Tree Physiology 24, 1147–1155

Drought tolerance and transplanting performance of holm oak (*Quercus ilex*) seedlings after drought hardening in the nursery

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Received March 21, 2003; accepted February 8, 2004; published online August 2, 2004

Summary Drought stress is the main cause of mortality of holm oak (*Quercus ilex* L.) seedlings in forest plantations. We therefore assessed if drought hardening, applied in the nursery at the end of the growing season, enhanced the drought tolerance and transplanting performance of holm oak seedlings. Seedlings were subjected to three drought hardening intensities (low, moderate and severe) for 2.5 and 3.5 months, and compared with control seedlings. At the end of the hardening period, water relations, gas exchange and morphological attributes were determined, and survival and growth under mesic and xeric transplanting conditions were assessed. Drought hardening increased drought tolerance primarily by affecting physiological traits, with no effect on shoot/root ratio or specific leaf mass. Drought hardening reduced osmotic potential at saturation and at the turgor loss point, stomatal conductance, residual transpiration (RT) and new root growth capacity (RGC), but enhanced cell membrane stability. Among treated seedlings, the largest response occurred in seedlings subjected to moderate hardening. Severe hardening reduced shoot soluble sugar concentration and increased shoot starch concentration. Increasing the duration of hardening had no effect on water relations but reduced shoot mineral and starch concentrations. Variation in cell membrane stability, RT and RGC were negatively related to osmotic adjustment. Despite differences in drought tolerance, no differences in mortality and relative growth rate were observed between hardening treatments when the seedlings were transplanted under either mesic or xeric conditions.

Keywords: Mediterranean, nonstructural carbohydrate, osmotic adjustment, plasmalemma stability, residual transpiration, root growth capacity, stomatal conductance, water stress.

Introduction

Drought is an important limiting factor for plant life in Mediterranean ecosystems. Woody plants have developed different mechanisms to cope with water stress. Some species tolerate drought (Kramer and Kozlowski 1979) by avoiding tissue desiccation through early and tight stomatal closure, low cuticular transpiration or foliage reduction. Other species tolerate drought by withstanding some degree of tissue dehydration without protoplasmic damage by enhancing the stability of cell membranes, osmotic adjustment and increasing cell wall elasticity (Morgan 1984, Turner 1986, Martin et al. 1987, Fan et al. 1994, Gebre et al. 1994). A variety of compounds can contribute to osmotic adjustment, but soluble carbohydrates, inorganic ions and amino acids predominate in most species (Gebre et al. 1994, Tschaplinski et al. 1995, Dichio et al. 2003). Osmotic adjustment and increased cell wall elasticity permit greater cell turgor at low tissue water potential, which may enable plants to maintain gas exchange and growth during drought (Bennet and Sullivan 1981, Morgan 1984, Seiler and Johnson 1988).

Holm oak (*Quercus ilex* L.) is an evergreen tree that dominates many forest communities in the central and western Mediterranean basin. Many abandoned croplands are being afforested with holm oak. Although mature holm oak trees in natural populations enhance their dehydration tolerance by osmotic adjustment in response to summer drought (Kyriakopoulos and Richter 1991), seedlings are vulnerable to transplanting stress and to summer drought, having high mortality and slow growth compared with other Mediterranean species (Baeza et al. 1991). Drought hardening applied in the nursery before planting may improve field establishment of holm oak seedlings by increasing their drought tolerance. In two conifer species, drought hardening increased seedling survival under xeric planting conditions, and this was related to morphological changes that occurred during nursery cultivation (van den Driessche 1991*a*). However, few studies have demonstrated that greater growth or survival of a plant species under dry conditions is related to enhanced dehydration tolerance induced by previous drought hardening (Morgan 1984, Pita and Pardos 2001).

Changes in drought tolerance depend on the degree of water stress, the length of the hardening period and the species (Abrams 1988, Zwiazek and Blake 1989, Edwards and Dixon 1995, Vilagrosa et al. 2003). Restricted watering applied during seedling cultivation can induce osmotic adjustment, increase cell membrane stability to dehydration (Gebre et al. 1994, Collet and Ghuel 1997) and reduce both stomatal and residual transpiration (Edwards and Dixon 1995, Villar-Salvador et al. 1999).

We examined the response of holm oak seedlings to drought hardening applied in the nursery at the end of the growing season. Three hypotheses were tested: (1) moderate drought produces the largest increase in tolerance to water stress; (2) increased duration of hardening enhances drought tolerance; and (3) drought hardening increases the survival and growth of seedlings planted on xeric sites. To test these hypotheses, seedlings were subjected to different degrees of drought hardening for 2.5 or 3.5 months Water relations, gas exchange and growth attributes were measured, and the post-transplanting performance of seedlings under xeric and mesic conditions was assessed.

Material and methods

Plant material and experimental design

A total of 4200 seedlings were raised from acorns of holm oak collected in Spain from an inland provenance (La Alcarria-Serranía de Cuenca). The acorns were sown in November 1995 in Forest Pot300 trays (50 cavities of 300 ml per tray; Nuevos Sistemas de Cultivo S.L., Girona, Spain) containing an 80:20 (v/v) mixture of peat and vermiculite. To avoid late-spring frosts, seedlings were raised in a greenhouse until mid-June 1996 and then moved outdoors under ambient conditions. Seedlings were fertilized every 2 weeks with a 20:3:16, N,P,K, water-soluble fertilizer and irrigated every 1–3 days. By mid-August, each seedling had received 32.0, 2.4 and 25.2 mg of N, P and K, respectively, plus microelements.

