Eur J Forest Res (2012) 131:1091–1101
DOI 10.1007/s10342-011-0578-1

ORIGINAL PAPER

Two provenances of *Quercus ilex* ssp. *ballota* (Desf) Samp. nursery seedlings have different response to frost tolerance and autumn fertilization

Enrique Andivia · Manuel Fernández · Javier Vázquez-Piqué · Reyes Alejano

Received: 15 March 2011 / Revised: 8 September 2011 / Accepted: 11 October 2011 / Published online: 6 November 2011

Abstract Despite evidences that Holm oak has a high plasticity and great adaptability, there is limited or contradictory knowledge of the morphological and physiological variability of this species. Holm oak has been widely used for reforestation projects in Mediterranean areas, but has frequently shown poor field performance. We hypothesized that Holm oak has inter-population differences in physiological responses to abiotic stressors that could affect reforestation success. The influence of nursery culture on the characteristics of Holm oaks from different provenances has not been explored in depth. Thus, we studied the effect of nursery autumn fertilization on morphological traits, frost tolerance, root growth potential, and nutritional status of two Spanish provenances of Holm oak, La Alcarria (a region with inland Mediterranean climate) and Sierra Morena Occidental (a region with a warm coastal Mediterranean climate). There were significant differences between the provenances in frost tolerance, biomass allocation, and leaf nutrient content, suggesting a role of genetic factors. The leaves of seedlings from La Alcarria had less visual damage at −12°C than seedlings from the warmer provenance (45% vs. 92%). Seedlings from La Alcarria, compared to those from Sierra Morena, had higher leaf P concentration (0.17% vs. 0.15%), greater stem diameter (3.1 mm vs. 2.7 mm), lower shoot-to-root dry mass ratio (0.46 vs. 0.53), and lower slenderness (4.03 vs. 5.31). For both provenances, N autumn fertilization improved growth, root growth potential, cold hardiness, and nutritional status of seedlings. We suggest that forest reforestation programs should consider to a greater extent Holm oak provenances and their tolerances to different abiotic stressors.

Keywords Holm oak · Provenance · Reforestation · Adaptation · Cold hardiness · Autumn fertilization

Introduction

Quercus ilex L. (Holm oak) is a sclerophyllous evergreen tree widely distributed in the western Mediterranean Basin (Barbero et al. 1992) and is one of the most important species in these communities (Palacios et al. 2009). The use of this species for reforestation has increased significantly in recent years (MAPA 2006; Rodà et al. 1999). Nevertheless, Holm oak has poor field performance in comparison to other Mediterranean species, particularly in areas with unfavorable climatic conditions (Pausas et al. 2004). This and low plant quality are believed to be the main factors that limit plantation success (del Campo et al. 2010). In Spain, legislation about the sale and use of provenance regions (i.e. areas with similar ecological and climatic characteristics) for reforestation was really established in 2003, but these new laws are only recommendations, and are not legal obligations (Alía et al. 2005). Therefore, the extent to which the use of inappropriate provenances contributes to plantation failure is unknown.

Genetic adaptation and environmental acclimation are key processes that allow a tree to survive and compete successfully in different environments (Morin et al. 2007; Palmroth et al. 1999). The wide distribution of Holm oak and the heterogeneity of the environments that colonizes

---

Communicated by C. Ammer.

E. Andivia · M. Fernández (✉) · J. Vázquez-Piqué · R. Alejano
Departamento de Ciencias Agroforestales, Universidad de Huelva, Escuela Técnica Superior de Ingeniería, Campus Universitario de La Rábida Palos de la Frontera, 21819 Huelva, Spain
e-mail: manuel.fernandez@dcaf.uhu.es

suggest that this species has high phenotypic plasticity in terms of morpho-phenology (Gratani 1995), and that it has a great adaptation capacity (Valladares et al. 2000). Previous studies of physiological plasticity in oaks (Gimeno et al. 2009; Gratani et al. 2003; Laureano et al. 2008; Morin et al. 2007; Pesoli et al. 2003; Sánchez-Vilas and Retuerto 2007) are in disagreement about the main factors that contribute to stress tolerance. Tolerance may be due to the ability to acclimate to diverse environmental conditions (high plasticity within populations) and due to genetic adaptations of individual provenances leading to the emergence of locally adapted ecotypes. Michaud et al. (1995) observed a homogeneous genetic structure of Holm oak in the Mediterranean region with only slight geographic variations due to isolation, and that only a ten per cent of the total genetic diversity was accounted for by among-population variation. However, the physiological responses of different populations to stress factors, such as low temperature or drought, could be under strong genetic control (Gratani et al. 2003; Pesoli et al. 2003), and this fact has practical implications for the choice of seedlings for reforestation. Scenarios for climate change in Mediterranean areas predict an increase in temperature, a decrease in rainfall (Christensen et al. 2007), and increased frequency of extreme events, such as late season frosts (Gu et al. 2008). Analysis of the variability of ecologically important traits among populations may provide valuable information for the establishment of early selection criteria in breeding programs. Thus, the knowledge of the responses of different provenances to stressors, such as frost, is required for successful early establishment of seedlings (Terradas and Savé 1992). Such analysis is also essential to improve our understanding of the processes involved in the maintenance and divergence of species and to predict how species and ecosystems will respond to environmental change. There are contrasting reports on the degree of its morphological and physiological variability but very little research on the extent of the variability among populations (Sánchez-Vilas and Retuerto 2007).

Nursery practices, as well as genetic and environmental factors, affect the functional characteristics of seedlings and their field performance (Birchler et al. 1998). The adjustment of the availability of nutrients is a useful tool for the modification of seedling characteristics (Puttonen 1997). However, the effects of mineral nutrition on plant quality and field performance of *Quercus* species are still uncertain (Andivia et al. 2011a, b; Broncano et al. 1998; Oliet et al. 2009; Villar-Salvador et al. 2004a). Previous studies of *Quercus ilex* L. seedlings grown in the same nursery but subjected to different fertilization treatments with N, P, and K during autumn (Andivia et al. 2011a, b) found that N and K improved plant quality and frost hardiness. However, the role of provenance on responses to nutrient supply during the hardening phase and on subsequent plant quality is unknown.

In this study, we examined seedlings from two Spanish provenances of *Quercus ilex* ssp. *ballota*, one characterized by a continental climate with cold winters (La Alcarria, *ALC*) and the other by a warm climate with Atlantic influence (Sierra Morena Occidental, *HU*). We applied different doses of N and K to these provenances during the autumn to: (1) identify morphological traits and nutritional status at the end of the nursery phase of seedlings from each provenance, and (2) study the response to frost of seedlings from different provenances. As there are evidences of differences in structural and functional variability between Holm oak populations (Gratani et al. 2003; Jorrín Novo et al. 2004; Sánchez-Vilas and Retuerto 2007), we tested the hypothesis that seedlings from colder provenances have a higher resistance to frost events than seedlings from warmer provenances. Our results have important implications for the selection of acorn provenance for regeneration programs in different regions.

