Autumn fertilization of Quercus ilex ssp. ballota (Desf.) Samp. nursery seedlings: effects on morpho-physiology and field performance

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ORIGINAL PAPER

Autumn fertilization of *Quercus ilex* ssp. *ballota* (Desf.) Samp. nursery seedlings: effects on morpho-physiology and field performance

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Abstract
• ***Background*** The Holm oak (*Quercus ilex* ssp. *ballota* [Desf.] Samp.) is an evergreen tree widely distributed in the western Mediterranean Basin. Forest restoration programs using this species have enjoyed only limited success, and knowledge concerning the effect of fertilization on plant quality and post-transplantation response is sparse.
• ***Methods*** We assessed the effect of autumn fertilization using different doses of nitrogen, phosphorus, and potassium (70.0 mg N, 30.5 mg P and 58.1 mg K during the growing phase for all plants; and 30.0 vs 1.5 mg N, 13.1 vs 0.3 mg P and 24.9 vs 0.5 mg K during the hardening phase, depending on the fertilization treatment) on the seedling characteristics and field performance of Holm oak.
• ***Results and Conclusions*** Autumn fertilization, especially with N, did not decrease plant quality but improved overall growth, root growth capacity, cold hardiness, and the nutritional content of nursery-grown seedlings. However, autumn fertilization had only a small effect on field performance, which was affected only by K fertilization, probably because of the adequate N and P nutrient status of all the plants and the mild weather conditions of the field plot. In our site, which had a mild winter climate, late autumn out-planting was more successful than was mid-winter out-planting.

Keywords Holm oak · Nursery fertilization · Cold hardiness · Nutritional status · Field performance

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1 Introduction

Quercus ilex L. (Holm oak) is a late successional evergreen tree that dominates many types of woodlands in the western Mediterranean Basin (Ruiz de la Torre 2006), and is widely used for forest restoration in such ecosystems (Rodà et al. 1999). Nevertheless, this species shows a poor early out-planting performance compared to other Mediterranean species, particularly in sites with unfavorable climatic conditions (Pausas et al. 2004). This has been attributed to water stress (Villar-Salvador et al. 2004a, b), low site fertility (Pardos et al. 2005; Sanz-Pérez et al. 2007; Valdecantos et al. 2006), and poor seedling quality (del Campo et al. 2010). This indicates that Holm oak is very vulnerable to environmental stress during early life; this is especially true for the form *Q. ilex* ssp. *ballota* (Desf.) Samp., which occurs mainly in continental and inland areas (Ruiz de la Torre 2006).

Nursery practices, environmental conditions, and genetic factors affect the functional characteristics of seedlings and field performance after transplantation (Birchler et al. 1998). Manipulation of nutrient availability is one of the most powerful tools modifying plant characteristics (Puttonen 1997). However, its effect on seedling quality and field performance of Mediterranean *Quercus* spp. still remains uncertain (Broncano et al. 1998; Villar-Salvador et al. 2004a, 2005; Oliet et al. 2009a; Trubat et al. 2010).

During the hardening phase in the nursery, environmental factors (including chill temperature, short photoperiod, irrigation, and fertilization) can induce plant dormancy and improve hardiness (Colombo et al. 2003; Fernández et al. 2008). For instance, Holm oak seedlings have reached a typical cold hardiness (temperature causing 50 % leaf tissue damage) between −13° and −19°C, depending on the

growing conditions and seed provenance (Fernández et al. 2008; Heredia et al. 2009; Mollá et al. 2006). Morin et al. (2007) reported the 50 % tissue damage at −27°C, but this was measured in stem segments, not in leaf tissues. Some studies have found considerable cambium and root growth during this phase, causing dilution of tissue nutrients if additional amounts are not provided (Boivin et al. 2002). Therefore, fertilization during this phase could increase the level of reserves without significantly altering morphology, improving post-transplantation response in some instances (Puertolas et al. 2003; Rikala et al. 2004), but not in other ones (Birchler et al. 2001).

Nitrogen, phosphorus, and potassium are the three primary plant macronutrients that can improve, respectively, photosynthetic rate, root growth, and water use efficiency (Lambers et al.; 1990; Fernández et al. 2006); post-transplantation root growth (Oliet et al. 2005); and stomatal control, osmotic adjustment, and tolerance to drought and cold temperatures (Bogeat-Triboulot and Lévy 1998). Some workers have suggested that the relative doses of these nutrients could be more important than is the total dose (Fernández et al. 2003).

