

Plasticity and stress tolerance override local adaptation in the responses of Mediterranean holm oak seedlings to drought and cold

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Summary Plant populations of widely distributed species experience a broad range of environmental conditions that can be faced by phenotypic plasticity or ecotypic differentiation and local adaptation. The strategy chosen will determine a population's ability to respond to climate change. To explore this, we grew *Quercus ilex* (L.) seedlings from acorns collected at six selected populations from climatically contrasting localities and evaluated their response to drought and late season cold events. Maximum photosynthetic rate (A_{\max}), instantaneous water use efficiency (iWUE), and thermal tolerance to freeze and heat (estimated from chlorophyll fluorescence versus temperature curves) were measured in 5-month-old seedlings in control (no stress), drought (water-stressed), and cold (low suboptimal temperature) conditions. The observed responses were similar for the six populations: drought decreased A_{\max} and increased iWUE, and cold reduced A_{\max} and iWUE. All the seedlings maintained photosynthetic activity under adverse conditions (drought and cold), and rapidly increased their iWUE by closing stomata when exposed to drought. Heat and freeze tolerances were similarly high for seedlings from all the populations, and they were significantly increased by drought and cold, respectively; and were positively related to each other. Differences in seedling performance across populations were primarily induced by maternal effects mediated by seed size and to a lesser extent by idiosyncratic physiologic responses to drought and low temperatures. Tolerance to multiple stresses together with the capacity to physiologically acclimate to heat waves and cold snaps may allow *Q. ilex* to cope with the increasingly stressful conditions imposed by climate change. Lack of evidence of physiologic seedling adaptation to local climate may reflect opposing selection pressures to complex, multidimensional

environmental conditions operating within the distribution range of this species.

Keywords: *Quercus ilex*, thermal tolerance, water use efficiency.

Introduction

Broadly distributed tree species are submitted to a wide range of climatic conditions. There are different ways these species can follow to cope with this environmental variability, and the strategy chosen determines their vulnerability to climatic changes (Davis et al. 2005, Jump and Peñuelas 2005). Two possible, non-excluding, strategies are enhancing phenotypic plasticity, i.e., the ability of a given genotype of rendering different phenotypes under different environmental conditions (Bradshshaw 1965, Valladares et al. 2007) and adapting to local climate (ecotypic differentiation); i.e., evolving traits that provide an advantage under the local environmental conditions (Kawecki and Ebert 2004, Savolainen et al. 2007). Both are mechanisms of responding to climate change (Visser 2008) that sum up to migration and extinction (Davis et al. 2005). Mediterranean climate is characterized by a pronounced seasonality and a remarkable unpredictability of rainfall and extreme events (Valladares et al. 2002a, 2002b, Oliveira and Peñuelas 2004, Cowling et al. 2005). Summer drought is considered the main constraint to plant's biomass production in the Mediterranean and therefore the primary limit of their distribution and abundance (Larcher 2000, Gulías et al. 2002, Sánchez-Gómez et al. 2006). However, in Mediterranean-continental areas, cold temperatures also play a relevant, albeit less explored, role in limiting plant survival and growth. Evergreen species dwelling in these areas have to

overcome long periods of low temperatures and frosts (García-Plazaola et al. 1999a, 1999b, Thomashow 1999, Aranda et al. 2005, Cavender-Bares 2007). Furthermore, these areas are subjected to periods of extreme temperatures: in summer temperature can rise up to 45–50 °C and in winter it can drop to –10 °C (Martínez-Ferri et al. 2004, Corcuera et al. 2005, Valladares et al. 2008). Surviving under these conditions requires great stress tolerance, phenotypic plasticity or both. Protection against heat involves a series of molecular mechanisms responsible for general cell survival (Kozłowski 2002, Wang et al. 2004), and freeze protection is achieved through a series of biochemical and physiologic changes that are dependent on the expression of low-temperature-responsive genes (Novillo et al. 2004, Suping et al. 2005). All these adaptive and constitutive mechanisms provide protection against cell damage and maintenance of photosynthetic function, which may play a key role for survival and growth of evergreen Mediterranean plants during both summer and winter (Oliveira and Peñuelas 2004, Cavender-Bares 2007, Varone and Gratani 2007).

We have explored the ecophysiological responses to drought and cold events of seedlings from different populations of holm oak (*Quercus ilex* ssp. *ballota* [Desf.] Samp. [Fagaceae]), an evergreen tree widely distributed over the Mediterranean region. In the previous field and glasshouse experiments, *Q. ilex* and related species have shown a capacity to acclimate their morphology and physiology to changing environments (Gratani et al. 2003, Martínez-Ferri et al. 2004, Cavender-Bares et al. 2005, Sardans et al. 2006). However, phenotypic plasticity of evergreen Mediterranean oaks in response to light and nutrients was considered to be low when compared to that of tropical evergreen shrubs (Valladares et al. 2002a, 2002b). According to the predictions, for the Mediterranean region, climate change will involve temperature rise, decreased rainfall, increased climate variability (Lavorel et al. 1998, Ogaya and Peñuelas 2004, Christensen et al. 2007), and frequency of extreme events (such as heat waves and late season frosts, Gu et al. 2008). Under this new climatic scenario, we can expect that species with greater phenotypic plasticity will have an advantage over those more narrowly adapted (Davis et al. 2005, Parmesan 2006, Valladares et al. 2007). We expect that the contrasting climatic conditions derived from geographically distant populations may have exerted different selective pressures that would have led to the ecotypic differentiation in this widespread tree (Heschel et al. 2002, Gianoli and González-Teuber 2005, Nicotra et al. 2007, Savolainen et al. 2007). As the mechanisms involved in climate tolerance and acclimation capacity are gene-expression dependent, there is an opportunity for natural selection to act, leading to local adaptation. In fact, this is what has been found in certain studies of holm oak (Gratani et al. 2003, Sánchez-Vilas and Retuerto 2007) and other evergreen sclerophyllous species (Boorse et al. 1998, Balaguer et al. 2001, Zarter et al. 2006). Thus, we

hypothesized that *Q. ilex* seedlings from different populations with contrasting climates would respond differently to the exposure to drought and cold. Our specific questions were: (i) What are the constitutive and adaptive ranges of heat and freeze tolerances in this species? (ii) Are these two tolerances related? (iii) Do seedlings from contrasting populations differ in their ecophysiological performance and in their acclimation capacity according to the climate of the population of origin?