Materials and methods

Plant material and growth conditions

Acorns of *Q. ilex* ssp. *ballota* were collected during the autumn of 2008 from two locations with different climatic conditions. At each location, 300 acorns per tree were taken from 25 trees that were more than 80 m apart. The *ALC* location is in Cuenca (UTM, zone 30: X, 586871; Y 4420928; altitude 1,135 m), inside the provenance of La Alcarria and Serranía de Cuenca; the other location is in Sierra de Huelva (*HU*) (UTM, zone 29: X, 644288; Y, 4161376; altitude 160 m) inside the provenance of Sierra Morena Occidental (Jiménez-Sancho et al. 1996). Table 1 shows climatic conditions of both regions for the period 2001–2010. Daily climatic data for this period were obtained from two automatic weather stations belonging to the Regional Governments: “Cañete” (UTM, zone 30: X, 615300; Y, 4432004; altitude 1,053 m) for *ALC* and “La Puebla de Guzman” (UTM, zone 29: X, 654836; Y, 4157771; altitude 288 m) for *HU*.

At the beginning of 2009, 300 acorns per provenance (12 randomly selected healthy acorns per tree) were pre-germinated in a growth chamber at 20°C on wet perlite. All acorns from each location were pooled together, so the genetic variability was only considered at the provenance level, without taken into account the mother effect. The mean dry mass of acorns was significantly different ($P < 0.05$) between provenances: 2.61 ± 0.50 g in *ALC* and 3.89 ± 0.60 g in *HU* ($n = 50$ acorns). In the third week of February 2009, 120 acorns per provenance were

Table 1 Climatic conditions of the Alcarria (*ALC*) and Sierra Morena (*HU*) locations during the period 2001–2010

Location	*ALC*	*HU*
P (mm)	569	564
Tm (°C)	10.6	16.5
Tmax (°C)	36.7	43.6
Tmmax (°C)	31.1	33.8
Tmin (°C)	−18.6	−4.0
Tmmin	−5.0	5.2
R (MJ m^{-2} day^{-1})	16.6	18.3

P mean annual precipitation, *Tm* mean annual temperature, *Tmax* absolute maximum annual temperature, *Tmmax* mean of the maximum temperatures of the hottest month, *Tmin* absolute minimum annual temperature, *Tmmin* mean of the minimum temperatures of the coldest month, and *R* mean solar radiation

randomly taken and sown in 300 cm^3 Plasnor® containers (45 containers per tray) that contained sphagnum peat Kekkilä® B0, with pH corrected to 6.0 using Dolokal®. Pre-germination was performed to reduce germination time and to ensure that all cells in the tray will contain a plant. The 240 acorns were sown in 8 trays (each tray containing 15 acorns per provenance and three rows of five border acorns). Seedlings were grown in a nursery in Huelva (UTM, zone 29: X, 684708; Y, 4119184; altitude 23 m) under a shade-cloth that reduced radiation by ~50%. All containers were well watered and moved weekly to eliminate microsite effects. Mean air temperature inside the nursery during the study was 18.2°C, mean of the maximum temperatures was 25.4°C and mean of the minimum temperatures was 11.6°C, mean humidity was 69.7% and maximum photosynthetic photon flux density (PPFD) was 900 µmol m^{-2} s^{-1}.

During the first 28 weeks, a constant fertilization regimen was applied using a water-soluble fertilizer (Peters professional® 20-20-20) at a rate of 125 mg l^{-1} N (dose N_1), 54 mg l^{-1} P (dose P_1), and 104 mg l^{-1} K (dose (K_1), so that each seedling received a weekly dose of 20 ml of fertilized solution corresponding to 2.500 mg N (3.94% ammoniacal N, 6.05% nitrate N, 10.01% urea N, all w/w), 1.088 mg P, and 2.075 mg K (monoammonium phosphate, monopotassium phosphate and potassium nitrate). This corresponds to 70.0 mg N, 30.5 mg P, and 58.1 mg K over the first 28-week culture period. The doses N_1, P_1, and K_1 were close to those typically recommended in forest nurseries for Holm oak during the growing phase (Navarro-Cerrillo et al. 2009). Fertilizer was added to each seedling once per week and plants were watered as needed with tap water between two consecutive fertilizations.

For the next 12 weeks (first week of October to third week of December, the hardening phase), the doses of N and K were modified by a 2 × 2 factorial experiment, using two nutrients (*N*, *K*) with two levels each (N_1, $N_{1/20}$ and K_1, $K_{1/50}$). P remained constant (P_1). The doses of N_1, P_1, and K_1 were the same as in the previous 28 weeks, so it implied to maintain a constant fertilization treatment because autumn fertilization of Holm oak has reported favorable results (Andivia et al. 2011a; Oliet et al. 2011). The low nutrient doses $N_{1/20}$ (0.125 mg N) and $K_{1/50}$ (0.042 mg K) were chosen to apply reduced doses but preventing total nutrient deprivation (Boivin et al. 2002; Trubat et al. 2011). Therefore, four different fertilization treatments were tested ($N_1P_1K_1$, $N_1P_1K_{1/50}$, $N_{1/20}P_1K_1$ and $N_{1/20}P_1K_{1/50}$), and 60 seedlings (30 per provenance) received each treatment. Each tray contained two provenances and received a dose of N, so each treatment was applied to 60 seedlings (30 per provenance) that were distributed in two trays. Autumn fertilization was applied using a nutrient solution composed at the laboratory, using calcium nitrate and ammonium sulphate as nitrogen sources. In early November, plants showed no visual signs of shoot growth.

Morphological traits and nutritional status

The assessment of morphological traits and nutritional status was done in two dates: at the end of the first 28 weeks (at the beginning of October 2009) in eight seedlings per provenance (1 per tray) randomly selected, and at the end of the 12 weeks of autumn fertilization (at the end of December 2009) in six plants per treatment and provenance (three seedlings per provenance and tray) randomly selected. Shoots were cut at the cotyledon insertion point, leaves and stems were separated and roots were cleaned. Shoot height (*H*) was measured and stem diameter (*D*) was assessed at 0.5 cm above the cotyledon insertion point. Next, all samples were washed with distilled water, oven-dried at 65°C until no further mass reduction was observed, and the dry mass of leaves (LDW), stems (StDW) and roots (RDW) were measured. Shoot dry mass (ShDW = LDW + StDW) and the shoot-to-root dry mass ratio (SRDW = ShDW/RDW) were calculated.

Nutrients were only determined in leaves because Andivia et al. (2011a) reported that the differences in nutrient concentration and content due to different fertilization treatments were larger in leaves than in the other tissues. To obtain leaf samples sufficiently large for nutrient analysis, three groups (two plants randomly selected per group) were formed. For N analysis, an elemental analyzer (Termo Finnigan 1112 Series EA, Milan, Italy) was used. For P and K analyses, dry samples were treated at 550°C for approximately 7 h, subjected to acid digestion in 5M HCl, and analyzed by ICP-OES Jobin–Yvon Ultima 2 (Tokyo; Japan). Hydroalcoholic extraction and anthrone colorimetry (using the UV-1601 spectrophotometer; Shimadzu®, Tokyo, Japan) were used to determine soluble sugars (SS) (Spiro 1966). Acid hydrolysis followed by anthrone colorimetry was used to determine starch (St) (Rose et al. 1991). Nutrient and non-structural carbohydrates (NSC) contents (in mg) were calculated as the products of concentration (%) and dry mass (g). The same leaves were used for the analysis of N, P, K, SS, and St.

