along 3 years (2011-2014).

At the laboratory, 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). 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 dried, for each species on each sampling date all senesced and green leaves of the same age class collected from each individual were ground together to obtain a sufficient amount of sample for chemical analyses. Leaf N concentrations were determined with a CE-Instruments NA-2100 autoanalyzer (ThermoQuest, Milan, Italy). After the N analyses, the remaining material of the green leaves was used to analyze the fibre content (hemicellulose and cellulose) with an Ankom Analyzer (A220, New York, USA), following the method of Goering and Van Soest (1970). The nitrogen and fiber contents of leaves were expressed per unit dry mass (as milligrams of N or fiber per gram of leaf dry mass), and per unit of leaf area ( $\text{g m}^{-2}$ ), obtained as the concentration per unit dry mass multiplied by LMA.

### *Data analysis*

For each individual tree we obtained a value of the resorption efficiency for each date on

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which the leaf litter was collected as  $(100 * (N \text{ in green leaves} - N \text{ in shed leaves}) / N \text{ in green leaves})$ . For the N content in living leaves, we used that of the green leaves collected from the same tree, which was compared with that of the shed leaves of the same age class. Leaf mass loss during senescence was calculated according to a similar equation. Resorption efficiency (RE) was calculated from the nitrogen concentrations expressed both per unit leaf mass ( $RE_{\text{mass}}$ ) and per unit leaf area ( $RE_{\text{area}}$ ). A single resorption efficiency value was estimated for each individual tree as the average of the values obtained on all the dates on which abscission was recorded weighted by the number of leaves shed on each sampling occasion throughout the entire 3-year study period, after checking that there were no significant differences in the average resorption efficiency obtained between the different years of study (data not shown). Finally, a single resorption estimate was calculated for each species and site as the average of the values obtained for each individual tree. To estimate the N resorption proficiency of each species at each site and in each year of study we selected the absolute minimum N content value (expressed per unit mass and per unit leaf area) recorded among all shed leaves for each of the five individuals selected for that species and site. Finally, a single resorption proficiency value was obtained for each species at each site as an average of the fifteen data (five trees and 3 years), after checking that there were no significant differences in the average resorption proficiency obtained for the same species and site between the different years of study.

The relationships between resorption efficiency and proficiency and different temperature measurements were explored by means of linear regression analysis. We assigned a single value to each site for mean monthly temperatures and mean monthly maximum and minimum daily temperatures, obtained as an average of the values measured for each month along the study period. For each site, we also obtained a single value for the absolute maximum and minimum temperatures, the number of days with frost along the study period and the annual rainfall. We used the Emberger's pluviothermic index (Emberger 1930) to analyze the effects of water stress on resorption parameters:

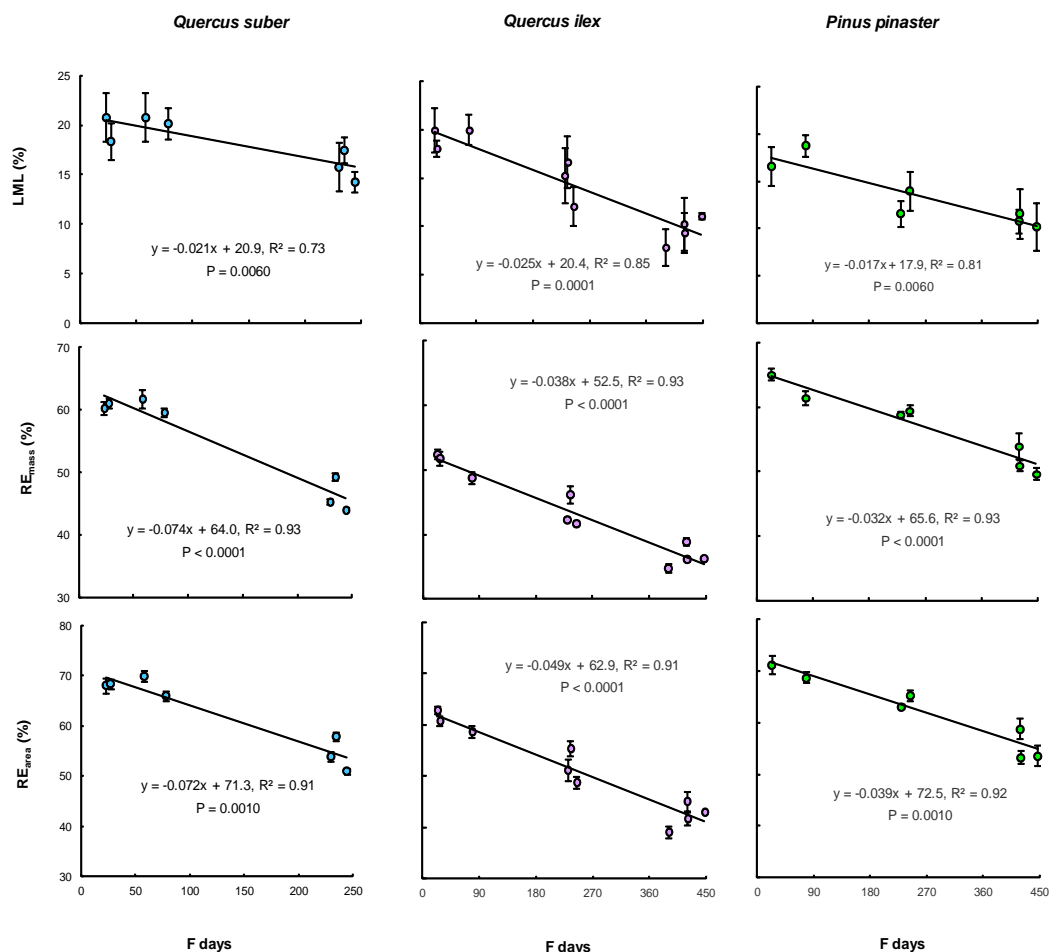
$$Q = \frac{1000 \times P}{(273 + \frac{T_X + T_N}{2}) \times (T_X - T_N)}$$

where  $P$  is the annual precipitation (mm),  $T_X$  is the average temperature of the hottest month (°C) and  $T_N$  is the average temperature of the coldest month (°C). This index is commonly used in Mediterranean climates (Kunstler et al. 2007). Similarly, to better explore the relationships between N resorption and the different leaf traits analyzed in leaves of the same age class, the data obtained for each trait in each species at each site were pooled over the entire study period, after checking that there were no significant differences in the values obtained for the same species and age class at each site in the different years of study. Linear regression analysis was also used to check the relationships between resorption efficiency and leaf traits. When necessary, data were log-transformed to linearize the relationships. All statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA).

## RESULTS

### *Patterns of leaf nitrogen resorption in relation to winter temperature*

Leaf mass loss during senescence ranged between approximately 8 and 20% of the “green” mass and was more marked in the warmer than in the colder environments for all three species (Fig. 1). Due to the decrease in leaf mass accompanying the decrease in N concentrations during senescence, the N resorption percentage was always higher when N contents were expressed on a leaf area basis than on a leaf mass basis. However, for both  $RE_{\text{mass}}$  and  $RE_{\text{area}}$  in all species the average resorption efficiency showed a clear negative relationship with the total number of days with frost along the study period (Fig. 1). Within a site, different individual trees tended to have very similar resorption efficiencies, as demonstrated by the low standard errors calculated (Fig. 1). Resorption efficiency per unit area was also related to other indicators of the intensity of winter harshness (mean monthly



**Fig. 1** Leaf mass loss during senescence (LML) and nitrogen resorption efficiency on a per unit leaf mass ( $RE_{mass}$ ) and per unit leaf area ( $RE_{area}$ ) basis as a function of the total number of days with frost along the study period (F days) at each site. Each data point is the mean and standard error of the 5 individual trees sampled at each site.