Materials and methods

Plant species and seed collection sites

Quercus ilex ssp. *ballota* (Desf.) Samp. (holm oak) is an evergreen, drought-adapted tree, found all over the Mediterranean region (Rodá et al. 1999). In the Iberian Peninsula, *Q. ilex* is found over a wide range of conditions spanning from extreme continental sites, with harsh winters and marked daily and annual thermal oscillations, to mild coastal areas. It is a monoecious tree, wind-pollinated and with animal-dispersed acorns. It grows in Mediterranean-continental areas, always under climatic conditions marked by dry and hot summers (Castroviejo et al. 1993).

We selected six populations to cover the main range of climatic conditions of *Q. ilex* distribution in the Iberian Peninsula. The six populations ranged from a maximum mean annual temperature of 16.25 °C to a minimum of 10.68 °C (Table 1; Figure 1). According to the aridity index of De Martonne (I_{ar} DM; De Martonne 1926), populations ranged from semiarid (El Pardo and Mérida), subhumid (Plasencia and Villuercas) to humid (Alto Tajo and Hurdes) (Table 1). Mean month air temperatures and rainfall were obtained from the closest weather station of the Spanish Institute of Meteorology. In autumn 2006, we collected acorns from 10 randomly chosen individuals, to obtain a random genetic sample of each population. All acorns from each population were pooled together and they were not labeled with the mother tree, as the genetic variability was only considered at the population level. Acorns were kept in a cold and dark room until they were sown.

Experimental design

In January 2007, we sowed 100 acorns per population on the same day. Seedlings were grown in a glasshouse during winter and spring 2007 (January–June). During the growth period, temperature and irradiance readings were recorded in the glasshouse every 5 min with a Hobo H08-006-04 data logger (Onset, Pocasset, MA). Mean, maximum and minimum daily temperatures were 19.74, 31.33 and 12.98 °C, respectively. Relative humidity on average was $27 \pm 11\%$. Photosynthetically active radiation (PAR) was measured with four quantum sensors cross-calibrated with a Li-Cor 190SA sensor (Li-Cor, Lincoln, NE). Mean daily total PAR available was $23.7 \pm 5.8 \text{ mol m}^{-2} \text{ day}^{-1}$

Table 1. Mean \pm SE annual temperatures (T), annual precipitation (P), temperature of the warmest (T_{\max}), and the coldest month (T_{\min}) of the closest meteorologic station to each of the six *Q. ilex* populations studied. De Martonne aridity index (I_{ar} DM) is the ratio: $P/(T + 10)$ and the type of climate is assigned according to De Martonne 1926. Population codes: AT: Alto Tajo, PAR: El Pardo, PLA: Plasencia, HUR: Hurdes, VIL: Villuercas, and MER: Mérida. Information on the altitude, longitude and latitude of each meteorologic station is given. Time series for climatic data: 1964–2007.

<i>Q. ilex</i> population	Meteorologic station	Altitude (m a.s.l.)	Longitude	Latitude	T (°C)	P (mm)	T_{\max} (°C)	T_{\min} (°C)	I_{ar} DM	Climate
AT	Zaorejas	1225	2°12'12" W	40°45'40" N	10.7 \pm 0.4	692.4 \pm 44.3	21.4 \pm 0.5	1.8 \pm 0.3	33	Humid
PAR	Madrid el Goloso	740	3°42'40" W	40°34'00" N	13.7 \pm 0.1	476.4 \pm 21.8	23.9 \pm 0.3	4.6 \pm 0.3	20	Semiarid
PLA	Plasencia	352	6°05'37" W	40°01'40" N	16.1 \pm 0.2	690.4 \pm 26.8	27.1 \pm 0.2	6.9 \pm 0.3	26	Subhumid
HUR	Villanueva de la Sierra	524	6°24'17" W	40°12'00" N	16.0 \pm 0.2	969.5 \pm 48.1	25.7 \pm 0.3	8.0 \pm 0.3	37	Humid
VIL	Garvín de la Jara	690	5°21'32" W	39°41'20" N	14.2 \pm 0.2	700.6 \pm 36.1	25.5 \pm 0.3	5.7 \pm 0.4	29	Subhumid
MER	Proserpina	256	6°22'00" W	38°58'06" N	16.3 \pm 0.9	548.9 \pm 27.6	27.0 \pm 0.2	8.1 \pm 0.4	20	Semiarid