Frost tolerance and root growth potential

A detached leaf test was used for evaluating cold hardiness, as previously described for *Eucalyptus globulus* L. (Fernández et al. 2007) and *Quercus ilex* L. (Andivia et al. 2011a, b). These tests were performed in the third week of December 2009 on two fully expanded leaves per temperature and plant (one leaf from the medium-higher part of the shoot and the other from the medium-lower part), and four plants per treatment and provenance. At each temperature 64 leaves from 32 plants were used, 16 plants per provenance (4 plants per fertilization treatment). The minimum tested temperatures were −8, −10, −12 and −14°C and were analyzed separately. There was a total of 239 h of total chilling hours (≤8°C) accumulated in the nursery at the time the tests were in progress.

Selected leaves were inserted into test tubes (2.2 cm diameter, 15 cm length) and placed in a programmable freezer (West® 4400; ISE Inc., Cleveland, OH) with two internal fans to remove air. The test began at 12°C, and the temperature was reduced by 3°C h^{-1} to the minimum, which was maintained for 3 h. Then, the temperature was increased by 5°C h^{-1} to 12°C. A complete cycle lasted 14–16 h. After completion of this temperature cycle, the leaves were removed from the freezer, distilled water (≤2 ml) was added until the cross-section of the short petiole was immersed, and the samples were maintained in a growth chamber (25°C day/17°C night; 12 h photoperiod; ≥70% relative humidity, 350 μmol m^{-2} s^{-1} PPFD).

Damage was visually assessed at 4, 24, 48, and 96 h after the end of the freezing test, by measurement of leaf percentage that suffered from visual damage (VD). VD was estimated as the percentage of leaf surface which suffered cell lysis, as determined by a particular dimming and/or browning. To estimate VD, each leaf lamina was observed by dividing it into two longitudinal parts considering each part as the 50% of the leaf surface (we did not account the central vein), and we estimated visually the surface of each part that was dimming and/or browning. The final value of VD was the average of the estimated percentage of damage of each part.

Root growth potential (RGP) was assessed in December 2009 (6 plants per treatment and provenance). Seedlings were carefully removed from containers, white root tips emanating from the plug were cut, and seedlings were planted with undamaged plugs in 2.5 l pots with wet perlite. The pots were randomly distributed on a heating table (20°C) inside a greenhouse for 4 weeks. The seedlings were irrigated every 2–3 days and given no fertilization. After 4 weeks, all seedlings were lifted, carefully cleaned from the medium, and all the new roots protruding from the root plug were excised, separated in fine roots (roots < 2 mm diameter) and thick roots (roots > 2 mm) and dried at 65°C for at least 48 h. RGP was determined as the fine root dry mass (FRW), and thick root dry mass (ThRW).

Data analysis

The effect of autumn fertilization and provenance on morphological traits, nutritional status and RGP of seedlings was evaluated with a general linear model (GLM):

$$\begin{aligned} y_{ijkl} = {} & \mu + N_i + K_j + PROk + NK_{ij} + NPRO_{ik} \\ & + KPRO_{jk} + NKPRO_{ijk} + e_{ijkl} \end{aligned} \tag{1}$$

where y_{ijkl} is the value of the dependent variable in plant l fertilized at a dose i of N ($i = 1, 2$), at dose j of K ($k = 1, 2$) and from provenance k ($k = 1, 2$); μ is the overall mean; N, K, and PRO are the fixed effects of nutrients and provenance; the double and triple terms represent interactions; and e is the error term with the hypothesis $e_{ijkl} \sim \mathrm{N}(0, \sigma_e^2)$. Significant differences between effects were assessed by Tukey's HSD test, with $\alpha = 0.05$.

The influence of fertilization and provenance on VD was estimated by a mixed model:

$$\begin{aligned} y_{ijklmno} = {} & \mu + \alpha_j + t_k + h_l + N_m + K_n + PRO_o \\ & + th_{kl} + NK_{mn} + NPRO_{mo} + KPRO_{no} \\ & + tN_{km} + tK_{kn} + tPRO_{ko} + tNK_{kmn} \\ & + tNPRO_{kmo} + tKPRO_{kno} + thN_{klm} \\ & + thK_{k\,\mathrm{ln}} + thPRO_{klo} + e_{ijklmnoq} \end{aligned} \tag{2}$$

where $y_{ijklmno}$ is arcsin $\sqrt{\mathrm{VD}(\%)/100}$ of leaf i of plant j, tested at temperature k, measured after l hours (24, 48, 72, or 96 h), at dose m of N, dose n of K and from provenance o; μ is the overall mean; α is a random plant effect with hypothesis $\alpha \sim \mathrm{N}(0, \sigma_p^2)$; t is the temperature fixed effect; h is the measurement time (fixed effect); N, K, and PRO are the fixed effects of nutrients and provenance; and e is the error term under the hypothesis of Normal distribution. The other terms describe interactions of the main effects. The analysis considered that (1) observations belonging to the same plant can be correlated, and (2) observations taken in the same leaf at different measurement time (24, 48, 72 and 96 h) can also be correlated and have heterogeneous variance. To test (1), we tested the significance of the plant random effect with a likelihood ratio test, as the reduction of the statistic $-2 \times$ log likelihood (-2LL), after introducing the plant random effect follows a χ^2 distribution with 1 degree of freedom. For testing (2), we tested different variance–covariance structures for the submatrix corresponding to the observations taken at the same leaf at the four different times. The structures tested were (see Wolfinger (1996) for a description of the variance–covariance structures): diagonal with homogeneous variance, unstructured with 1, 2, 3 and 4 bands, toeplitz with 1, 2, 3 and 4 bands, heterogeneous toeplitz with 1, 2, 3 and 4 bands, autoregressive heterogeneous and first-order ante-dependence. Comparison of different structures has been done with Akaike's information criteria (AIC, the smaller the better). Variance components for each structure were estimated by restricted maximum likelihood (REML). Least squares means estimates of the different levels of the significant fixed effects and their standard errors were calculated to account for significant differences.

Results

Morphological traits and nutritional status of seedlings

At the end of the first 28 weeks, the seedling characteristics of both provenances as a whole were: height (9.45 ± 0.44 cm), stem diameter (2.38 ± 0.07 mm), shoot dry mass (1.19 ± 0.07 g), root dry mass (2.46 ± 0.28 g), shoot-to-root dry mass ratio (0.58 ± 0.04), and 0.99% N, 0.09% P, 0.63% K in leaves. At this time, there were only significant differences between the provenances for height ($P = 0.049$), with *HU* seedlings taller than *ALC* seedlings (11.41 cm vs. 8.87 cm).

After the autumn fertilization, Holm oak seedlings fertilized with high N dose (N_1) had greater H, D, ShDW, and RWD, but not SRDW, than those given low N dose ($N_{1/20}$) (Table 2). Provenance also had a significant effect on leaf dry mass ($P = 0.031$; 1.17 g [*HU*] vs. 1.02 g [*ALC*]), and slenderness (H/D) ($P < 0.001$; 5.12 ± 0.31 [*HU*] vs. 4.03 ± 0.19 [*ALC*]). K and the interactions of fixed effects were not significant for any measured parameters.