The three major objectives of the current study, with *Quercus ilex* ssp. ballota seedlings subjected to different N–P–K doses during autumn fertilization, were to determine: (i) the morphological and nutritional status at the end of the nursery period, (ii) the effect of autumn fertilization on cold hardiness and root growth capacity on two dates (late autumn and mid-winter), and (iii) the effect of changes in plant characteristics resulting from autumn fertilization on field performance.

2 Materials and methods

2.1 Seedling growth conditions

Early in February 2007, acorns of *Quercus ilex* ssp. *ballota* (Desf.) Samp. collected in the Spanish provenance region of Sierra Morena Occidental were pre-germinated in a growth chamber at 20°C, on wet perlite. Pre-germination was performed to reduce germination time and intra-population variability. On the third week of February 2007, 1,395 healthy acorns were randomly sown in 300 cm^3 Plasnor® containers (45 containers per tray, 31 trays) containing sphagnum peat Kekkilä® B0 (with pH corrected to 6.0 using Dolokal®). Seedlings were grown in a greenhouse until May 2007, and next moved to a nursery under shadecloth that reduced radiation by 50%. All trays were well-watered, moved weekly, and rotated to eliminate microsite effects during all the study period.

During the first 28 weeks, a constant fertilization regimen was applied using the water-soluble fertilizer Peters Professional® 20–20–20 at a solution rate of 125 ppm N, 54 ppm P and 104 ppm K, with each seedling receiving a weekly dose of 2.500 mg N (3.94% ammoniacal nitrogen, 6.05% nitrate nitrogen, 10.01% urea nitrogen, all w/w), 1.088 mg P and 2.075 mg K (monoammonium phosphate, monopotassium phosphate and potassium nitrate), corresponding to 70.0 mg N, 30.5 mg P, and 58.1 mg K over the entire 28-week period. The desired amount of fertilizer was added to each seedling once per week dissolved in distilled water, and between two consecutive fertilizer application dates, plants were watered as needed with tap water. At the end of the first 28 weeks, seedling characteristics were: height (15.23±0.87 cm), stem diameter (3.03±0.16 cm), shoot dry weight (1.74±0.13 g), root dry weight (4.02±0.26 g), shoot-to-root dry weight ratio (0.43±0.03), and 0.93 %N, 0.09 %P, 0.56 %K in leaves. For the next 12 weeks (from the first week of October to the third week of December), during the hardening phase, the doses of N, P and K were modified by a balanced three-way experiment, using three nutrient effects (*N*, *P*, *K*) with two levels each: *N* (N_1, $N_{1/20}$), *P* (P_1, $P_{1/50}$), and *K* (K_1, $K_{1/50}$). Therefore, eight different fertilization treatments were tested (Table 1), and 120 randomly chosen seedlings received each treatment (30 plants per treatment and tray). Plants showed new sprouts since late summer to the end of October, without significant differences between treatments in the number of sprouted plants. From early November onwards, no plant showed visual signs of shoot growth. The doses of N_1, P_1, and K_1 were the same as in the previous 28 weeks, and close to those typically recommended in forest nurseries for Holm oak during the growing phase (Navarro et al. 2009), although nursery fertilization programs still need to be refined for this species (Jacobs et al. 2009). The low nutrient doses ($N_{1/20}$, $P_{1/50}$ and $K_{1/50}$) were chosen because we tried to avoid total nutrient deprivation (Boivin et al. 2002) but to

Table 1 Total amounts of N, P and K applied to each seedling during the 12-week hardening phase of the study period

Nutrient treatment				mg during last 12 weeks		
				N	P	K
1	N_1	P_1	K_1	30.0	13.1	24.9
2	N_1	$P_{1/50}$	K_1	30.0	0.3	24.9
3	N_1	P_1	$K_{1/50}$	30.0	13.1	0.5
4	N_1	$P_{1/50}$	$K_{1/50}$	30.0	0.3	0.5
5	$N_{1/20}$	P_1	K_1	1.5	13.1	24.9
6	$N_{1/20}$	$P_{1/50}$	K_1	1.5	0.3	24.9
7	$N_{1/20}$	P_1	$K_{1/50}$	1.5	13.1	0.5
8	$N_{1/20}$	$P_{1/50}$	$K_{1/50}$	1.5	0.3	0.5

apply a significant low dosage (our previous unpublished experiences with this species showed us that 1/5 to 1/10 N doses and 1/5 to 1/20 P and K doses did not result in significant differences in seedling growth and leaf nutritional status).