Drought hardening was imposed on 3600 seedlings through drought cycles and three drought hardening regimes to which the seedlings were randomly assigned. The drought hardening regimes were: low (L), moderate (M) and severe (S) drought, in which seedlings were irrigated when the weight of the containers was reduced to 30, 40–45 and 45–50% of weight at saturation, respectively. Six hundred seedlings (12 trays) were assigned to a control (C) treatment and were kept well watered by irrigating every 1–3 days. After the first drought cycle, the predawn xylem water potentials (Ψ_{pd}) of seedlings in the C, L, M and S drought treatments were: –0.28 ± 0.01, –0.61 ± 0.14, –1.28 ± 0.09 and –1.63 ± 0.12 MPa (mean ± SE, $n = 5$), respectively.

Drought hardening was applied for 2.5 and 3.5 months. In the latter case, half of the seedlings assigned to the L, M and S hardening treatments (600 seedlings per treatment) began hardening in mid-August 1996. For the remaining seedlings, hardening began in mid-September 1996. In each case, hardening was completed by late-November 1996. Six treatments resulted from the drought hardening regime × hardening duration combination ($L_{2.5}$, $L_{3.5}$, $M_{2.5}$, $M_{3.5}$, $S_{2.5}$, $S_{3.5}$) plus one control. Treatments were arranged in a randomized complete block design with three blocks.

Control seedlings and those water stressed for 3.5 months, received 1.6 g per seedling of slow-release fertilizer (14:6:12, N,P,K, 3–4 months at 21 °C) in mid-August, whereas seedlings hardened for 2.5 months received the slow-release fertilizer in mid-September. At the end of the hardening period in November, all seedlings were well watered and left to recover for 3 days before physiological and morphological analyses. At the end of the cultivation period, seedlings in all of the treatments fulfilled the morphological quality standards specified by European Union legislation for 1-year-old seedlings of this species (Directive 1999/105/EC).

Mean air temperature during the hardening period varied from 23 °C in August to 7 °C at the end of November. The first frost occurred at the beginning of October and frosts were frequent in November.

Shoot water relations

Pressure–volume (P–V) curves were made before the hardening period and again 5–10 days after the end of the hardening period, following the free-transpiration method (Koide et al. 1989). Shoot xylem water potential was determined with a home-built pressure chamber. Eight randomly sampled seedlings per treatment were watered the afternoon before and maintained in the dark until morning shoot sampling. As petioles of holm oak leaves are short, we used shoots containing 8–10 leaves. From each curve, the osmotic potential at the turgor loss point ($\Psi\pi_{tlp}$), the osmotic potential at saturation ($\Psi\pi_{sat}$), the relative water content at turgor loss point (RWC_{tlp}), the modulus of elasticity (ε) and the symplasm volume fraction (V_s/V_t) were calculated (Koide et al. 1989). Plateaus in some P–V curves were detected. In these cases, the shoot weight at full saturation was calculated following the method described in Kubiske and Abrams (1990). Osmotic adjustment was defined as the difference between $\Psi\pi_{sat}$ of the water-stressed treatments and $\Psi\pi_{sat}$ of control seedlings.

Residual transpiration, electrolyte leakage and stomatal conductance

Residual transpiration (RT) was measured 4 days after the end of the hardening period. Seedlings ($n = 10$) were watered and enclosed in an opaque plastic bag to ensure saturation overnight. In the morning, shoots were excised and left to dry in a ventilated growth chamber in which mean temperature, water vapor pressure deficit and photosynthetic photon flux (PPF) were 22.2 °C, 0.6 kPa and 150 μmol m^{-2} s^{-1}, respectively. Shoot fresh mass was measured to the nearest 1 mg at intervals of 0.5–1 h. By plotting shoot fresh mass against time, a curvilinear relationship was obtained, in which the linear portion represents water loss from seedling surfaces after stomatal closure. The rate of RT of each shoot was calculated on a mass basis as the ratio of the slope of the linear portion to the shoot mass.

Three weeks after the end of the hardening period, seedlings in all treatments were subjected to a new drought cycle. Forty seedlings per treatment were selected, watered and placed

in a greenhouse for 2 months without irrigation. Periodically, five seedlings per treatment were randomly sampled for water content (WC), electrolyte leakage (EL) and stomatal conductance (g_s). Seedlings were randomly sampled on the first three sampling dates, but subsequent sampling was directed to obtain an ample range of WC, EL and g_s values in each treatment. We calculated WC (%) as (leaf fresh mass – leaf dry weight)100/leaf dry mass, where leaf dry mass was measured after drying at 80 °C for 48 h.