Table 3 shows that autumn N fertilization significantly increased leaf nitrogen concentration and content (1.53%N [N_1] vs. 1.31%N [$N_{1/20}$] and 18.54 mgN [N_1] vs. 12.83 mgN [$N_{1/20}$]), and that provenance had a significant effect on leaf N content (17.04 mgN [*HU*] vs. 14.29 mg N [*ALC*]). Provenance also had an effect, albeit not significant, in leaf K concentration ($P = 0.073$), with *HU* seedlings showing higher leaf K concentration. However, autumn fertilization with K had no significant effect on leaf nutrient concentration or content. The interaction $N \times K$ had a significant effect on leaf K concentration (Fig. 1a), in that

Table 2 Morphological parameters of *Q. ilex* seedlings (measured in December 2009) cultivated with different doses of N (N_1 y $N_{1/20}$) and from different provenances (*ALC* or *HU*)

	N			*PRO*		
	N_1	$N_{1/20}$	*P*	*ALC*	*HU*	*P*
H (cm)	14.43	11.61	**<0.001**	12.53	13.41	0.153
D (mm)	3.15	2.68	**<0.001**	3.14	2.69	**<0.001**
ShDW (g)	1.71	1.33	**0.007**	1.45	1.58	0.395
RDW (g)	3.43	2.79	**0.002**	3.20	3.01	0.284
SRDW	0.51	0.48	0.253	0.46	0.53	**0.005**

The significance of differences (*P* values) is indicated. *H* height, *D* diameter, *ShDW* shoot dry mass, *RDW* root dry mass, *SRDW* shoot-to-root dry mass ratio

Bold values show significant differences ($P < 0.05$)

Table 3 *P* values derived from the general linear model that evaluated the effect of autumn fertilization (*N*, *K*), provenance (*PRO*), and their interactions on leaf concentrations and content of N, P, and K of *Q. Ilex* seedlings (December 2009)

	N	*K*	*PRO*	*N·K*	*N·PRO*	*K·PRO*	*N·K·PRO*
[N]	**0.003**	0.322	0.229	0.970	0.995	0.238	0.758
[P]	**0.006**	0.060	**0.007**	0.812	**0.012**	0.119	0.749
[K]	**0.037**	0.070	0.073	**0.039**	0.715	0.300	0.446
Ncont	**<0.001**	0.395	**0.005**	0.550	0.449	0.102	0.500
Pcont	**<0.001**	0.125	0.266	0.966	**0.005**	0.140	0.257
Kcont	**0.003**	0.176	**0.023**	0.191	0.913	**0.037**	0.324

Bold values show significant differences ($P < 0.05$)

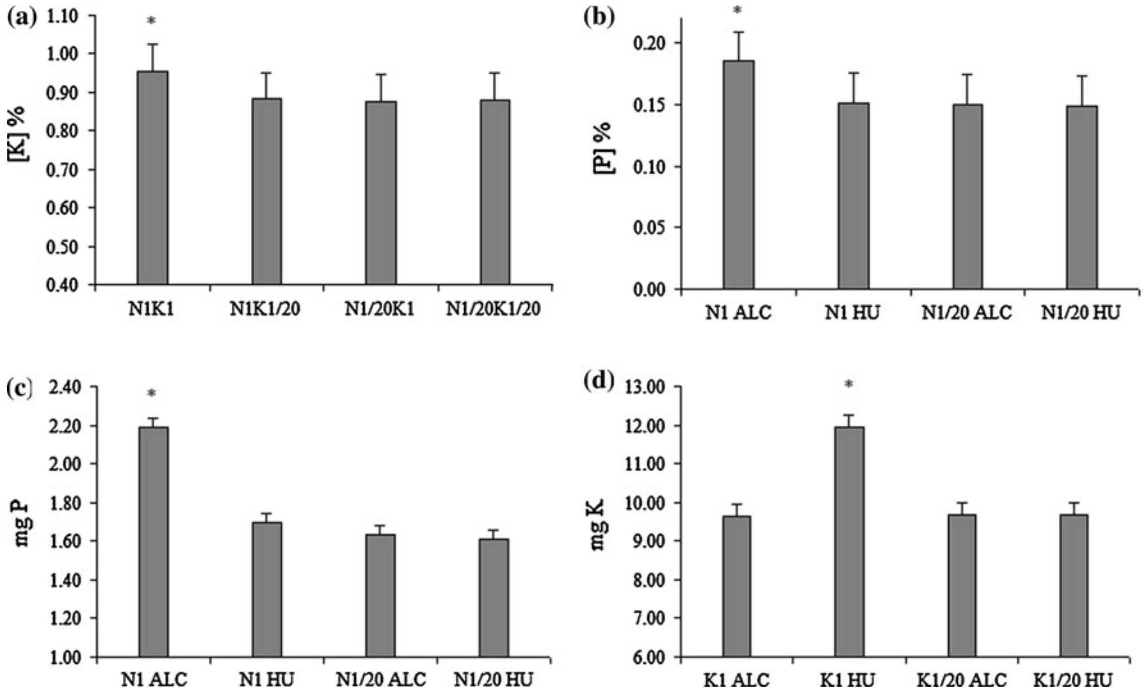

Fig. 1 Significant interactions derived from the general linear model that evaluated the effect of nutrient autumn dose (N_1, $N_{1/20}$, K_1 and $K_{1/20}$) and provenance (*ALC* and *HU*) on leaf concentrations and content of nutrients. **a** shows the effect of $N \times K$ interaction on K leaf concentration, **b** shows the effect of $N \times PRO$ interaction on P leaf concentration, **c** the effect of $N \times PRO$ interaction on P leaf content, and **d** shows the effect of $K \times PRO$ interaction on K leaf content. *Asterisks* indicate significant differences ($P < 0.05$)

leaf K concentration increased significantly in seedlings fertilized with higher doses of both nutrients (N_1 and K_1). The interaction $N{\times}PRO$ was also significant for leaf P concentration and content (Fig. 1b, c), in that seedlings from *ALC* and fertilized with N_1 had the highest values of leaf P concentration and content. The interaction $K{\times}PRO$ also had a significant effect on leaf K content (Fig. 1d): seedlings from *HU* and fertilized with K_1 had the highest values of leaf K content.

Fertilization treatment had no significant effect on leaf SS and St concentration (4.97 and 25.05%, respectively) or content (56.40 and 276.45 mg, respectively). However, provenance had a significant effect on leaf SS content ($P = 0.043$), and was higher in *HU* seedlings than *ALC* seedlings (69.21 mg vs. 43.60 mg, respectively). Provenance also had a small non-significant effect ($P = 0.122$) in leaf SS concentration, with *HU* seedlings showing higher leaf SS concentration.

Frost tolerance and root growth potential

The selected model for the analysis of the influence of autumn fertilization and provenance on leaf visual damage (VD) after freezing tests indicates a significant plant random effect ($P = 0.013$). The selected variance–covariance matrix of observations from the same leaf measured at different times was the first-order ante-dependence, hence indicating correlation between measurements from the same leaf and heterogeneous variance at different measurement times. Table 4 shows the significance of the fixed effects in the selected model. Our results indicate significantly more VD at lower temperatures except at temperatures of −10 and −12°C. Furthermore, VD was significantly greater as the time after the freezing test increased (38% at 24 h, 55% at 48 h, 68% at 72 h and 76% at 96 h). Autumn N fertilization and provenance had significant effect on VD, but autumn K fertilization had not. Seedlings given low dose N ($N_{1/20}$) had greater VD than seedlings given high dose of N (N_1) for all temperatures except −14°C (Fig. 2, above). *HU* seedlings had significantly greater VD than *ALC* seedlings at −10 and −12°C (Fig. 2, below).