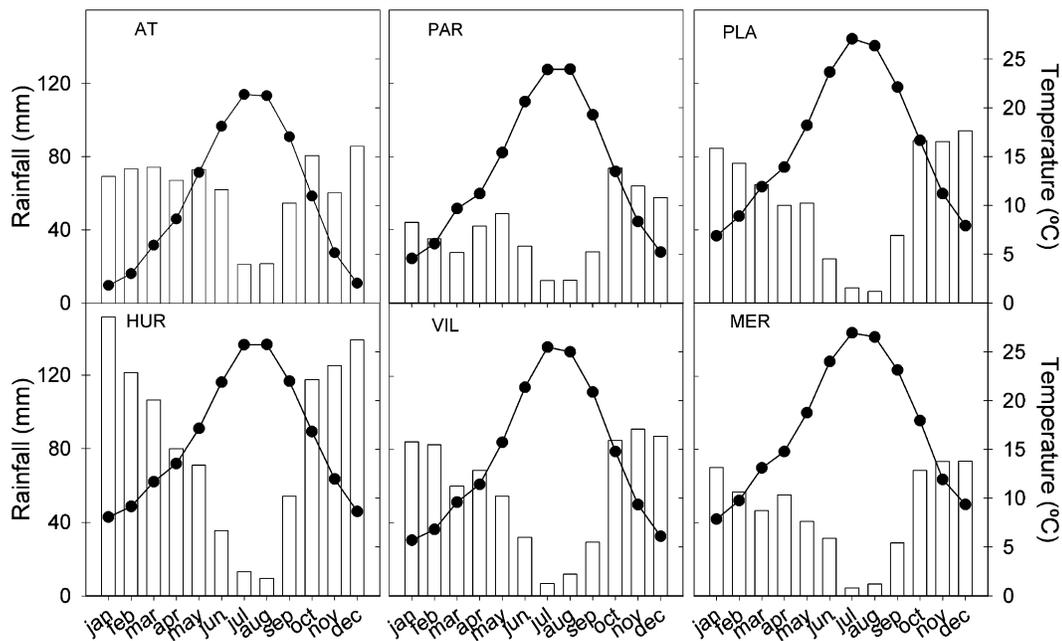


Figure 1. Average monthly rainfall (bars) and temperature (black line and dots) for the meteorologic stations closest to each population studied (population codes: AT: Alto Tajo, PAR: El Pardo, PLA: Plasencia, HUR: Hurdes, VIL: Villuercas, MER: Mérida). Data are the mean for a 20–57-year period.

($n = 115$ days). Acorns were germinated in forest multi-pot containers of 360 cm³ per pot. Soil substrate consisted of a mixture of 75% peat (2020 Natur Bow, Kekkilä, Finland) and 25% vermiculite (Vermiculita Tipo 3, Projar, Madrid, Spain). After 3 months we randomly selected 35 seedlings per population, among those healthy ones, and transplanted them to individual pots (3000 cm³) with a soil substrate composed of 25% peat (4460 B6 Forestal, Kekkilä, Finland) and 75% washed, river sand. They were watered to full soil capacity three times a week.

Transplanted seedlings were randomly assigned to one of the three experimental treatments: control, drought and cold. Seedlings that were subjected to control and drought treatments were kept under ambient light intensity in the glasshouse. Control plants were watered regularly to maintain a minimum, volumetric, relative water content of

15–20%. In the drought treatment, we interrupted watering and monitored the soil moisture, until we reached a low volumetric water content (6–10%) with a time domain reflectometer (TDR 100; Fieldscout, Spectrum Technologies, Inc., IL). Previous studies, in similar substrate, with *Q. ilex* have shown that seedlings experience water stress when the relative soil water content drops $< 10\%$ (Valladares et al. 2005, Sánchez-Gómez et al. 2006). The relative soil water content was maintained $< 10\%$ for 10 days. Pots were weighed and water was added to compensate for evapotranspiration losses imposing soil desiccation beyond the desired level. For the cold treatment, seedlings were placed in a refrigerated room, for 10 days, under an average temperature of 6.2 °C, under an average PAR of 277 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in a 12-h photoperiod. In the cold room, the light source had to be artificial but we tried to get as much light as possible to

minimize the confounding effects of different PAR. *Quercus ilex* also can deal with low light, and most of the studies revealed that photosynthesis saturates at rather low PAR values (Valladares et al. 2000, Gómez-Aparicio et al. 2006). Beside, PAR for control and drought exposed plants was not measured under full sunlight, as filtering and shade by nearby buildings generated only about 70% of full sunlight. We estimate that the difference in PAR between plants in the cold room and in the greenhouse was < 30% and it was for PAR well above saturation so the effects can be expected to be minimal. In total, the six different populations exposed to three different treatments gave rise to 18 experimental groups, with 8–12 plants per group.

Emergence, morphology and physiologic measurements

Each acorn was weighed to the nearest 0.001 g before sowing. Emergence rates for each population were recorded at four different times during the initial growing period, which extended over 3 months. Once transplanted to large pots, seedlings were randomly assigned to one of the three treatments, and the height of 5-month-old seedlings was recorded.

Tolerance to heat and to freezing temperatures was determined by calculating T_{15} and T_{50} ; critical temperatures at which 15% and 50% reduction of the initial maximum photochemical efficiency, estimated by chlorophyll fluorescence, are observed, based on the well-known decline of the maximum photochemical efficiency of photosystem II with either increasing or decreasing temperatures (Li et al. 1996, Larcher 2000, Kozłowski 2002, Cui et al. 2006). We quantified the ratio of variable to maximum fluorescence (F_v/F_m) with an FMS2 fluorometer (Hansatech, UK) in the leaf samples as the temperature gradually changed in dim light ($PAR < 3 \mu\text{mol m}^{-2} \text{s}^{-1}$). Two similar-sized disks of mature and healthy leaves of each plant were cut and placed over a moist filter paper and covered with a plastic, transparent film. The three layers (filter paper, leaf disk and plastic film) were placed over a thermally controlled aluminum plate. We placed another aluminum plate over the plastic film to ensure good contact of all leaf disks with the thermally controlled plate. This upper plate had holes and hence the leaves could be reached for fluorescence measurements with the fluorimeter fiber-optic at a 60° angle. Leaf temperature was registered with a thermocouple placed at the leaf level between the moist filter paper and the plastic film was connected to a DT-610B thermocouple thermometer (CEM, Germany). The device used (manufactured by OCHE Control y Equipamientos, Zaragoza, Spain) included a temperature digital control unit (Syros, DITEL, Barcelona, Spain) connected to a plate with resistances and a Peltier unit that permitted a tightly controlled linear variation in temperature of $0.5 \text{ }^\circ\text{C min}^{-1}$. For the heat tolerance measurements, the initial F_v/F_m value was taken at ambient temperature (around $25 \text{ }^\circ\text{C}$), and for freezing tolerance the initial F_v/F_m value was taken