minimum daily temperatures or absolute minimum winter temperature) (Table 2). Maximum temperatures, by contrast, either had no influence or the effect was less significant than that of winter harshness (Table 2). In most cases, the rainfall levels and the Emberger's pluviothermic index did not reveal significant effects on resorption efficiencies. Total soil nitrogen contents showed no trend along the temperature gradient (Table 1). Accordingly, there was no relationship between the N resorption values at each site and the levels of N recorded in the soils of the same sites for any of the species (Table 2).

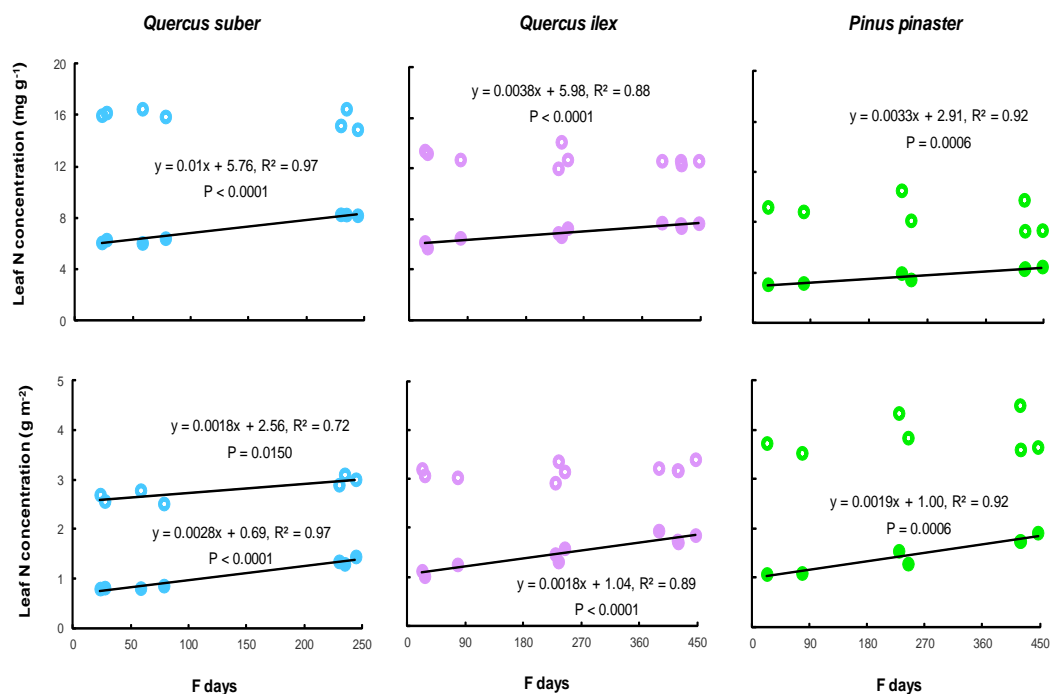
These differences in resorption efficiency in response to winter harshness were due mainly to differences in the amount of N in leaf litter, since no trend was observed for the nitrogen

**Table 2.** Summary of linear regressions relating N resorption efficiency per unit area ( $RE_{area}$ , %) and per unit mass ( $RE_{mass}$ , %) to different environmental characteristics and to leaf N content.