Autumn N fertilization also had a significant effect on RGP. Seedlings fertilized with N_1 had more new roots at the end of the test than seedlings fertilized with $N_{1/20}$ (59.8 mg ThRW [N_1] vs. 20.8 mg ThRW [$N_{1/20}$]); 7.6 mg FRW [N_1] vs. 4.2 mg FRW [$N_{1/20}$]. There were no significant effects of autumn K fertilization or provenance, and no significant interactions between fixed effects for any of the measured variables.

Table 4 F and *P* values derived from the selected mixed model used for studying the influence of fertilization and provenance on leaf visual damage after freezing tests

Effect	F value	Pr > F
PRO	68.19	<0.0001
N	44.86	<0.0001
K	0.35	0.5548
Time	195.85	<0.0001
Temp	172.09	<0.0001
PRO × N	0.14	0.7036
PRO × K	1.51	0.2190
PRO × Time	2.76	0.0413
PRO × Temp	11.57	<0.0001
N × K	0.05	0.8263
N × Time	5.69	0.0007
N × Temp	3.74	0.0109
K × Time	1.99	0.1143
K × Temp	0.40	0.7497

PRO, N, K, Time and Temp are the fixed effects of provenance, dose of N, dose of K, measurement time and temperature

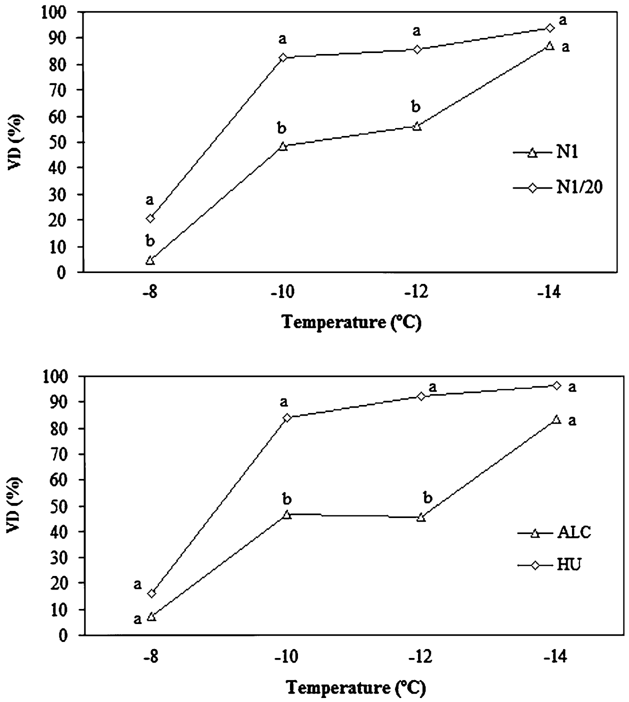

Fig. 2 Least squares means estimates of seedlings fertilized with two doses of N (N_1 and $N_{1/20}$) (*above*) and of seedlings from different provenances (*ALC* and *HU*) (*below*) at different temperatures. *Different letters* indicate significant differences between N doses ($P < 0.05$) and between provenances ($P < 0.05$) for each temperature tested

Discussion

Our results indicated that autumn N fertilization is an effective tool for modifying the morphological parameters (*H*, *D*, ShDW and RDW) of Holm oak seedlings. In particular, at the end of the growth phase in the nursery, the values of these parameters were about 20% higher in seedlings fertilized with N_1 than $N_{1/20}$. Our observation of higher values of *H*, *D*, and ShDW is in agreement with previous studies (Broncano et al. 1998; Villar-Salvador et al. 2004a), and our observation of greater root growth following N fertilization confirms the positive effect of autumn N fertilization on the development of the root system in Holm oak grown in warm autumn nurseries (Andivia et al. 2011a, b). Our results also indicated that N fertilization was the main factor affecting leaf nitrogen concentration and content: more autumn supplement of N increased significantly concentration and content of N. In addition, the application of higher N and K doses was necessary to increase leaf K concentration suggesting that it is necessary to apply higher doses of both nutrients to avoid nutrient dilution during the hardening phase. Our carbohydrate analysis indicated that, although N_1 increased plant growth, leaf NSC concentrations and content were unaffected by N dose. Autumn fertilization avoided the dilution effect in leaf concentration of NSC, despite the highest growth of seedlings given N_1 dose and therefore the higher carbon consumption and demand of carbohydrates of these plants (Fernández et al. 2008; Sanz-Pérez et al. 2007). These data confirm that Holm oak seedlings can take up mineral nutrients from their growing medium and accumulate them during the autumn, as reported by Andivia et al. (2011a). Nevertheless, the lack of nutrient and NSC dilution could be influenced by the reduced growing conditions of the hardening phase compared, for instance, with the growing phase.

In spite of the differences in the acorns mass from *HU* and *ALC*, total dry mass of the seedlings was not significantly different for both provenances 7–10 months after germination in the nursery. However, we observed a significant effect of provenance on *D*, LDW, SRDW and *H*/*D*. In particular, *HU* seedlings had greater increases in height and foliar tissue than *ALC* seedlings, and *ALC* seedlings tended to a greater allocation of biomass to the roots than *HU* seedlings. As acorns from each provenance were pooled together, we cannot analyze the possibility of maternal effects mediated by seed size, as acorn and seedling size are usually correlated (Gimeno et al. 2009; Leiva and Fernández-Alés 1998). We found the biggest acorns in the provenance with the warmer climatic conditions as a likely consequence of a greater window of time for growth and reproduction, and this would confer survival and competitive advantages to the emerging seedlings (Gimeno et al. 2009). Nevertheless, our results suggest that morphologic differences in early seedling performance between the two provenances are also induced by genetic effects other than seed size. In addition, the effect of acorn biomass on seedling size is not the main effect after the first flush of growth (García-Cebrián et al. 2003; Villar-Salvador et al. 2010), as mother effects other than seed size and environmental conditions can affect seedling growth (Andersson and Frost 1996; Leiva and Fernández-Alés 1998; Miao 1995; Puértolas et al. 2009). Interactions between fertilization and provenance indicated that in *ALC* seedlings, leaf P concentration and content increased considerably in seedlings fertilized with dose N_1. P dynamics is affected by fall fertilization, occurring during the hardening phase a partial translocation of P to the shoots (Oliet et al. 2011). Our data suggest that higher N dose increases P fall uptake and/or P translocation to shoots probably due to the higher remobilization and absorption of nutrients expected in large seedlings (Cuesta et al. 2010). However, the higher leaf P concentrations and contents in seedlings given higher N dose have been reported only for *ALC* seedlings. P is an important constituent of phospholipids (Marschner 1995), and frost-induced damage first occurs in phospholipid membranes (Heino and Palva 2003). Cold hardening is associated with increased membrane fluidity (Levitt 1980), and this is determined by different concentrations of saturated and unsaturated phospholipids, proteins, and sterols (Livonen et al. 2004). Thus, the higher accumulation of P in *ALC* seedlings could allow them to better withstand winter frosts, which are more frequent and severe in this region.