at $10 \text{ }^\circ\text{C}$. After an increase, or decrease, of $2 \text{ }^\circ\text{C}$, new F_v/F_m values were obtained from each leaf disk on the plate. We stopped the measurements for heat tolerance when a temperature giving no fluorescence signal was reached. We stopped the measurements for freezing tolerance at $-11 \text{ }^\circ\text{C}$. Heat tolerance was measured in control (no stress) conditions and after the drought treatment and freezing tolerance in control (no stress) and after the cold treatment.

Measurements of leaf gas exchange were carried out on healthy, fully developed leaves, using a Li-Cor 6400 gas exchange system (Li-Cor, Inc., NE). Leaves were exposed to an atmospheric CO_2 concentration of 400 ml l^{-1} using the built-in Li-Cor 6400 CO_2 controller unit. Measurements were taken at ambient temperatures ($20\text{--}25 \text{ }^\circ\text{C}$) for the control and drought treatments, and at $6 \text{ }^\circ\text{C}$ for the cold treatment. Ambient relative humidity of the air was 45–50% for all treatments. The PAR for the determination of maximum photosynthetic rate (A_{max}) and stomatal conductance (g_s) was set to $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ and measurements were done on 2 consecutive days, for each treatment, between 8.00 am and 11.00 am. Instantaneous water use efficiency (iWUE) was calculated by the ratio of A_{max} and g_s (Gulías et al. 2002).

Statistical analysis

We used one-way ANOVA to test for significant differences in acorn mass and seedling's height among populations. Both normality and homogeneity of variances were checked before performing the analysis (Zar 1999). As a post hoc test, we used Tukey's HSD. We used the Kaplan–Meier product-limit method to estimate the emergence function of seedlings of each population from the data obtained in the periodic censuses. We performed a χ^2 -multiple comparison test to test for significant differences among populations and, afterwards, Cox's F test was used to test for differences in emergence functions among populations for all possible pair-wise combinations (Pyke and Thompson 1986). Responses of F_v/F_m with temperature were analyzed using a sigmoidal fit model for the data obtained for each plant. Critical temperatures that promoted 15% and 50% reduction of initial F_v/F_m values (T_{15} and T_{50}) were determined in each fitted curve. We used general linear model (GLM) to test for significant differences in critical temperatures (T_{15} and T_{50}), iWUE, g_s and A_{max} among populations and treatments; seed and seedling size effects were included as covariates when significantly correlated to the response variables. Both drought and cold treatments were compared to the control. Simple linear regression was performed to check for a significant relation between heat and freezing tolerances measured in the same individuals kept at optimum conditions (control). All analyses were performed after checking for normality and homogeneity of variances. As post hoc test, we used Tukey's HSD. All the statistical analyses were performed using STATISTICA Version 6.0 (Statsoft Inc., Tulsa).

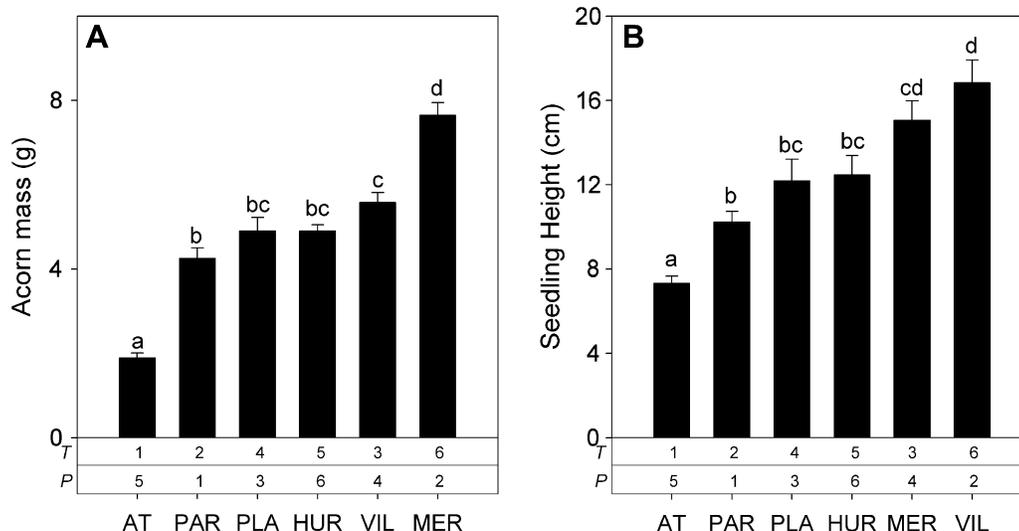


Figure 2. (A) Mean acorn mass (\pm SE) and (B) mean height (\pm SE) of 5-month-old *Q. ilex* seedlings from each population. Different letters indicate significant differences among populations after Tukey's HSD test. A score is given to each population according to the temperature of the coldest month (T) from the coldest (1) to the warmest (6) and according to the total annual precipitation (P) from the driest (1) to the wettest (6). See Figure 1 caption for population codes.