Dependent variable	Independent variables	<i>Q. suber</i> ( <i>n</i> = 7)				<i>Q. ilex</i> ( <i>n</i> = 10)				<i>P. pinaster</i> ( <i>n</i> = 7)			
		Intercept	Slope	R <sup>2</sup>	P	Intercept	Slope	R <sup>2</sup>	P	Intercept	Slope	R <sup>2</sup>	P
$RE_{area}$	Mean <i>m min T</i> (°C)	36.68	2.79	0.889	0.0015	33.94	2.42	0.880	0.0001	48.53	2.07	0.868	0.0023
$RE_{area}$	Min <i>T</i> (°C)	77.66	2.65	0.899	0.0012	65.67	1.57	0.867	0.0001	75.67	1.32	0.876	0.0019
$RE_{area}$	Mean <i>m max T</i> (°C)	-32.03	4.23	0.596	0.0420	-21.45	3.45	0.743	0.0013	11.17	2.47	0.680	0.0224
$RE_{area}$	max <i>T</i> (°C)	-27.07	2.13	0.371	0.1465	-59.61	2.69	0.387	0.0549	-9.94	1.76	0.234	0.2716
$RE_{area}$	Annual rainfall (mm)	38.81	0.043	0.918	0.0007	31.78	0.039	0.336	0.0791	54.16	0.0161	0.057	0.6070
$RE_{area}$	Emberger's index	46.54	0.241	0.416	0.1179	62.81	-0.195	0.076	0.4394	88.55	-0.42	0.574	0.0484
$RE_{area}$	Total soil N content (%)	48.21	172.9	0.519	0.0677	46.62	50.72	0.044	0.5599	58.84	42.11	0.059	0.5996
$RE_{mass}$	$N_{mass}$ (mg g <sup>-1</sup> )	-88.36	8.99	0.493	0.0785	-38.84	6.38	0.340	0.0767	40.67	2.03	0.138	0.3644
$RE_{area}$	$N_{area}$ (g m <sup>-2</sup> )	135.6	-26.31	0.561	0.0527	108.3	-18.19	0.095	0.3873	85.72	-5.83	0.101	0.4423

*mean m min T* Mean monthly minimum daily temperature, *min T* Minimum winter temperature, *mean m max T* Mean monthly maximum daily temperature, *max T* Maximum summer temperature,  $N_{mass}$  N concentration per unit mass in green leaves,  $N_{area}$  N content per unit area in green leaves

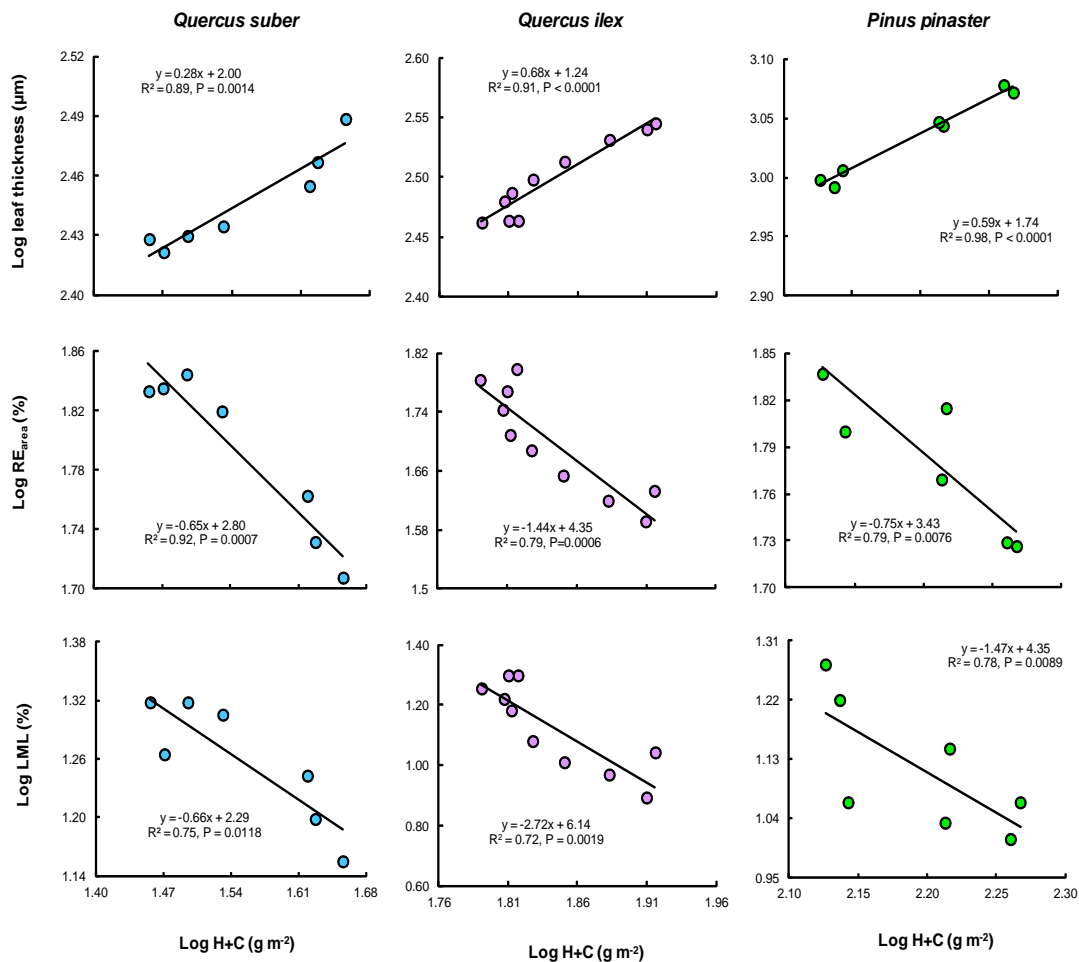
content in green leaves in response to the number of frosts, with the exception of N content per unit area in *Q. suber* (Fig. 2). By contrast, N concentrations in leaf litter showed a clear positive relationship with the number of frosts at each site (Fig. 2).