We measured EL as described by Earnshaw (1993). Briefly, two leaves per seedling were cut in square 2–3 mm^2 pieces and washed twice in distilled water for 20 min. The pieces were placed in a vial with 20 ml of distilled water on an illuminated bench at room temperature and periodically shaken. The EL was determined as EC_i100/EC_f (%), where EC_i is electroconductivity of the water bathing the leaf pieces after 24 h and EC_f is electroconductivity of the same water after autoclaving the vials for 10 min at 120 °C (producing tissue destruction and complete release of cell solutes).

Stomatal conductance was measured 22, 34 and 47 days after the start of the new drought cycle with a porometer (LI-1600, Li-Cor, Lincoln, NE). Measurements were taken around midday (1100–1300 h, solar time). Temperature, relative humidity and PPF during measurements varied between 19–22.5 °C, 38–49% and 116–210 μmol m^{-2} s^{-1}, respectively.

Morphology, nonstructural carbohydrates, nutrient and root growth capacity determinations

Four days after the end of the hardening period, 36 seedlings per treatment (12 seedlings per block) were randomly sampled early in the morning and immediately frozen to –30 °C until processed. Once defrosted, shoots were cut at the cotyledon insertion point and separated into leaves and stems. Total leaf area per seedling was measured with an image analysis system (Dias, Delta-T Devices, Cambridge, U.K.). Root plugs were washed from the medium, rinsed in distilled water, dried at 60 °C for 48 h and weighed. Seedling mass, the shoot to root mass ratio (S/R) and specific leaf mass (SLM; total leaf mass to leaf area) were determined.

To determine mineral nutrient (K, Na and Mg) and nonstructural carbohydrate (starch and soluble carbohydrates) concentrations, leaves and roots of seedlings sampled in a block (n = 12) were pooled separately and finely ground. Concentrations of K, Na and Mg were determined by vacuum inductively coupled plasma emission spectroscopy (Optima 2000, Perkin Elmer, Wellesley, MA) after sample digestion in a microwave with HNO_3. Storage carbohydrates were extracted as described by Spiro (1966). Soluble carbohydrate (SC) and starch concentrations were determined by the anthrone and perchloric acid methods, respectively (Spiro 1966, Rose et al. 1991).

A root growth capacity (RGC) test (Ritchie 1985) was implemented 2 weeks after the end of the hardening period. White preexisting roots were removed from 20 seedlings per treatment and seedlings were individually transplanted to 3-l pots containing perlite. Seedlings were randomly arranged in a controlled environment room providing a 16-h photoperiod with a photosynthetic photon flux of 190 μmol m^{-2} s^{-1} and a day/night temperature of 25/20 °C. Relative humidity varied between 40 and 80% and seedlings were watered every other day. After 28 days, seedlings were lifted, cleaned from the medium and all new roots protruding more than 1 cm from the root plug were excised. The RGC of each seedling was determined as total dry mass of new roots.

Transplanting performance

Transplanting survival and growth of seedlings subjected to the different hardening treatments were assessed under xeric and mesic conditions in a ventilated greenhouse 15 days after the end of the hardening period. Eight 0.6 m^3 containers (1 m^2 surface and 0.6 m depth) were filled with a 1:1 (v/v) mixture of sand and peat. In each container, all treatments were randomly assigned to one of seven rows of eight seedlings. After transplanting, all containers were irrigated and half (n = 4) were assigned to a mesic environment and the remainder to a xeric environment. The experimental design was a split plot with four blocks. Soil water content in the mesic environment was maintained between 20 and 25%, whereas containers in the xeric environment were watered when soil water content was reduced to 3%. The latter value represented severe water stress as many seedlings showed symptoms of foliar necrosis. Soil water availability in both environments was assessed weekly by time domain reflectometry (Trime System I, Soil Moisture Equipment, Santa Barbara, CA). A waveguide 20 cm in length was inserted vertically in the middle of each container with the upper part of the waveguide remaining at 15 cm depth. First irrigation in the xeric planting environment was made 5 months after planting. Seedling height and root collar diameter were measured 5 weeks after planting and again at the end of the first growing season in early October 1997. Stem volume (mm^3) reflected seedling shoot size and was calculated as $0.33\pi(0.5 \times \text{root collar diameter})^2 \times \text{shoot height}$, assuming the stem to be a cone. Growth was defined as relative growth rate (RGR, $month^{-1}$) of stem volume and determined as $(\ln SV_2 - \ln SV_1)/(t_2 - t_1)$, where SV_1 and SV_2 are the stem volume of seedlings measured 1.5 months (t_1) and 10 months after transplanting (t_2), respectively.

Data analyses

All data, except EL, g_s and transplanting performance, were analyzed by a two-way analysis of variance (ANOVA) with a hanging control group. We considered results significant when $P \leq 0.05$. The two factors considered were drought hardening (L, M and S) and hardening duration (2.5 and 3.5 months). The least significance difference (LSD) method was employed for multiple comparisons of means. Planned comparisons assessed the differences between the hanging control group and other treatments. For the statistical analyses of morphology, nutrient and storage carbohydrate concentration data, the experimental unit was the block; however, its influence on variables was not assessed. For the remainder of seedling variables, the experimental unit was the seedling.