On the other hand, *HU* seedlings showed a higher leaf N, K and SS contents than *ALC* seedlings, due to a combination of increased leaf biomass with a higher, albeit not significant, leaf N, SS and K concentration (especially for leaf K concentration) in *HU* seedlings, indicating differences in the accumulation of N, K and SS of plants from these two provenances. This and the differences in morphological parameters could be related to climatic differences between the two regions (Gratani et al. 2003). The absence of frosts and the longer and wetter growing season in *HU* could have led to their greater N accumulation and shoot growth, which would provide them with a competitive advantage. *HU* has a longer and drier summer drought period than *ALC* and K plays an important role in controlling stomatal aperture and cell osmotic adjustment (Hsiao and Läuchli 1986). Thus, higher leaf K concentration of *HU* seedlings could be an adaptation to summer drought that is common in the *HU* region. However, it is necessary to conduct further studies that consider a greater number of provenances from diverse climatic conditions to clarify the effect of provenance on seedling morphology and nutrient and carbohydrates accumulation.

Previous studies of other species (Fernández et al. 2007, 2008; Puértolas et al. 2005) and Holm oak (Andivia et al. 2011a, b) have shown that leaf VD is an effective indicator for assessing frost-induced plant damage. Our data suggest that autumn N fertilization in a warm autumn nursery significantly improved the cold tolerance of Holm oak seedlings. Previous studies have shown a positive effect of increased SS concentration on cold tolerance for several *Quercus* species (Fernández et al. 2008; Mollá et al. 2006; Morin et al. 2007). Although our data show similar concentrations and content of SS in seedlings given different N doses, autumn fertilization with N nonetheless improved cold tolerance. Accumulation of SS is only one mechanism that prevents frost damage, and other molecules and solutes also have important roles in improving cold tolerance (Larcher 2000; Zwiazek et al. 2001). In fact, Villar-Salvador et al. (2004a, b) suggest that individual sugars or other compounds such as organic acids in Holm oaks, as well as cell membrane stability, may be more important in osmotic adjustment than the total concentration of soluble sugars. K content is inversely related to frost damage and P is needed for membrane synthesis (Marschner 1995) that are the first structures damaged by low temperature stress (Elstner 1982). In our study, K and P leaf concentrations were higher in seedlings fertilized with N_1, and these seedlings also had greater cold tolerance.

Provenance was a significant factor in cold tolerance at the intermediate temperatures tested (−10 and −12°C), where seedlings showed the limit of tolerance in this study. Just at the end of autumn (3rd week of December), before the colder winter period, *ALC* seedlings (from the cooler region) had VD values of ~40%, whereas *HU* seedlings (from the warmer region) had VD values of ~80–90%. Thus, our data suggest that these two provenances are adapted to local climatic conditions. Frost protection is partially achieved by changes in the expression of genes sensitive to low temperatures (Novillo et al. 2004; Suping et al. 2005). Physiological adaptations among provenances have also been studied for other species, such as *Pinus taeda* (Bongarten and Teskey 1986), *Picea abies* (Oleksyn et al. 1998), *Pinus sylvestris* (Palmroth et al. 1999), *Fagus sylvatica* (Peuke et al. 2002), but data for Mediterranean sclerophyllous hardwood species are sparse and contradictory. Some studies of *Quercus ilex* (Gratani et al. 2003; Sánchez-Vilas and Retuerto 2007) and *Quercus suber* (Ramírez-Valiente et al. 2009) have reported an adaptation of seedlings from xeric provenances to drought, whereas other studies of seedlings from cold provenances reported better response to frosts in different *Quercus* species (Aranda et al. 2005; Morin et al. 2007).

By contrast, Gimeno et al. (2009) concluded that physiological responses to drought and frost are more dependent on acclimation than adaptation to local conditions. The differences between our study and that of Gimeno et al. (2009), who reported no differences in cold tolerance among provenances, might be due to different durations and conditions of the hardening phase (all in the autumn and nursery conditions in our study, 10 days in a growth chamber subjected to cold temperatures and a PPFD < 300 μmol m^{-2} s^{-1} in the Gimeno et al. study). The short period of time and the low PPFD could have limited plants from Gimeno et al. study for developing their full frost tolerance potential. Cold acclimation by exposure to suboptimal temperatures is known to improve cold tolerance (Thomashow 1999; Puértolas et al. 2005). The temperatures during the hardening phase in our nursery were exceptionally warm for this assay (only 239 accumulated chilling hours [$\leq$8°C]). Hence, the acclimation of our plants was less than in other studies which used cooler weather conditions (Mollá et al. 2006; Andivia et al. 2011a). However, genetic heterogeneity among Holm oak provenances could explain variations in functional and structural characteristics of populations (Jorrín Novo et al. 2004; Sánchez-Vilas and Retuerto 2007).

RGP is considered a measure of seedling vigor, and a proxy for photosynthetic efficiency or the amount of stored carbohydrates (Ritchie 1985). Our results indicated that autumn N fertilization (N_1) increased RGP and shoot growth. Previous studies have used RGP as a predictor of transplantation performance of forest species, and RGP is frequently correlated with cold hardiness (Simpson and Ritchie 1996). In our study, N fertilization (N_1) increased RGP and cold hardiness, in agreement with Mollá et al. (2006). A well-developed root system is essential for successful reforestation. In Mediterranean summers, for instance, roots need to access water that is deeper in the soil (Palacios et al. 2009; Querejeta et al. 2001), and previous studies (Andivia et al. 2011a; Palacios et al. 2009) showed that in warm winter areas, early winter root growth could improve water status in the dry season. Differences in RGP of different populations may be due to adaptations to xeric conditions. In our study, there were no differences in RGP between the warm winter provenance (*HU*) and the cold winter provenance (*ALC*). Testing of additional provenances, including acorns from xeric areas, could clarify how different provenances respond to different types of environmental stress.

Conclusions

Autumn fertilization of Holm oak seedlings with 30 mg of N per plant during the hardening phase in the nursery (plus 70 mg N during the growing phase) improved seedling quality, modified seedling morphology, allowed seedlings to accumulate mineral nutrition, improved seedling response to frost, and improved root growth potential compared to autumn fertilization with 1.5 mg N.

Our results also indicate different uptake and/or accumulation of nutrients, especially for N, K, and SS of different provenances. Genetic factors control physiological responses, such as cold tolerance, and these would be expected to differ for plants from different climatic regions. Clearly, further studies of more provenances with more culture conditions and a more frequent measurement of seedlings characteristics (such as freezing tolerance, nutrients content, and NSC) on several dates are needed to establish the effect of intra- and inter-population variability and plasticity in cold tolerance and other physiological responses.

Our results suggest that Holm oak provenance should be considered in forest restoration programs. Even though acclimation is a key factor in forest plant cultivation, provenance should be included in the design of forest restoration programs, particularly considering the forecasted climatic changes for Mediterranean areas. This especially applies to *Quercus ilex* L., in which field establishment is difficult compared with other Mediterranean species.

Acknowledgments This work has been financed by MEC of Spain (Ref. AGL2006-12609-C02-01/FOR). In addition, the work was partially supported by University of Huelva and Andalucía Research Programs to promote research groups activity. First author is benefiting from a doctoral grant from the Ministry of Education of Spain. We gratefully acknowledge the support of the laboratory of research and Agrifood control (LICAH) of the University of Huelva for carrying out chemical analysis.