Results

Acorn mass, seedling height and emergence rate

We found significant differences among populations for acorn mass ($F = 75.24$, $P < 0.01$) and for the height of 5-month-old seedlings ($F = 24.96$, $P < 0.01$). Acorns from Alto Tajo population were the smallest and rendered the shortest seedlings (mean \pm SE: 1.90 ± 0.11 g and 7.32 ± 0.35 cm), followed by those from El Pardo, Plasencia, Hurdes, Villuercas and Mérida, the latter with the biggest acorns and tallest seedlings (7.65 ± 0.30 g and 15.7 ± 0.92 cm) (Figure 2). The emergence rate was significantly different among populations ($\chi^2_{df=5} = 188.73$, $P < 0.01$). The acorns that had the lowest final emergence percentage were those coming from Alto Tajo population, the coldest of all populations. However, for the rest of the populations, emergence rates did not follow the pattern observed for the acorn and seedling sizes (Figure 3).

Thermal tolerances and critical temperatures (T_{15} and T_{50})

As expected, drought had a significant effect on heat tolerance (T_{15} and T_{50} , Table 2), with values for these two critical temperatures being higher in plants exposed to drought ($T_{15} = 43.0 \pm 0.3$ °C and $T_{50} = 46.2 \pm 0.3$ °C) than those from the control treatment ($T_{15} = 39.9 \pm 0.3$ °C and $T_{50} = 42.6 \pm 0.3$ °C) (Figure 4). In turn, cold treatment significantly increased freezing tolerance (Table 2), with critical temperatures for seedlings exposed to cold being lower ($T_{15} = -7.3 \pm 0.2$ °C and $T_{50} = -11.4 \pm 0.3$ °C) than the control ($T_{15} = -5.2 \pm 0.3$ °C and $T_{50} = -8.1 \pm 0.2$ °C; Figure 4). Seed and seedling size had no significant effect on any of the thermal tolerances.

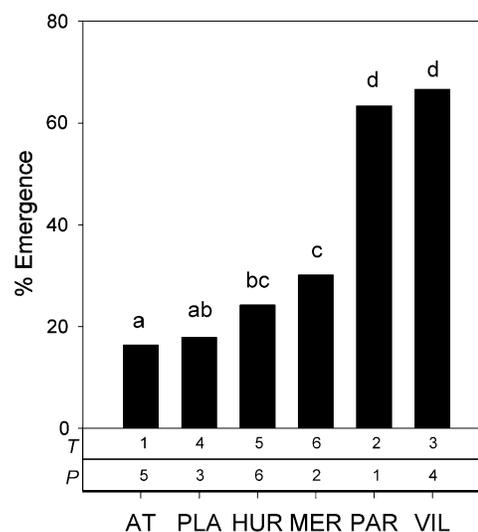


Figure 3. Final emergence rate after 84 days of *Q. ilex* acorns from the six populations studied. Different letters indicate significant differences according to the Kaplan–Meier analysis of emergence over time after multiple comparisons followed by the Cox–Mantel test. See Figure 1 caption for population codes and Figure 2 caption for the T and P scores.

No significant population \times treatment interaction was found indicating a similar effect of both drought and cold treatments on all the populations studied (Table 2). Linear regression revealed a significant and positive relationship between heat and freezing tolerances, which was observed in both T_{15} and T_{50} (Figure 5). Individual regressions for each population were not significant (data not shown).

Table 2. Results of the GLM for the ecophysiological variables measured in *Q. ilex* seedlings. Main factors were treatment (T) (control, drought and cold treatments) and population (P). T_{15} and T_{50} : critical temperatures that reduced 15% and 50% initial F_v/F_m ; iWUE, instantaneous water use efficiency; A_{max} : maximum photosynthetic rate; and g_s : stomatal conductance.

		Optimum drought			Optimum cold		
		DF	F	P	DF	F	P
T_{15} (°C)	T	1	196.4	< 0.001	1	31.78	< 0.001
	P	5	1.9	0.887	5	0.78	0.568
	T*P	5	7.3	0.255	5	0.72	0.608
T_{50} (°C)	T	1	53.85	< 0.001	1	66.34	< 0.001
	P	5	0.46	0.804	5	1.03	0.405
	T*P	5	0.81	0.543	5	1.44	0.218
iWUE	T	1	16.49	< 0.001	1	51.49	< 0.001
	P	5	2.89	0.019	5	3.65	0.005
	T*P	5	0.36	0.877	5	0.87	0.507
A_{max}	T	1	61.07	< 0.001	1	129.9	< 0.001
	P	5	1.18	0.328	5	1.7	0.142
	T*P	5	1.49	0.203	5	1	0.425
g_s	T	1	89.02	< 0.001	1	52.94	< 0.001
	P	5	2.13	0.07	5	1.03	0.404
	T*P	5	1.29	0.275	5	2.37	0.047