Analysis of g_s data was made by two-way ANOVA where hardening treatment (7 levels) and measurement date (3 levels) were the main factors. Differences in plasmalemma stability

were analyzed by comparing EL of seedlings in hardening treatments at a particular water content. For each treatment, a quadratic predictive model relating EL (dependent variable) and WC (independent variable) was fitted. Coefficients of determination of fitted quadratic models ranged from 0.67 to 0.96. A predicted EL value and its confidence interval were estimated at 65% WC, which was the WC limit at which seedlings started to die (about −5.5 MPa). Confidence intervals were utilized to calculate the standard error of each EL prediction and thus assess, by minimum significant difference (*t*-method for unequal sample sizes), if EL differences among treatments were statistically significant (Sokal and Rohlf 1995). Mortality in the xeric transplanting was evaluated by ANOVA, the factors being block (4 levels) and hardening treatment (7 levels). We evaluated the RGR data by a two-way ANOVA, the experimental design being a split plot with four blocks. Transplanting environment (xeric and mesic) was the main plot factor, and hardening (7 levels) was applied to subplots. Statistical analyses were performed with STATISTICA 5.1 (StatSoft, Tulsa, OK).

Results

Water relations and gas exchange

Seedlings in the M hardening treatment (M seedlings), especially those conditioned for 2.5 months, had a significantly lower $\Psi\pi_{sat}$ and $\Psi\pi_{tlp}$ than C seedlings; no differences in these parameters existed between C and S and L seedlings (Table 1). Hardening treatments had no effect on RWC_{tlp}, ε and V_s/V_t. Before the start of the hardening treatments in mid-August, $\Psi\pi_{sat}$, $\Psi\pi_{tlp}$ and ε were –2.11, –2.43 and 20.1 MPa, respectively. A significant seasonal reduction occurred in these traits in C seedlings from mid-August to late November (0.40 and 0.79 MPa for $\Psi\pi_{sat}$ and $\Psi\pi_{tlp}$, respectively, $P < 0.001$; 4.5 MPa for ε, $P = 0.028$). In mid-August, RWC_{tlp} was 87.6% and there was no significant seasonal variation in this variable. The C and the $S_{2.5}$ seedlings had the highest EL values, whereas the $M_{2.5}$ and $L_{2.5}$ seedlings had the lowest. Seedlings in the latter treatments differed significantly from seedlings in the former treatments but did not differ from seedlings in the other hardening treatments (Table 1). Drought hardening reduced RT ($P = 0.019$), the main differences being between C and M and S seedlings. Neither RT nor any of the parameters obtained from the P–V curves was influenced by the length of the hardening period.

There was a significant interaction between measurement date and treatment on g_s ($P = 0.036$, Figure 1). The S seedlings had a lower g_s than C and L seedlings and seedlings hardened for 3.5 months had a higher g_s than seedlings hardened for 2.5 months. This pattern was apparent on Days 22 and 34 but not on Day 43, where C and $L_{3.5}$ seedlings had lower g_s values and $L_{2.5}$ seedlings had higher g_s values.

Morphology, root growth capacity, and nutrient and nonstructural carbohydrate concentration

Drought hardening did not affect seedling mass, but C seed-

Table 1. Components of plant water relations, electrolyte leakage (EL) and residual transpiration (RT) of holm oak seedlings subjected to different regimes and durations of drought hardening. Values are means ± 1 SE. Means with different letters are significantly different ($P \leq 0.05$). Abbreviations: $\Psi\pi_{sat}$ = osmotic potential at saturation; $\Psi\pi_{tlp}$ = osmotic potential at the turgor loss point; RWC_{tlp} = relative water content at turgor loss point; ε = modulus of elasticity; and V_s/V_t = symplasm volume fraction. Treatment abbreviations: low (L), moderate (M) and severe (S) drought hardening regimes.

Hardening regime	Duration (months)	$\Psi\pi_{sat}$ (MPa)	$\Psi\pi_{tlp}$ (MPa)	RWC_{tlp} (%)	ε (MPa)	V_s/V_t	EL (%)	RT (μmol kg^{-1} s^{-1})
Control	3.5	−2.60 ± 0.05 a	−3.22 ± 0.07 a	89.3 ± 0.84 a	15.4 ± 1.23 a	0.54 ± 0.033 a	28.7 ± 1.60 a	459 ± 42 a
L	2.5	−2.74 ± 0.07 ab	−3.43 ± 0.04 ab	90.3 ± 1.19 a	16.8 ± 1.87 a	0.55 ± 0.026 a	24.1 ± 1.05 b	479 ± 45 a
L	3.5	−2.64 ± 0.07 ab	−3.21 ± 0.08 ab	88.9 ± 1.02 a	16.3 ± 1.84 a	0.48 ± 0.018 a	26.6 ± 1.25 ab	418 ± 26 ab
M	2.5	−2.91 ± 0.11 b	−3.51 ± 0.09 b	88.0 ± 0.79 a	17.5 ± 3.60 a	0.58 ± 0.04 a	22.5 ± 0.67 b	368 ± 44 ab
M	3.5	−2.80 ± 0.08 b	−3.45 ± 0.06 b	88.9 ± 0.84 a	17.8 ± 1.79 a	0.61 ± 0.027 a	26.5 ± 0.87 ab	334 ± 28 b
S	2.5	−2.69 ± 0.07 ab	−3.29 ± 0.08 ab	89.9 ± 0.83 a	18.5 ± 2.06 a	0.55 ± 0.034 a	27.4 ± 1.35 a	379 ± 25 ab
S	3.5	−2.76 ± 0.06 ab	−3.34 ± 0.07 ab	90.0 ± 0.56 a	18.8 ± 1.56 a	0.58 ± 0.057 a	25.4 ± 1.35 ab	364 ± 30 b