References

Alía R, Alba N, Agúndez D, Iglesias S (2005) Manual para la comercialización y producción de semillas y plantas forestales: materiales de base y reproducción. Serie Forestal. Dirección General para la Biodiversidad, Madrid, Spain, p 384

Andersson C, Frost I (1996) Growth of *Quercus robur* seedlings after experimental grazing and cotyledon removal. Acta Botanica Neerlandia 45:85–94

Andivia E, Fernández M, Vázquez-Piqué J (2011a) Autumn fertilization of *Quercus ilex* ssp *ballota* (Desf.) Samp. nursery seedlings: effects on morpho-physiology and field performance. Ann For Sci 68:543–553

Andivia E, Márquez-García B, Vázquez-Piqué J, Córdoba F, Fernández M (2011b) Autumn fertilization with nitrogen improves nutritional status, cold hardiness and the oxidative stress response of holm oak (*Quercus ilex* ssp. *ballota* [Desf.] Samp) nursery seedlings. Trees Struct Funct. doi:10.1007/s00468-011-0593-3

Aranda I, Castro L, Alía R, Pardos JA, Gil L (2005) Low temperature during winter elicits differential responses among populations of the Mediterranean evergreen cork oak (*Quercus suber*). Tree Physiol 25:1085–1090

Barbero M, Loisel R, Quézel P (1992) Biogeography ecology and history of Mediterranean *Quercus ilex* ecosystems. Vegetation 99–100:19–34

Birchler T, Rose RW, Royo A, Pardos M (1998) La planta ideal: revisión del concepto, parámetros definitorios e implementación práctica. Inv Agrar-Sist Rec F 7:109–121

Boivin JR, Miller BD, Timmer VR (2002) Late-season fertilization of *Picea mariana* seedlings under greenhouse culture: biomass and nutrient dynamics. Ann For Sci 59:255–264

Bongarten BC, Teskey RO (1986) Water relations of loblolly pine seedlings from diverse geographic origins. Tree Physiol 1:265–276

Broncano MJ, Riba M, Retana J (1998) Seed germination and seedling performance of two Mediterranean tree species, holm oak (*Quercus ilex* L.) and Aleppo pine (Pinus halepensis Mill.): a multifactor experimental approach. Plant Ecol 138:17–26

Christensen JH, Hewitson B, Busuioc A et al (2007) Contribution of working group I to fourth assessment report of the intergovernmental panel of climate change. In: Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Miller MTHL (eds) Climate change 2007: the physical science. Cambridge University Press, Cambridge

Cuesta B, Villar-Salvador P, Puértolas J, Jacobs DF, Rey Benayas JM (2010) Why do large, nitrogen rich seedlings better resist stressful transplating conditions? A physiological analysis in two functionally contrasting Mediterranean forest species. For Ecol Manage 260:71–78

del Campo AD, Navarro-Cerillo RM, Ceacero CJ (2010) Seedling quality and field performance of comercial stocklots of containerized holm oak (*Quercus ilex*) in Mediterranean Spain: an approach for establishing a quality standard. New For 39:19–37

Elstner EF (1982) Oxygen activation and oxygen toxicity. Ann Rev Plant Physiol 33:73–96

Fernández M, Marcos C, Tapias R, Ruiz F, López G (2007) Nursery fertilisation affects the frost-tolerance and plant quality of *Eucalyptus globulus* Labill cuttings. Ann For Sci 68:865–873

Fernández M, Alejano R, Domínguez L, Tapias R (2008) Temperature controls cold hardening more effectively than photoperiod in four Mediterranean broadleaf evergreen species. Tree For Sci Biotech 2:43–49

García-Cebrián F, Esteso-Martínez J, Gil-Pelegrín E (2003) Influence of cotyledon removal on early seedling growth in *Quercus robur* L. Ann For Sci 60:69–73

Gimeno TE, Pías B, Lemos-Filhos JP, Valladares F (2009) Plasticity and stress tolerance override local adaptation in the response of Mediterranean Holm oak seedlings to drought and cold. Tree Physiol 29:87–98

Gratani L (1995) Structural and ecophysiological plasticity of some evergreen species of the Mediterranean maquis in response to climate. Photosynthetica 31:335–343

Gratani L, Meneghini M, Pesoli P, Crescente MF (2003) Structural and functional plasticity of *Quercus ilex* seedlings of different provenances in Italy. Trees 17:515–521

Gu L, Hanson PJ, Post WM, Kaiser DP, Yang B, Nemani R, Pallardy SG, Meyers T (2008) The 2007 Eastern US spring freeze: increased cold damage in a warming world? Bioscience 58:253–262

Heino P, Palva ET (2003) Signal transduction in plant cold acclimation. In: Hirt H, Shinozaki K (eds) Plant responses to abiotic stress. Top Curr Gen, vol 4. Springer, Berlin, pp 151–185

Hsiao TC, Läuchli A (1986) Role of potassium in plant–water relations. In: Tinker B, Laüchli A (eds) Advances in plant nutrition, vol II. Praeger Scientific, New York, pp 281–312

Jiménez-Sancho P, Díaz-Fernández PM, Iglesias-Sauce S, de Tuero M, Gil L (1996) Regiones de procedencia de *Quercus ilex* L en España. MAPA, ICONA

Jorrín Novo JV, Navarro-Cerillo JM, Jorge I, Gómez E, Ariza Mateos D (2004) Aplicación de la proteómica a la caracterización de procedencias de *Quercus ilex* subsp. *ballota* (Desf.) Samp. Cuad Soc Esp Cien For 17:57–61

Larcher W (2000) Temperature stress and survival ability of Mediterranean sclerophyllous plants. Plant Biosyst 134:279–295

Laureano RG, Lazo YO, Linares JC, Luque A, Martínez F, Seco JI, Merino J (2008) The cost of stress resistance: construction and maintenance costs of leaves and roots in two populations of *Quercus ilex*. Tree Physiol 28:1721–1728

Leiva MJ, Fernández-Alés R (1998) Variability in seedling water status during drought within a *Quercus ilex* subsp *ballota* population, and its relation to seedling morphology. For Ecol Manage 111:147–156

Levitt J (1980) Responses of plants to environmental stresses. Academic Press, New York

Livonen S, Saranpää P, Sutinen ML, Vapaavuori E (2004) Effects of temperature and nutrient availability on plasma membrane lipid composition in Scots pine roots during growth initiation. Tree Physiol 24:437–446

MAPA (2006) Forestación de tierras agrícolas. Ministerio de Agricultura y Pesca, Madrid, 373 p

Marschner H (1995) Mineral nutrition of higher plants. Academic Press Limited, London

Miao S (1995) Acorn mass and seedling growth in *Quercus rubra* in response to elevated CO_2. J Veg Sci 6:670–700

Michaud H, Toumi L, Lumaret R, Li TX, Romane F, Di Giusto F (1995) Effect of geographical discontinuity on genetic variation in *Quercus ilex* L. (holm oak). Evidence from enzyme polymorphism. Heredity 74:590–606

Mollá S, Villar-Salvador P, García-Fayos P, Peñuelas-Rubira JL (2006) Physiological and transplanting performance of *Quercus ilex* L holm oak) seedlings grown in nurseries with different winter conditions. For Ecol Manage 237:218–226