**Fig. 2** Nitrogen contents in green (open symbols) and senesced (closed symbols) leaves as a function of the total number of days with frost along the study period (F days) at each site. Error bars are omitted for clarity

### Relationships between nitrogen resorption and different leaf traits

In the three species, LMA, leaf thickness and the structural carbohydrate content (cellulose + hemicellulose) showed a pronounced response to changes in temperature, increasing between the sites with the intensity of their winter harshness (higher total number of days with frost and lower minimum temperatures) (data not shown). Accordingly, the reinforcement of the leaves with a greater LMA as the harshening of winter conditions progresses seems to occur through leaf thickening as a result of a greater amount of structural carbohydrates. Indeed, there was a strong positive relationship between the structural carbohydrate content and the leaf thickness for the three species (Fig. 3). This



**Fig. 3** Green leaf thickness, nitrogen resorption efficiency on a per unit leaf area (RE<sub>area</sub>) basis and leaf mass loss during senescence (LML) and as a function of hemicellulose and cellulose (H + C) contents in green leaves at each site. Error bars are omitted for clarity.

leaf reinforcement at the coldest sites resulted in significant negative effects of the structural carbohydrate content on N resorption efficiency and leaf mass loss during senescence (Fig. 3). Since no trend was observed for the nitrogen content in green leaves in response to temperature, no significant relationships between leaf N content and resorption efficiency were observed (Table 2). Minimum N concentrations in leaf litter increased (proficiency decreased) with the increase in LMA and the structural carbohydrate content in all three species (Table 3). No significant relationships were observed between the N concentration in green leaves and the resorption proficiency for any of the species (Table 3).

**Table 3.** Linear regression parameters for N content ( $\text{mg g}^{-1}$ ) in leaf litter depending on leaf mass per unit area (LMA,  $\text{g m}^{-2}$  hemicellulose + cellulose content (H + C,  $\text{mg g}^{-1}$ ) and N content ( $\text{mg g}^{-1}$ ) in green leaves.