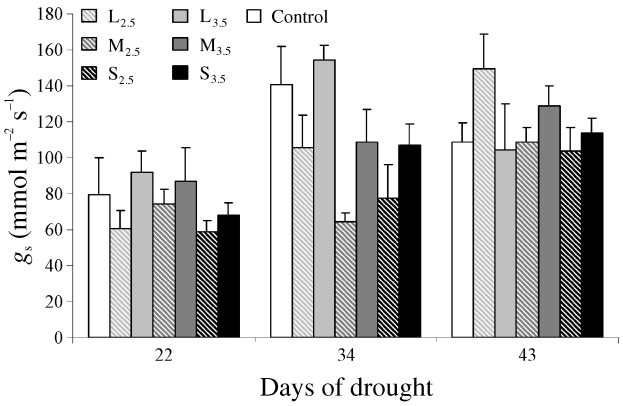

Figure 1. Stomatal conductance to water vapor (g_s) measured on three dates after holm oak seedlings were subjected to drought hardening. Seedlings were subjected to four drought hardening regimes (control, low (L), moderate (M) and severe (S)) for 2.5 or 3.5 months. Columns are means and error bars represent one SE (n = 5).

lings had higher masses than L and S seedlings, although there were no differences between C and M seedlings (Table 2). Hardening duration did not affect seedling size. Root mass was considerably higher than shoot mass in all treatments (data not shown) resulting in a low *S*/*R*. Hardening had no effect on *S*/*R* and no significant difference in *S*/*R* was observed between C seedlings and seedlings in the other hardening treatments. Hardening duration, however, increased *S*/*R* in M and S seedlings but not in L seedlings (drought hardening × hardening duration interaction, P = 0.047). Specific leaf mass was unaffected by either hardening intensity or its duration. Control seedlings had a higher RGC than L, M and S seedlings, though no differences were found among seedlings in the L, M and S treatments. Hardening duration had no influence on RGC.

Of the shoot mineral nutrients measured, K concentration was the highest and Na the lowest (data not shown). No significant effect of hardening was found on shoot nutrient pool (K + Na + Mg) concentration, but duration of hardening reduced mineral nutrient concentration (P < 0.001) (Table 2). Shoot SC was significantly reduced by hardening (P = 0.013) only in S seedlings. Duration of hardening did not affect SC. Shoot starch was significantly lower in M and L seedlings than in C and S seedlings. However, there was a marginally significant interaction between drought hardening and its duration on shoot starch concentration (P = 0.057). Thus, an increase in hardening duration reduced shoot starch concentration, but the response was greater in S seedlings than in M and L seedlings. Accumulation of nonstructural carbohydrates in the root was unaffected by either hardening regime or duration of hardening (Table 2).

Relationships between variables

Significant negative linear relationships were found between $\Psi\pi_{tlp}$, EL and RT and osmotic adjustment (Figure 2). Root growth capacity was also negatively related to osmotic adjustment but the relationship was curvilinear. Shoot SC and mineral solute concentration were not related to either osmotic adjustment (r^2 = 0.008, P = 0.54 for SC and r^2 = 0.007, P = 0.56 for mineral solutes) or EL (r^2 = 0.10, P = 0.48 for SC and r^2 = 0.36, P = 0.16 for mineral solutes). Stomatal conductance was positively related to RT (Figure 3).

Transplanting performance

Seedlings in the xeric environment began to show symptoms of foliage necrosis 5 months after planting. No mortality occurred in the mesic environment, whereas mortality in the xeric environment varied from 12.5% in the $M_{2.5}$ and $S_{2.5}$ seedlings to 3.0% for the C and L seedlings, although the differences were not statistically significant. The RGR of seedlings in the xeric environment was half of that of seedlings in the mesic environment (P = 0.002). Hardening treatments did not affect RGR (Figure 4).

Table 2. Morphology, new root growth capacity (RGC) and concentration of mineral nutrients and storage carbohydrates in holm oak seedlings subjected to different regimes and durations of drought hardening. Values are means ± 1 SE. Means with different letters indicate significant differences ($P \le 0.05$). Significant differences ($P \le 0.05$) between hardening durations are indicated with an asterisk.