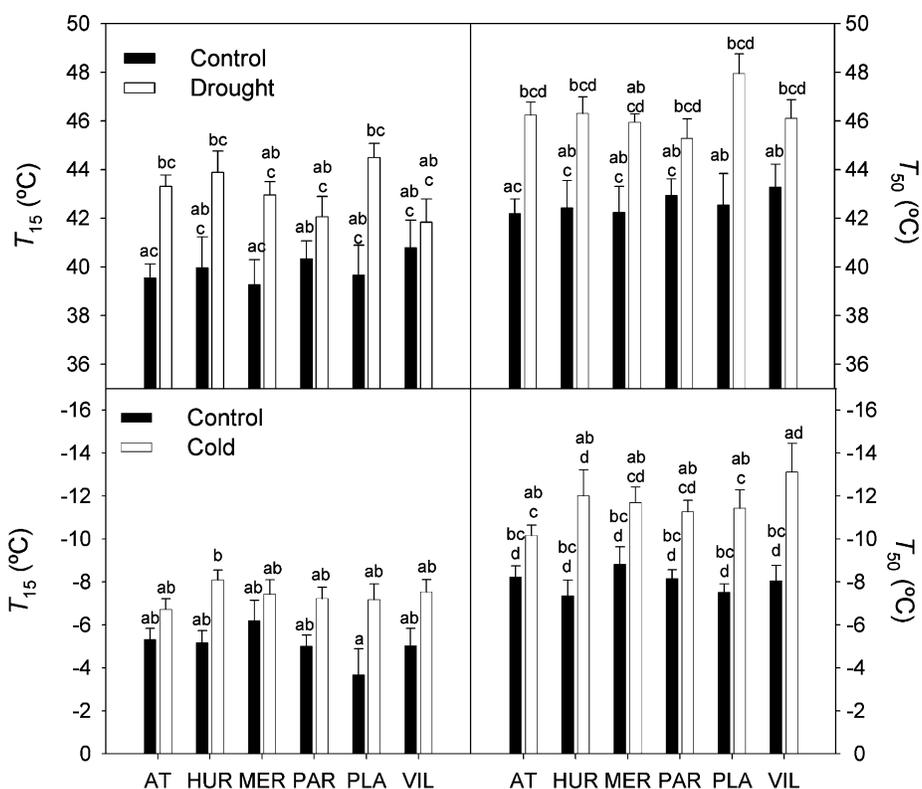


Figure 4. Thermal tolerance (mean \pm SE) expressed as critical temperatures reducing 15% (T_{15}) and 50% (T_{50}) of the initial F_v/F_m . Values are given for both heat (upper panels) and freezing (lower panels) tolerances of *Q. ilex* seedlings from the six populations studied and their change in response to drought and cold treatments. Different letters indicate significant differences after Tukey's HSD test. See Figure 1 caption for population codes.

Gas exchange measurements

Drought significantly decreased the A_{max} (Figure 6; Table 2) and increased the iWUE (Table 2; Figure 6). Cold treatment significantly reduced both A_{max} and iWUE (Table 2; Figure 6). No significant population \times treatment interaction was found for either A_{max} or iWUE, again revealing a similar effect of the treatments on seedlings from

all the populations (Table 2; Figure 6). The iWUE was significantly influenced by the population in both drought and cold treatments (Table 2). Post hoc test revealed that seedlings from Alto Tajo had significantly lower iWUE than those from Mérida, Plasencia and Villuercas but the differences were not clearly related to the climatic conditions at each locality; for instance, populations from drier sites showed no increased iWUE. A similar result was

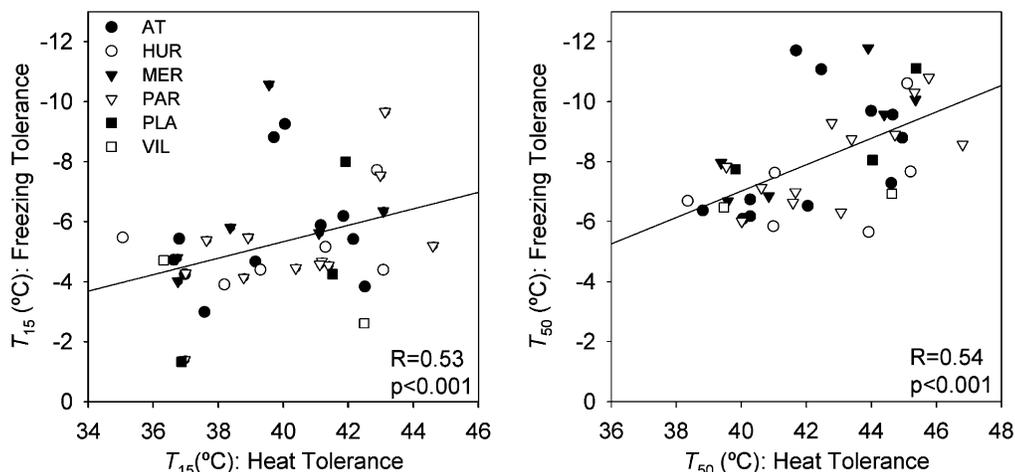


Figure 5. Linear regression between heat and freezing tolerances expressed as critical temperatures reducing 15% (T_{15}) and 50% (T_{50}) of initial F_v/F_m . Each data point is the mean of two leaf disks. See Figure 1 caption for population codes.