Dependent variable	Independent variables	<i>Q. suber</i> (n = 7)			<i>Q. ilex</i> (n = 10)			<i>P. pinaster</i> (n = 7)		
		y	R <sup>2</sup>	P	y	R <sup>2</sup>	P	y	R <sup>2</sup>	P
N (leaf litter)	LMA	0.06x - 2.94	0.82	0.0050	0.05x - 4.70	0.82	0.0003	0.01x - 1.13	0.59	0.0449
	H + C	0.04x - 1.48	0.72	0.0162	0.03x - 1.81	0.51	0.0197	0.03x - 5.91	0.60	0.0424
	N (green leaves)		NS			NS			NS	

## DISCUSSION

All the leaves studied underwent a decrease in mass per unit area during senescence. Mass loss can be due to several processes, including resorption, leaching and in situ decomposition between the time of leaf abscission and the collection of the fallen leaves (Vergutz et al. 2012). In the present case, losses due to decomposition and leaching were unlikely, because most leaves were shed during the dry season and remained suspended in the air until they were sampled thanks to the retention clips used. Accordingly, the losses of biomass during senescence must essentially be the consequence of the remobilization of carbon, which -as demonstrated by several authors- represents an important source for growth and reproduction (Eckstein et al. 1998; Karlsson 1994; Vergutz et al. 2012). Evidently, these leaf mass losses affect the total content of N of the senescent leaves and, accordingly, also the estimation and interpretation of the resorption data (Fife et al. 2008; Franklin and Ågren 2002; van Heerwaarden et al. 2003). This is because if leaf mass declines during senescence, nutrient resorption, expressed as a percentage of mass, is underestimated (Chapin and Kedrowski 1983; van Heerwaarden et al. 2003). Indeed, in our study in all cases the N resorption percentages proved to be higher when expressed on a leaf area basis. However, regardless of the mode of expression in all cases a decrease was observed in the N resorption efficiency in the colder environments. In addition, leaf mass losses during senescence were also more marked in warmer environments, which exacerbated the between-site differences in N resorption when expressed per unit leaf area. Killingbeck (1996) proposed that resorption is highly proficient in plants that have reduced

their leaf N during senescence to concentrations lower than  $7 \text{ mg g}^{-1}$ . Thus, according to this proposed resorption threshold, only *P. pinaster* would reach complete N resorption along the whole temperature gradient, with minimum N values in senesced leaves significantly lower than those of the other species. In both *Quercus* species, however, minimum N concentrations in senesced leaves were only below this threshold in the warmer environments, incomplete resorption efficiency being observed at the cooler sites. The lower resorption efficiency at colder sites was mainly due to the effect of minimum winter temperatures. Between-site differences in winter temperatures and in the frequency and intensity of frosts were much more marked than the differences in summer maximum temperatures. As a consequence summer temperatures showed much weaker effects on resorption efficiencies. Differences in the intensity of drought stress is one of the factors previously reported as determinants of the differences in resorption efficiency (Del Arco et al. 1991; Pugnaire and Chapin 1992). Higher summer temperatures could thus have an effect on N resorption if they determine more severe drought at the warmer sites. However, in the present study the warmer sites also receive greater rainfall, which could reduce the differences in drought stress. In fact, the Emberger's pluviothermic index was not related to summer temperatures (Table 1). Accordingly, rainfall levels and the pluviothermic index tended to have weak and inconsistent effects on resorption, which suggests that the between-site differences in N withdrawal were a direct effect of minimum winter temperatures and not of differences in the levels of drought stress during summer.

According to our results, in environments subjected to a higher intensity of winter harshness the leaves of the three species were reinforced with a greater LMA due to the increased thickness achieved with greater amounts of structural carbohydrates. The increased leaf thickness and cell wall content appear to be necessary to reduce the risk of freezing in leaves in cold environments (Ball et al. 2002; Rajashekar and Lafta 1996). Unlike other studies, which have reported increases in foliar N contents with decreases in temperature (Jian et al. 2009; Reich and Oleksyn 2004; Weih and Karlsson 2001), we did not find any



trend in the amounts of this nutrient in green leaves associated with changes in temperature, as in fact has also been observed in other cases (Chen et al. 2013; Hultine and Marshall 2000; Premoli and Brewer 2007). The changes in leaf morphology and chemical composition were also accompanied by a significant decrease in N resorption efficiency and proficiency with lower winter temperatures.