	Drought hardening regime				Drought hardening duration	
	Control	Low	Moderate	Severe	2.5 months	3.5 months
Seedling mass (g)	5.20 ± 0.43 a	4.19 ± 0.24 b	4.78 ± 0.24 ab	4.38 ± 0.19 b	4.25 ± 0.19	4.66 ± 0.18
Shoot/root	0.54 ± 0.091 a	0.61 ± 0.019 a	0.60 ± 0.033 a	0.61 ± 0.049 a	0.56 ± 0.022	0.65 ± 0.025 *
Specific leaf mass (g m^{-2})	176 ± 5.5 a	173 ± 5.6 a	177 ± 5.2 a	168 ± 5.8 a	170 ± 2.7	176 ± 5.6
RGC (mg)	298 ± 30.6 a	204 ± 12 b	174 ± 14.4 b	197 ± 16.8 b	183 ± 11.3	200 ± 12.4
Shoot mineral nutrient (mg g^{-1})	6.7 ± 0.59 a	8.0 ± 0.56 a	7.2 ± 0.42 a	7.8 ± 0.33 a	8.4 ± 6.91	6.9 ± 0.22 *
Shoot soluble carbohydrates (mg g^{-1})	75.9 ± 2.48 a	71.7 ± 3.25 a	76.9 ± 4.12 a	62.2 ± 2.35 b	50.2 ± 5.6	48.9 ± 4.2
Shoot starch (mg g^{-1})	27.0 ± 5.74 a	21.8 ± 2.32 b	16.6 ± 1.21 b	35.3 ± 5.94 a	29.0 ± 4.71	20.2 ± 2.48 *
Root soluble carbohydrates (mg g^{-1})	56.6 ± 2.08 a	41.5 ± 6.00 a	56.6 ± 2.31 a	50.5 ± 7.36 a	50.2 ± 5.60	48.9 ± 4.2
Root starch (mg g^{-1})	115 ± 29.2 a	123 ± 22.0 a	104 ± 8.5 a	131 ± 19.3 a	132 ± 16.6	106 ± 10.1

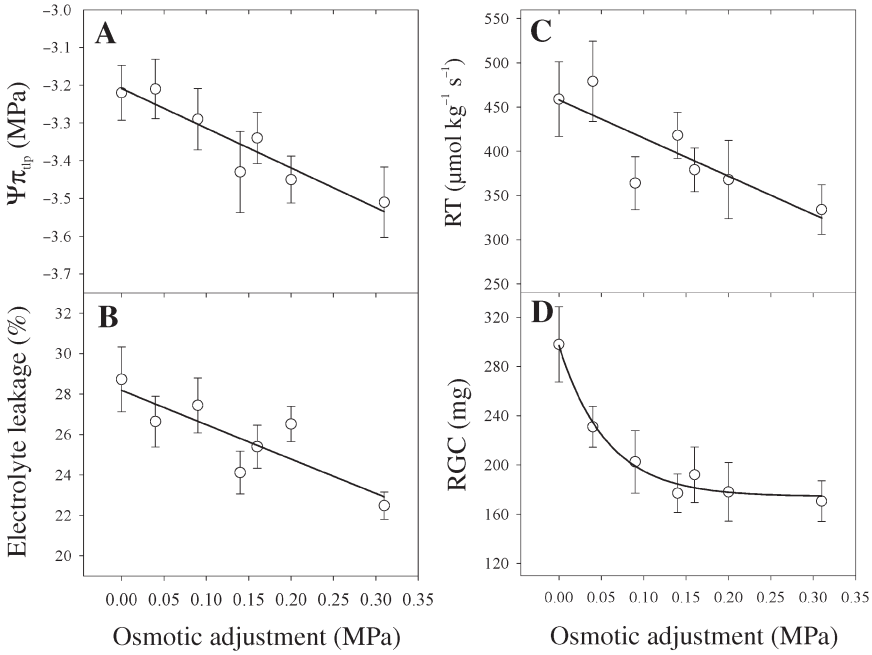

Figure 2. Relationships between osmotic adjustment in holm oak seedlings and: (A) osmotic potential at the turgor loss point ($\Psi\pi_{tlp}$); (B) electrolyte leakage estimated at 65% water content; (C) residual transpiration (RT); and (D) new root growth capacity (RGC). Error bars represent 1 SE. Regression equations are: (A) $y = -3.208 - 1.0529x$ ($r^2 = 0.87$, $P = 0.002$); (B) $y = 28.187 - 17.002x$ ($r^2 = 0.71$, $P = 0.017$); (C) $y = 458.01 - 430.96x$ ($r^2 = 0.70$, $P = 0.019$); and (D) $y = 174.27 + 122.66(e^{(-17.761x)})$ ($r^2 = 0.98$, $P < 0.001$).

Discussion

Drought hardening applied in the nursery increased drought tolerance of holm oak seedlings primarily by affecting physiological traits rather than morphological attributes. Hardening promoted osmotic adjustment, reduced $\Psi\pi_{tlp}$, RT and g_s, and enhanced plasmalemma stability. These responses were most prominent in M seedlings. Although the L drought-hardening treatment was probably too mild to induce a response, the S treatment may have inhibited drought tolerance enhancement as reported previously (Gebre and Khuns 1993).