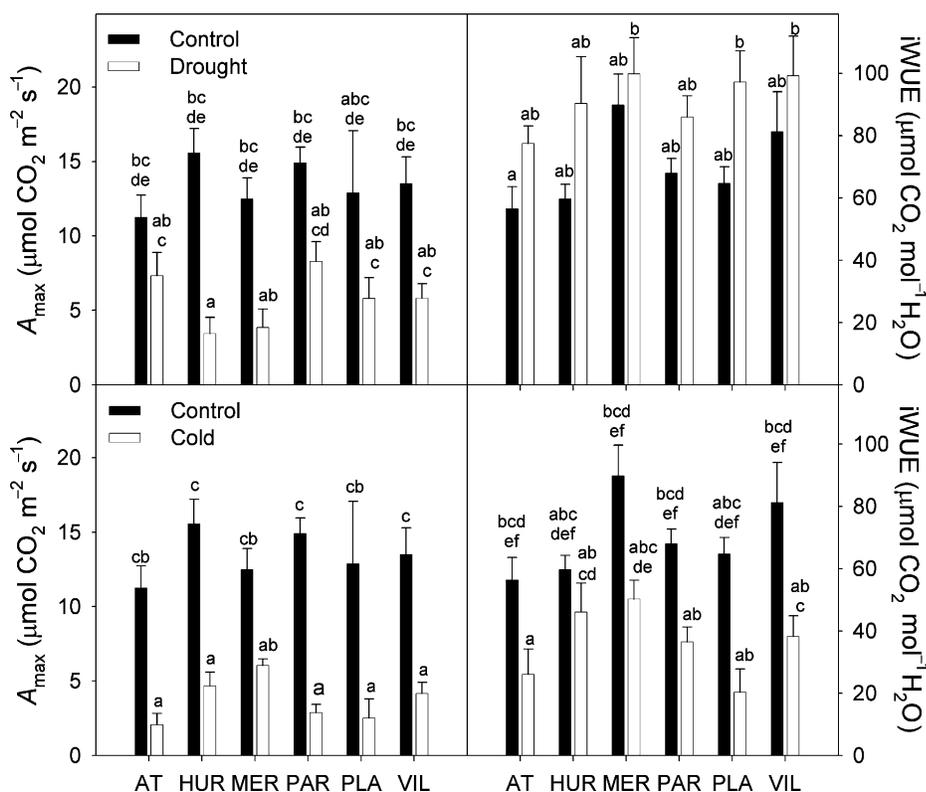


Figure 6. Maximum photosynthetic rate (A_{max} , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and instantaneous water use efficiency (iwUE, $\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$) (mean \pm SE) of *Q. ilex* seedlings from the six populations studied and their change in response to drought (upper panels) and cold (lower panels) treatments. Different letters indicate significant differences after Tukey's HSD test. See Figure 1 caption for population codes.

found for the comparison between the cold treatment and the control, with population differences not being related to mean or minimal temperatures at each locality. Both drought and cold significantly reduced g_s , and the interaction term (population \times treatment) was also significant for the cold treatment (Table 2); the post hoc test revealed that this was due to the dissimilar response of seedlings from Merida whose g_s was significantly lower in control conditions and was not significantly reduced by cold exposure (data not shown). Seed and seedling size had no significant effect on either iwUE, A_{max} or g_s .

Discussion

Stress tolerance, plasticity and functional differences among holm oak populations

Collectively, our results revealed both a great thermal tolerance and a remarkable acclimation capacity to drought and low temperatures in all *Q. ilex* seedlings without a clear and consistent pattern in the differences among populations. All seedlings showed high thermal resistance to both heat and freezing temperatures, which was significantly increased by exposure to drought and cold temperature, respectively.

Maximum photosynthetic rates were similar across populations under control conditions and there were no statistically significant differences among populations in the shape of their photosynthetic responses to periods of drought and low temperature; however, the great variability of the response variables and the limited sample size could be obscuring differences among populations. For iWUE, we found significant differences between populations, but the shape of the response to the experimental treatments was the same for all populations, in contrast with results by Gratani et al. (2003) showing that certain populations of *Q. ilex* from drier habitats could have enhanced iWUE. These results do not confirm the expected local adaptation patterns: seedlings from different populations with contrasting climates would show different ecophysiological responses. This suggests in turn that the plasticity observed is more relevant than the local adaptation in the responses of *Q. ilex* seedlings to the contrasting climatic conditions experienced along its distribution range. *Quercus ilex* seedlings have been reported not to be highly plastic in response to light and nutrient availabilities, which was interpreted as a conservative resource use strategy (Valladares et al. 2002a, 2002b). However, their plasticity in response to periods of drought and cold was relatively high for the ecophysiological parameters studied; that provides a safety margin to cope with droughts and late season frosts that are expected to become more frequent and harmful in the most likely climate change scenarios (Christensen et al. 2007, Gu et al. 2008). The importance of extreme climatic events has been highlighted by Gutschick and BassiriRad (2003) as they can act as directional selection drivers. Therefore, the ability of different species to respond to this type of events is key to predict future potential distributions of species under climate change scenarios.

It is becoming well established that plasticity for physiologic parameters in general is greater than that for morphologic features (Valladares et al. 2002a, 2002b, Niinemets and Valladares 2004). In a study of seedlings from three Italian populations, Gratani et al. (2003) found that the largest differences in plasticity among populations were found for morphologic and anatomical traits, and in contrast to our study, leaf ecophysiology and functional morphology could be related to the climate of the provenance. Functional differences as evidence of ecotypic differentiation were found in another study of *Q. ilex* seedlings from three populations (Sánchez-Vilas and Retuerto 2007). Ecotypic differentiation was also observed for seedlings of a closely related evergreen oak (*Quercus coccifera* L.) by Balaguer et al. (2001) but again the study included a combination of both morphologic and physiologic variables, and it was based on only three populations. We argue that leaf morphology and plant architecture are more involved in local adaptation of evergreen Mediterranean oaks than leaf physiology, that seems to be plastic enough to cope with the environmental ranges. We suggest that trends revealing adaptation to local climate of functional

and ecophysiological traits might become less clear when more populations are added to the study. Unclear patterns in adaptation to local climate may arise from the co-occurrence of multiple constraints, as is the case of Mediterranean-continental ecosystems, and from the existence of complex interactions among environmental factors (winter frosts together with severe summer drought) that can explain departure from optimality in plant form and function and in phenotypic plasticity for adaptive traits (Niinemets and Valladares 2004, Valladares et al. 2007).