2.2 Morphological and nutritional status

At the end of December 2007, 12 plants per treatment were randomly selected (three seedlings per tray) for assessment of morphological and nutritional status. Shoots were cut at the cotyledon insertion point, leaves and stems were separated, and roots were cleaned. Shoot height was measured, and stem diameter was assessed 0.5 cm above the cotyledon insertion point. Next, all samples were washed with distilled water, oven-dried at 65°C until no further weight reduction was observed, and the dry weights of leaves (LDW), stems (StDW), and roots (RDW) were measured. Shoot dry weight (ShDW = LDW+StDW) and the shoot-to-root dry weight ratio (SRDW = ShDW/RDW) were calculated.

To obtain samples sufficiently large for nutrient analysis, four groups (of three plants per group) were chosen. 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 5 M HCl, and analyzed by ICP-OES Jobin Yvon Ultima 2 (Tokyo, Japan). Nonstructural carbohydrates (NSC) were also determined: hydroalcoholic extraction and anthrone colorimetry (using a spectrophotometer; UV-1601, Shimadzu ®, Tokyo, Japan) was used to determine soluble sugars (SS) (Spiro 1966); acid hydrolysis followed by anthrone colorimetry was employed to analyze starch (St) (Rose et al. 1991). Nutrient and NSC contents (in mg) were calculated as the products of concentration (%) and dry weight (g). The same plant material was used for analyzing N, P, K, SS and St (leaves and roots), and also stems for nutrient analyses.

2.3 Frost tolerance and root growth capacity

A detached leaf test was conducted to evaluate cold hardiness, as previously described for *Eucalyptus globulus* L. (Fernández et al. 2007). This test allows collection of data without sacrifice of the whole plant, and allows several experiments to be conducted at different temperatures using leaves of the same plant. Selected leaves were inserted into test tubes (2.2 cm in diameter, 15 cm in length), and placed in a freezer featuring temperature programming (West ® 4400; ISE Inc., Cleveland, OH, USA); two internal fans were used to remove air. The test commenced at 12°C, and the temperature was next reduced by 3°C h^{-1} to the minimum temperature evaluated. The temperature was maintained at the minimum for 3 h, and next increased by 5°C h^{-1} to 12°C. A complete cycle lasted 14–16 h. After completion of temperature cycling, leaves were removed from the freezer, distilled water (≤ 2 ml) was added to each test tube until the cross-section of the short petiole was immersed, and samples were maintained in a growth chamber (25°Cday/17°C night; 12 h photoperiod; ≥ 70% relative humidity; 350 μmol m^{-2} s^{-1} photosynthetic photon flux). Damage was visually assessed at 4 h and 24 h after the end of the freezing test, by estimation of the leaf percentage that suffered from visual damage (VD). VD was estimated as the percentage of leaf surface which suffered cell lysis (recognized by a particular dimming and/or browning of the leaf) after the freeze. Chlorophyll fluorescence parameters were simultaneously measured, in the same leaves on which VD was measured, using a portable fluorometer (Fim 1500; ADC, London, UK). For fluorescence measurements, leaves were maintained in darkness for 20 min, and minimal fluorescence from antenna pigments (Fo), the maximal fluorescence (Fm), the variable fluorescence (Fv=Fm - Fo), and the Fv/Fm ratio were then determined.

The tests were performed in the third week of December 2007 and the first week of February 2008. In December 2007, two fully expanded leaves per temperature and plant (one from the medium-higher part of the shoot and other from the medium-lower part), and four plants per treatment, were sampled, and tested at temperature minima of −6°C, −7°C, −8°C, and −10°C. In February 2008, two leaves per plant and three plants per treatment were sampled and tested at temperature minima of −7°C, −8°C, and −10°C. The chilling hours (≤ 8°C) accumulated in the nursery at the time of the freezing tests were 425 (December) and 950 (February).

Root growth capacity (RGC) was assessed in December 2007 (12 plants per treatment) and February 2008 (eight plants per treatment). Seedlings were carefully removed from containers, the white root tips emanating from the plug were cut, and seedlings were planted with undamaged plugs in 2.5 l pots containing wet perlite. Pots were randomly distributed on a heating table (20°C) inside a greenhouse for 4 weeks. Seedlings were irrigated every 2–3 days and received no fertilization. After 4 weeks, seedlings were cleaned, and the new fine root weight (FRW; roots<2 mm diameter) and thick root weight (ThRW; roots>2 mm diameter) of plugs were measured.