Seedlings subjected to $M_{2.5}$ drought-hardening attained the highest osmotic adjustment at 0.31 MPa. Similar osmotic adjustment values have been reported for two natural populations of holm oak differing in drought stress (Sala and Tenhunen 1994); however, this value was lower than those reported for many North American oak species, which typically adjust at 0.4–0.5 MPa (Abrams 1990). Independent of hardening

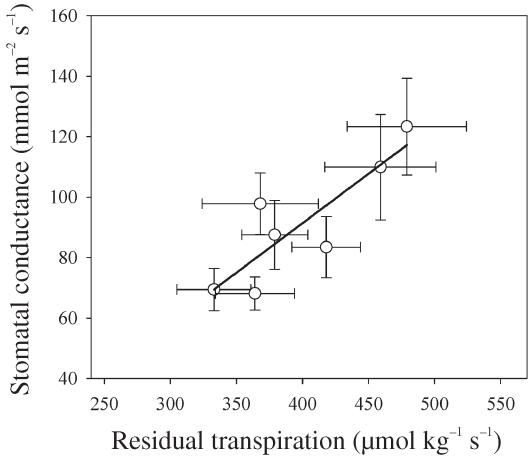

Figure 3. Relationship between stomatal conductance to water vapor and residual transpiration in holm oak seedlings. Vertical and horizontal error bars represent 1 SE. Regression equation: $y = -39.467 - 0.3271x$ ($r^2 = 0.74$, $P = 0.013$).

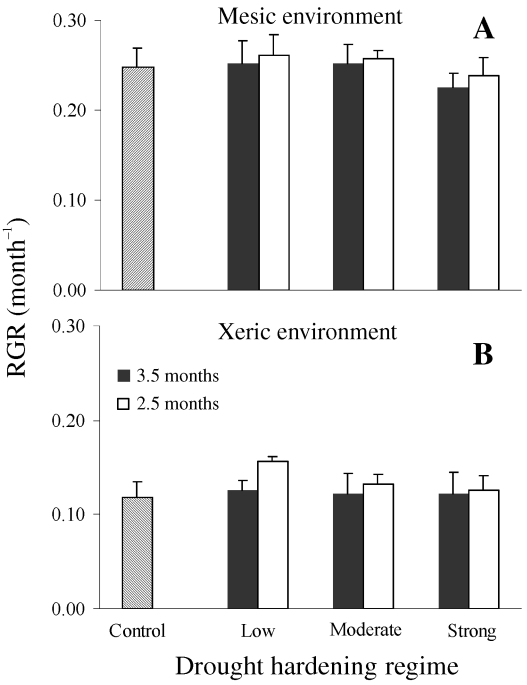

Figure 4. Stem volume relative growth rate (RGR) of drought-hardened holm oak seedlings 10 months after being planted in (A) a mesic or (B) a xeric environment. Seedlings were subjected in the nursery to four drought-hardening regimes (control, low, moderate and severe) for 2.5 or 3.5 months before transplanting. Columns are means and error bars represent 1 SE. No significant differences were detected among any treatment in either the mesic or xeric environment.

treatment, seedlings exhibited a seasonal osmotic adjustment of 0.49 MPa that was not induced by drought, as inferred by the reduction in $\Psi\pi_{sat}$ of C seedlings from midsummer to late November. Thus, drought hardening had an additive effect on seasonal osmotic adjustment reducing $\Psi\pi_{sat}$ by 50% in M seedlings. Seasonal osmotic adjustment, which is not induced by water stress, is primarily attributed to ontogenetic changes or cold hardening during autumn, or both (Bigras et al. 2001). Because seedlings in the various treatments did not differ in symplasm volume fraction, osmotic adjustment was a consequence of active solute accumulation. In *Quercus petraea* Liebl and *Q. robur* L., SC and K, respectively, play important roles in osmotic adjustment (Épron and Dreyer 1996, Vivin et al. 1996). We found that osmotic adjustment was unrelated to either shoot SC or mineral solute concentration. Two reasons might explain these results. First, SC may contribute to osmotic adjustment in holm oak but not all compounds that form the SC pool contribute. The increase of some osmotically active SC could be offset by a reduction in others (Green et al. 1994), explaining the absence of a relationship between $\Psi\pi_{sat}$ and SC observed in our study. Second, compounds other than SC, such as amino acids or organic acids, may be the primary contributors to osmotic adjustment (Tschaplinski and Tuskan 1994).

Variations in $\Psi\pi_{tlp}$ can be attributed to changes in $\Psi\pi_{sat}$ or ε or both (Turner 1986). Our treatments did not significantly affect ε, so the reduction in $\Psi\pi_{tlp}$ in M seedlings was attributed to a diminution in $\Psi\pi_{sat}$ (Figure 2). A decrease in $\Psi\pi_{tlp}$ may enable seedlings to maintain gas exchange and cell elongation at lower water potentials (Bennet and Sullivan 1981). Plasmalemma stability was enhanced by drought hardening, particularly for $L_{2.5}$ and $M_{2.5}$ seedlings. A similar result was reported for *Sorghum bicolor* L. Moench (Premachandra et al. 1992), but this response was not observed in nursery-grown drought-hardened seedlings of *Pinus halepensis* Mill. (Villar-Salvador et al. 1999). The M and S drought-hardened seedlings showed a reduction in RT relative to C seedlings. Water stress also reduced RT in two Mediterranean pines (Rook 1973, Villar-Salvador et al. 1999), but no drought-induced reduction in RT was observed in three Mediterranean shrubs (Vilagrosa et al. 2003). A lower RT might be attributed to a greater resistance to water vapor diffusion through the external epidermal walls as a result of enhanced cuticle thickness or deposition of hydrophobic compounds, or both (Premachandra et al. 1992). Increase in plasmalemma stability and reduction in RT occurred in parallel to osmotic adjustment (Figure 2). The increase in plasmalemma stability with osmotic adjustment is consistent with previous studies (Gebre et al. 1994) and has been attributed to accumulation of SC (Santarius 1973), which concomitantly reduces $\Psi\pi_{sat}$. However, we found no relationship between EL and SC concentration, reinforcing the contention that individual sugars in holm oak, or other compounds such as organic acids, may be more important in osmotic adjustment and enhancing plasmalemma stability than the total concentration of soluble sugars.