Maternal effects mediated by seed size were not accounted for in previous comparisons of the performance of evergreen oaks from different populations (e.g., Gratani et al. 2003, Sánchez-Vilas and Retuerto 2007), despite their potential importance for understanding local adaptation (Roach and Wulff 1987). We found the biggest acorns in the populations with the mildest climatic conditions (warmer mean annual temperature) as a likely consequence of a greater window of time for growth and reproduction of maternal plants at these locations (Díaz-Fernández et al. 2004). Larger acorns gave rise to the highest seedlings as it can be expected since early seedling growth relies on the materials stored in the seed (Roach and Wulff 1987). Furthermore, seed and seedling size can influence seedling physiologic performance (e.g., Kennedy et al. 2004, Wang et al. 2006). However, in our experiment we found no effect of seed size or seedling height on the physiologic variables that were measured. The direct relationship between acorn size and seedling size found here agrees with the previous studies with other oak species (Guo and Werger 1999, Purohit et al. 2003, Quero et al. 2007). Our results suggest that the climatically less restricting sites produce bigger acorns, and this confers survival and competitive advantages to the emerging seedlings (Guo and Werger 1999). Emergence timing also differed between populations, with acorns collected at the coldest location (Alto Tajo) being not only the smallest but also the latest to emerge. This delayed emergence could be an adaptive strategy to avoid late season frosts, but it remains to be explored whether it merely is a by-product of limited seed reserves or not. Thus, our study suggests that morphologic differences in early seedling performance across populations are primarily induced by maternal effects mediated by seed size. Besides, these differences were influenced by local climate, whereas no clear patterns in relation to local climate were found for the physiologic differences among populations.

General physiologic responses to drought and cold temperatures

Maximum photosynthetic rates were decreased by both drought and cold temperatures, whereas iWUE was increased by drought and decreased by cold temperatures in a similar manner in seedlings from all the populations compared. The increase in iWUE, driven by a decrease in stomatal conductance, is consistent with similar findings

that support that *Q. ilex* might have evolved to optimize resource use under low water conditions (Gulías et al. 2002, Valladares et al. 2002a, 2002b, Valladares et al. 2005). The decreased photosynthesis under cold temperatures is associated with a reduction of the electron transport rate and a decrease in the activity of the enzymes involved in photosynthesis (Cavender-Bares et al. 1999, García-Plazaola et al. 1999a, 1999b). The maintenance of the photosynthetic function under low, suboptimal temperatures is important for evergreen species from Mediterranean areas, as during the winter they can benefit from moister soil conditions. This strategy can give evergreen species a competitive advantage over deciduous plants, that might otherwise overcompete evergreen species due to their higher growth rate (e.g., Bonfil et al. 2004). Our experiment showed that, despite the significant reduction in photosynthetic capacity, all *Q. ilex* seedlings, regardless of their origin, were able to maintain carbon gain under low, suboptimal temperatures and water availability, a stress-tolerance strategy that is in agreement with other studies of the species (Martínez-Ferri et al. 2004, Corcuera et al. 2005, Valladares et al. 2008).

The remarkable capacity of holm oak to modify the thermal tolerance according to ambient temperature found here agrees with the study of Daas et al. (2008) showing similar results for oak species from contrasting climates. We found a significant increase in tolerance to heat and freeze induced by drought and cold exposure, which is also in agreement with the previous findings (Valladares and Pearcy 1997, Ghouil et al. 2003). This result supports the notion that the mechanisms developed during acclimation to drought are related to those protecting plants against heat, as both involve synthesis of heat shock proteins (Li et al. 1996, Thomashow 1999, Sun et al. 2002). In Mediterranean-continental areas, heat tolerance has great importance, as temperature can rise up to 45–50 °C at mid-day during the summer. Mediterranean summer is also characterized by drought; therefore, tolerance to heat might be further increased under natural conditions due to drought exposure, which might be particularly important to cope with unpredictable heat waves (Ghouil et al. 2003).

Cold exposure increased freezing tolerance in all populations. This ability of *Q. ilex* seedlings to rapidly acclimate to low temperatures could confer them an advantage to survive their first winter after emergence, and the forthcoming late-season frosts that are common in Mediterranean-continental-type ecosystems (Chaar and Colin 1999, Valladares et al. 2008). Cold acclimation is a well-known mechanism that increases freeze resistance by exposure to low-suboptimal temperatures (Thomashow 1999). Acclimation involves a number of molecular and physiologic processes, devoted to protect plant tissues against plant ice formation and to maintain the photosynthetic apparatus (Larcher 2000); such as accumulation of sugars, synthesis of protective proteins, and enhanced transcription of mRNA (Novillo

et al. 2004, Suping et al. 2005). Increased resistance of photosynthetic function to cold is a common feature in evergreen species (Boorse et al. 1998, Cavender-Bares et al. 1999, 2005).

The significant relationship found between constitutive tolerance to heat and freeze could be due to the common mechanisms involved in protection against damage by extreme temperatures. Most of them aim to avoid cell-dehydration and protect functional properties of the membranes (Larcher 2000). For instance, intracellular sugar accumulation, driven by both drought and cold exposure, plays a major role in avoiding cell dehydration as it rapidly decreases leaf-water potential (Hüve et al. 2006) and also synthesis of heat shock proteins; in fact, some studies have found that the synthesis of heat shock proteins is enhanced by chilling temperatures (Sabehat et al. 1998). This simultaneous protection against heat and freeze damage has great importance for evergreen species inhabiting continental areas, where they are subjected to strong daily and seasonal thermal oscillations.

Conclusions

Our experiment showed that holm oak seedlings have great tolerance to both cold and heat. Furthermore, we have found that these two are related, suggesting that there are common mechanisms involved, and that drought enhanced both iWUE and thermal tolerance. Climate change is expected to bring to Mediterranean regions a decrease in water availability, an increased frequency of extreme climatic events, and a higher overall climate variability both within and between years (Ogaya and Peñuelas 2004, Christensen et al. 2007, Gu et al. 2008). Tolerance to multiple stresses and the capacity to physiologically acclimate to heat waves and cold snaps may allow *Q. ilex* to cope with these stressful climatic conditions. Lack of evidence of physiologic adaptation of seedlings to their local climate may reflect that within the climatic range considered in this experiment, plasticity prevails over local adaptation in terms of seedling performance under a range of environmental conditions. This could reveal opposing selection pressures to complex, multi-dimensional environmental conditions operating within the distribution range of this species.

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