2.4 Field performance

Planting was conducted in an experimental flat plot at the University of Huelva (37º12´N, 6º54´W; 10 m above sea

level) on 22 December 2007 and 15 February 2008, using 20 randomly selected plants per treatment per planting date. The field plot was outwardly homogeneous; nevertheless, the seedlings were planted in ten lines, with a separation of 1 m between plants and 2 m between lines. In each line, two plants per treatment and planting date were randomly distributed. The effect of line was not significant, so it was excluded from the subsequent statistical analysis. During the study period, the mean temperature was 16.5°C, and the mean maximum temperature of the hottest month and mean minimum temperature of the coldest month were 29.9°C and 4.8°C. Absolute maximum and minimum temperatures were 36.5°C and −3.0°C respectively. Mean annual rainfall during the study period was 484 mm, and the summer drought period extended for 4 months. Seedlings were planted, and height, stem diameter, and vigor were measured on five dates over the course of 2 years. Vigor was characterized using a scale from 0 to 3 (0, dead; 1, more than 50% defoliation or dry leaves; 2, less than 50% defoliation; and 3, completely healthy), and was assessed before commencement of the dry season (4 July 2008) and well after the dry season (2 December 2008). Stem diameter growth (SDI) and height growth (HI) were used to estimate overall plant growth from the time of planting. SDI and HI were calculated as diameter or height measured on a particular date, divided by the days that had elapsed between consecutive measurements. Predawn leaf water potential (Ψ) was measured near the middle (July 15) and end (September 10) of the first summer after transplantation, which presumably represented the most challenging periods for seedlings, using a pressure chamber (Model 1000; PMS Instruments, Corvallis, OR). Measures were done in four randomly selected seedlings per treatment and planting date. Two fully expanded leaves per plant from the center of the main shoot were taken. The plot was not irrigated during the entire study period, and undesirable plants were periodically eliminated.

2.5 Data analysis

The effect of autumn fertilization on seedling morphology and nutritional status was assessed using the following general linear model (GLM):

$$y_{ijkl} = \mu + N_i + P_j + K_k + NP_{ij} + NK_{ik} + PK_{jk} + NPK_{ijk} + e_{ijkl} \quad (1)$$

where y_{ijkl} is the value of the dependent variable in plant, l fertilized at dose i of N (i=1, 2), a dose j of P (j=1, 2), and a dose k of K (k=1, 2); μ is the overall mean; N, P, and K are nutrient fixed effects; the double and triple terms represent interactions; and e is the error term for the hypothesis $e_{ijkl} \sim N(0, \sigma_e^2)$. When the dependent variable was a percentage, the transformation arcsin $\sqrt{\text{var}(\%)/100}$ was used. Significant differences between effects were assessed by Tukey's HSD test, with $\alpha = 0.05$. RGC analysis followed the same GLM approach, but included measurement dates (December, February); and all interactions with the terms of model 1.

For assessment of frost damage, the relationship between VD and Fv/Fm was assessed using the Pearson correlation coefficient. The influence of fertilization on VD was estimated by a mixed model:

$$\begin{aligned} y_{ijklmnoq} = {} & \mu + \alpha_j + d_k + t_l + h_m + N_n + P_o + K_q \\ & + th_{lm} + NP_{no} + NK_{nq} + PK_{oq} + tN_{\ln} \\ & + tP_{lo} + tK_{lq} + tNP_{\ln o} + tNK_{\ln q} + tPK_{loq} \\ & + thN_{lmn} + thP_{lmo} + thK_{lmq} + e_{ijklmnoq} \end{aligned} \quad (2)$$

where $y_{ijklmnoq}$ is the arcsin $\sqrt{VD(\%)/100}$ of leaf i of seedling j, tested on date k (December, February), at temperature l, measured after m hours (4 h, 24 h), at dose n of N, dose o of P, and dose q of K; μ is the overall mean; α is a random seedling effect; d is the test date fixed effect; t is the temperature fixed effect; h is the measurement time; N, P, and K are nutrient fixed effects; and e is the error term. The other terms describe interactions of the main effects. The initial variance–covariance structure regarded variances in observations at different temperatures and measurement times to be different, and also assumed that covariance in observations of the same leaf at different times of measurement were not null and different for each temperature.

Plant vigor in the field was analyzed with the dependent variable considered to reflect a nominal response, following a multinomial distribution. The following generalized linear mixed model, incorporating a cumulative logit link function, was employed:

$$\begin{aligned} \log\left(\frac{\Pr(y_{ijklmn}) \le t}{\Pr(y_{ijklmn}) > t}\right) = {} & \mu + \alpha_i + d_j + m_k + N_l + P_m \\ & + K_n + NP_{lm} + NK_{\ln} \\ & + PK_{mn} + dN_{kl} + dP_{km} \\ & + dK_{kn} + dNP_{lm} + dNK_{\ln} \\ & + dPK_{mn} + dm_{jk} + e_{ijklmn} \end{aligned} \quad (3)$$

where y_{ijklmn} is the vigor (scaled at 0, 1, 2, or 3) of seedling i planted on date j (December, February) evaluated on date k (July, December) with a dose l of N, a dose m of P, and a dose n of K; t is vigor (scaled at 0, 1, 2, or 3); α is a random seedling effect; d is the plantation date fixed effect; m is the evaluation date fixed effect; N, P, and K are the nutrient fixed effects; and e is the error term.