Recent studies have reported lower resorption efficiencies in plant communities occupying warmer environments (Tang et al 2013; Vergutz et al. 2012; Yuan and Chen 2009). Yuan and Chen (2009) suggested that this response of resorption efficiency to temperature can be explained mainly in terms of changes with temperature in the N concentrations in green leaves, which decrease in warmer environments, with relatively smaller changes in the N concentrations in senesced leaves. Their results are consistent with those reported in the literature suggesting that resorption efficiency is modulated by the N status of plants (Chapin and Kedrowski 1983; Escudero et al. 1992; Fife et al. 2008), with more resorption in plants with more nitrogen in green leaves, probably because the amount of highly retranslocatable N is higher than that found in N-poor leaves (Chapin and Kedrowski 1983; Milla et al. 2005). In the present study, however, the differences in resorption efficiency between environments were related to changes in the N content in senesced leaves, with no associated trends in the N content in green leaves. Thus, the lower efficiency within a same species found in colder environments may instead be explained by the higher amounts of N immobilized in the greater amount of cell wall needed to cope with lower winter temperatures in these environments. Our results therefore support the hypothesis that the potential for N resorption varies with the amount of cell wall in the leaves, decreasing with a higher fraction of N associated with structural proteins occluded in the cell wall matrix (Hikosaka 2004; Niinemets and Tamm 2005). The need for stronger leaf reinforcement in colder areas inevitably leads to lower N resorption efficiency. Changes in leaf chemical composition in response to temperature must therefore be incorporated into the range of factors that can be invoked to explain the intraspecific variations in N resorption efficiency.

The differences in structural carbohydrate contents along the temperature gradient also explain the differences in leaf mass loss during senescence, since C resorption is determined by the availability of retranslocatable nonstructural C fractions (Vergutz et al. 2012).

Our results are therefore not in agreement with the conclusions of previous analyses of geographic patterns of nutrient resorption. These discrepancies might be explained by the fact that most previous studies describe resorption differences among different plant communities, which may be affected by differences in the leaf traits of the different species, such as differences in leaf life span (Vergutz et al. 2012), whereas in the present study temperature effects were measured within a same species. However, Oleksyn et al. (2003) also found a trend towards higher resorption efficiencies in northern latitudes in Scots pine populations, i.e., a result opposite to that found in the present study. In principle, we do not have any explanation for this discrepancy. The resorption efficiencies in Oleksyn et al. (2003) were expressed on a concentration per unit mass basis, which may lead to biased results if, as observed here, leaf mass loss during senescence varies with temperature. In addition, the survey of those authors was performed in colder areas than those addressed in the present study and along broader geographical gradients. These differences might involve additional uncontrolled factors that could explain the mentioned discrepancy.

Although it is difficult to extrapolate the responses observed here to a new scenario of climate change, it is evident that all these temperature-related changes must have profound ecological implications. The evergreen habit demands that leaves must be present throughout the year, overcoming unfavorable seasons, among them winter. As suggested by our observations, evergreen species must reinforce their leaves to a greater extent at the coldest sites in order to maintain their leaf longevity. This necessary greater leaf reinforcement at the coldest sites results in lower N resorption efficiency, thereby increasing the dependence on soil N. This must contribute to increasing the costs of the evergreen habit at the coldest sites. If climate change eventually leads to an increase in temperatures

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and a decrease in winter harshness (IPCC 2007), this could reduce the unfavorable effects associated with the leaf traits shown by evergreen species under conditions of low temperatures. It is clear in any case that the effect of temperature on the resorption process must also be incorporated into predictive models of the implications of climate change on vegetation.

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