Morin X, Ameglio T, Ahas R, Kurz-Besson C, Lanta V, Lebourgeois F, Miglietta F, Chuine I (2007) Variation in cold hardiness and carbohydrates concentration from dormancy induction to bud burst among provenances of three European oak species. Tree Physiol 27:817–825

Navarro-Cerillo RM, Pemán J, del Campo AD, Moreno J, Lara MA, Díaz JL, Pousa F, Piñón FM (2009) Manual de especies para la forestación de tierras agrarias en Andalucía. Junta de Andalucía, Consejería de Agricultura y Pesca, Sevilla

Novillo F, Alonso JM, Ecker JE, Salinas J (2004) CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in *Arabidopsis*. Proc Natl Acad Sci 101:3985–3990

Oleksyn J, Modrzynski J, Tjoelker MG, Zytkowiak R, Reich PB, Karolewski P (1998) Growth and physiology of *Picea abies* populations from elevational transects: common garden evidence for altitudinal ecotypes and cold adaptation. Funct Ecol 12: 573–590

Oliet JA, Tejada M, Salifu KF, Collazos A, Jacobs DF (2009) Performance and nutrient dynamics of holm oak (*Quercus ilex* L.) seedlings in relation to nursery nutrient loading and post-transplant fertility. Eur J For Res 128:253–263

Oliet JA, Salazar JM, Villar R, Robredo E, Valladares F (2011) Fall fertilization of Holm oak affects N and P dynamics, root growth potential, and post-transplanting phenology and growth. Ann For Sci 68:647–656

Palacios G, Navarro-Cerillo RM, del Campo A, Toral M (2009) Site preparation stock quality and planning date effect on early establishment of Holm oak (*Quercus ilex* L.) seedlings. Ecol Eng 35:38–46

Palmroth S, Berninger F, Nikinmaa E, Lloyd J, Pulkkinen P, Hari P (1999) Structural adaptation rather than water conservation was observed in Scots pine over a range of wet to dry climates. Oecologia 121:302–309

Pausas JG, Blade C, Valdecantos A, Seva JP, Fuentes D, Alloza JA, Vilagrosa A, Bautista S, Cortina J, Vallejo R (2004) Pines and oaks in the restoration of Mediterranean landscapes of Spain:

new perspectives for an old practice—a review. Plant Ecol 171:209–220

Pesoli P, Gratani L, Larcher W (2003) Response of *Quercus ilex* from different provenances to experimentally imposed water stress. Biol Plant 46:577–581

Peuke AD, Schrami C, Hartung W, Rennenberg H (2002) Identification of drought-sensitive beech ecotypes by physiological parameters. New Phytol 154:373–387

Puértolas J, Gil L, Pardos JA (2005) Effects of nitrogen fertilization and temperature on frost hardiness of Aleppo pine (*Pinus halepensis* Mill.) seedlings assessed by chlorophyll fluorescence. Forestry 78:502–511

Puértolas J, Disante K, Fuentes D, Valdecantos A, Monerris J, Cortina J, Benito LF, Peñuelas JL (2009) Ritmos de acumulación de biomasa en *Quercus ilex* durante el primer año de cultivo en contenedor: efectos maternos y ambientales. In: Sociedad Española de Ciencias Forestales, Junta de Castilla y León (eds) Proceedings V Congreso Forestal Español, Ávila, Spain, Ref.: 5CFE01-278, p 10. ISSN: 978-84-936854-6-1

Puttonen P (1997) Looking for the "silver-bullet": can one test do it? New For 13:9–27

Querejeta J, Roldán A, Albadalejo J, Castillo V (2001) Soil water availability improved by site preparation in a *Pinus halepensis* afforestation under semiarid climate. For Ecol Manag 149: 115–128

Ramírez-Valiente JA, Valladares F, Gil L, Aranda I (2009) Population differences in juvenile survival under increasing drought are mediated by seed size in cork oak (*Quercus suber* L.). For Ecol Manag 257:1676–1683

Ritchie GA (1985) Root growth potential: principles, procedures and predictive ability. In: Duryea ML (ed) Proceedings evaluating seedling quality: principles, procedures, and predictive abilities of major tests. Forest Research Laboratory, Oregon State University, Corvallis, pp 93–105

Rodà F, Retana J, Gracia C, Bellot J (1999) Ecology of Mediterranean evergreen oak forests. Springer, Berlin

Rose R, Rose CL, Omi SK, Forry KR, Durral DM, Bigg WL (1991) Starch determination by perchloric acid versus enzymes: evaluating the accuracy and precision of six colorimetric methods. J Agric Food Chem 39:2–11

Sánchez-Vilas J, Retuerto R (2007) *Quercus ilex* shows significant among-population variability in functional and growth traits but maintains invariant scaling relations in biomass allocation. Int J Plant Sci 168:973–983

Sanz-Pérez V, Castro-Díez P, Valladares F (2007) Growth versus storage: response of Mediterranean oak seedlings to changes in nutrient and water availabilities. Ann For Sci 64:201–210

Simpson DG, Ritchie GA (1996) Does RGP predict field performance? A debate. New For 13:249–273

Spiro RG (1966) Analysis of sugars found in glycoproteins. In: Neufeld EF, Ginsburg V (eds) Methods in ezymology. Complex carbohydrates, vol VIII. Academic Press, New York, pp 3–26

Suping Z, Sauvé RJ, Mmbaga MT (2005) Adaptation of *Pachysandra terminalis* Sieb & Zucc. to freezing temperatures by the accumulation of mRNA and cold-induced proteins. Hort Sci 40:346–347

Terradas J, Savé R (1992) The influence of summer and winter stress and water relationships on the distribution of *Quercus ilex* L. In: Romane F, Terradas J (eds) *Quercus ilex* L. ecosystems: function dynamics and management. Kluwer, Dordrecht, pp 137–145

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50:571–599

Trubat R, Cortina J, Vilagrosa A (2011) Nutrient deprivation improves field performance of woody seedlings in a degraded semi-arid shrubland. Ecol Eng 37:1164–1173

Valladares F, Martínez-Ferri E, Balaguer L, Pérez-Corona E, Manrique E (2000) Low leaf-level response to light and nutrients in Mediterranean evergreen oaks: a conservative resource-used strategy? New Phytol 148:79–91

Villar-Salvador P, Planelles R, Enriquez E, Peñuelas-Rubira JL (2004a) Nursery cultivation regimes, plant functional attributes, and field performance relationships in the Mediterranean oak *Quercus ilex* L. For Ecol Manage 196:257–266

Villar-Salvador P, Planelles R, Oliet J, Peñuelas-Rubira JL, Jacobss DF, González M (2004b) Drought tolerance and transplanting performance of holm oak (*Quercus ilex*) seedlings after drought hardening in the nursery. Tree Physiol 24:1147–1155

Villar-Salvador P, Heredia N, Millard P (2010) Remobilization of acorn nitrogen for seedling growth in holm oak (*Quercus ilex*), cultivated with contrasting nutrient availability. Tree Physiol 30:257–263

Wolfinger RD (1996) Heterogeneous variance-covariance structures for repeated measures. J Agric Biol Environ Stat 1:205–230

Zwiazek J, Renault S, Croser C, Hansen J, Beck E (2001) Biochemical and biophysical changes in relation to cold hardiness. In: Bigras FJ, Colombo SJ (eds) Conifer cold hardiness. Kluwer, Dordrecht, pp 165–186