Stomatal conductance following drought conditioning was lower in M seedlings and particularly lower in S seedlings than in C and L seedlings. A reduction in g_s after drought recovery has been reported for other species (Zwiazek and Blake 1989) and can be explained by a lower hydraulic conductance or changes in stomatal behavior induced by ABA accumulation during the drought period or both (Turner 1986, Rieger 1995). In contrast, van den Driessche (1991*b*) observed no effect of drought hardening on g_s in three conifer species. We found that g_s was positively related to RT (Figure 3) indicating that seedlings exhibiting lower stomatal transpiration also had reduced transpiration after stomatal closure. These responses might help drought-conditioned seedlings to expend less water and maintain better water status when transplanted to the field.

The hypothesis that a longer period of hardening would increase drought tolerance was not supported by our results. Most variables were unaffected by the length of the hardening period. Only *S*/*R*, mineral nutrients and g_s were influenced and they increased with increasing hardening duration. Albouchi et al. (1997) reported that osmotic adjustment in *Acacia cyanophylla* Lindl. occurred only when seedlings were subjected to more than 3 months of drought. In holm oak, a period longer than 3.5 months might be needed to enhance drought tolerance.

Formation of new roots is critical for seedling establishment after transplanting (Kaushal and Aussenac 1989). Root growth capacity is considered a measure of seedling vigor. Low RGC reflects poor functional integrity and therefore RGC frequently predicts outplanting performance of seedlings (Simpson and Ritchie 1997). Tinus (1996) affirmed that new root growth is sensitive to the drought stress history experienced by the seedling. This contention is supported by our results where hardened seedlings produced fewer new roots than the C seedlings. Negative effects of drought hardening on RGC have also been observed previously (Kaushal and Aussenac 1989, Villar-Salvador et al. 1999). We found that RGC was negatively associated with osmotic adjustment. This finding does not support the hypothesis of Ritchie (1985), who proposed that RGC predicts outplanting performance because it is positively related to drought and frost resistance of seedlings. The negative relationship between RGC and osmotic adjustment observed in this study together with the results of previous studies suggests that there may be a trade-off between drought tolerance and root growth capacity.

Transplanting results did not support our hypothesis that drought hardening would enhance seedling survival and growth under xeric conditions (Figure 4). The treatments had no significant effect on survival and growth of the seedlings even though they differed in drought tolerance. This finding contrasts with results reported by van den Driessche (1991*a*) that drought hardening increased seedling survival in two conifer species planted under xeric conditions. Similarly, Arnott et al. (1993) found that drought-hardened yellow cypress (*Chamaecyparis nootkatensis* (D. Don) Spach) steklings grew faster under both xeric and mesic conditions than unhardened seedlings. The lack of improved survival and growth of our drought-hardened holm oak seedlings may be associated with the loss of physiological differences among seedlings in the various treatments several weeks after hardening, because at 2.5 months after the end of the hardening period, no differences in $\Psi\pi_{sat,}$

$\Psi\pi_{tlp}$ and RT existed among the treated seedlings (Villar-Salvador et al. 1998). Reversibility of physiological traits might be accelerated when seedlings are not limited by water immediately after transplanting as probably occurred in the xeric environment in our experiment, where irrigation was first applied five months after transplanting. Morphological traits, such as low *S*/*R*, might confer resistance to drought (van den Driessche 1991*a*) but, unlike physiological traits, they are not reversible in the short term; however, our treatments had no effect on either *S*/*R* or SLM.

We conclude that drought hardening, especially of moderate intensity, increases drought tolerance of holm oak seedlings. Enhancement of drought tolerance resulted in reductions in both $\Psi\pi_{tlp}$ and RT and an increase in plasmalemma stability, and these changes occurred in parallel with osmotic adjustment. Moderate and severe conditioning also reduced stomatal conductance and new root growth after drought stress recovery. Drought conditioning did not improve transplanting performance under either xeric or mesic conditions. The potential performance advantage in the field of drought-hardened holm oak seedlings is probably limited to those conditions where seedlings experience water stress immediately after planting.

Acknowledgments

We are grateful to Professor Jose A. Pardos of the Universidad Politécnica de Madrid for the use of the growth chamber. Help of Doris Barreto and Francisco Artero with RGC determination and P–V curves is appreciated. Comments made by Pilar Castro and Fernando Valladares improved an early version of the manuscript. The research was supported by funds of the Ministerio de Medio Ambiente and of the Projects SC94-111 (INIA), Ren2000-0163-P4-05 and AGL-2001-1061 (Ministerio de Ciencia y Tecnología).

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