Analysis of growth of seedling height and diameter in the field was evaluated using the following model:

$$y_{ijklmn} = \mu + d_i + m_j + N_k + P_l + K_m + NP_{kl} + NK_{km} + PK_{lm} + mN_{jk} + mP_{jl} + mK_{jm} + dm_{ij} + mNP_{jkl} + mNK_{jkm} + mPK_{jlm} + e_{ijklmn} \quad (4)$$

where y_{ijklmn} is the diameter or height increase of seedling n planted on date i (December, February) and measured on date j (five measurements) with a dose k of N, a dose l of P, and a dose m of K; d is the plantation date fixed effect; m is the measurement date fixed effect; N, P, and K are the nutrient fixed effects; and e is the error term. The initial variance–covariance matrix was considered to be unstructured for observations of the same seedling.

The effect of fertilization on seedling water status was evaluated as described for model 1, but also considered planting date (December, February) and measurement date (July, September) as fixed effects and explored interactions of these two effects with the effects already present in model 1.

3 Results

3.1 Morphological and nutritional status

Autumn N fertilization had a significant effect on all measured morphological parameters (Table 2). Table 2 also shows that K_1 increased significantly both height and shoot weight. SRDW decreased during the hardening period for all treatments; nevertheless, N_1, P_1, and K_1 maintained higher SRDW values than $N_{1/20}$, $P_{1/50}$, and $K_{1/50}$, respectively. During the 12-week hardening phase, roots increased in dry weight between 25 and 56 %, depending on fertilization treatment, whereas shoots only increased between 6 and 34 %. Interactions between nutrients were not significant ($p>0.05$).

Table 3 shows that autumn P and K fertilization significantly increased leaf N concentration (1.03 %N [P_1], 0.88 %N [$P_{1/50}$], and 1.06 %N [K_1], 0.86 %N [$K_{1/50}$]), and that N fertilization elevated leaf P concentration (0.15 %P [N_1], 0.11 %P [$N_{1/20}$]). There were not more significant differences caused by the main effects, and the mean values for all treatments as a whole of the rest of tissue nutrient concentrations were: 0.75 %K for leaves; 0.47 %N, 0.11 %P and 0.65 %K for roots; and 0.44%N, 0.09 %P and 0.46 %K for stems. With regard to nutrient contents, autumn N and K fertilization significantly increased leaf N, P, and K content, and P fertilization increased leaf N content (Tables 2 and 3). N and K fertilization increased significantly root N content (28.4 mg N [N_1], 23.8 mg N [$N_{1/20}$], and 27.8 mg N [K_1], 24.5 mg N [$K_{1/50}$]). In the stem, only N fertilization significantly increased N content (3.7 mg N [N_1], 2.2 mg N [$N_{1/20}$]) and P content (0.7 mg P [N_1], 0.4 mg P [$N_{1/20}$]). The mean values for all treatments as a whole of the rest of tissue nutrient contents were 6.0 mg P and 36.0 mg K for roots, and 3.1 mg K for stems. Therefore, total seedling N content was about 48 mg and 35 mg for N_1 and $N_{1/20}$ treatments respectively. The N·P and P·K interactions were significant only for leaf N concentration and leaf N content ($0.001 \le p \le 0.027$). The N·K interaction was significant for leaf N concentration ($p<0.001$) and root N concentration ($p=0.029$); and for leaf, root, and stem N content ($0.001 \le p \le 0.010$). The significances of these interactions were attributable to the effect of the two doses of each applied nutrient; the effect of N was always greater than that of P or K (Table 2 shows an example of this). As a result, the N·P and N·K interactions indicated that it was necessary to apply higher doses of both nutrients (N_1 P_1, or N_1 K_1 respectively) to obtain an increase in leaf N concentration. Therefore, P and K fertilization also affected leaf N concentration. Leaf N level was also influenced by the P·K interaction, and seedlings with lower doses of both nutrients ($P_{1/50}$ $K_{1/50}$) had the lowest leaf N concentration, significantly less than plants given P_1 and K_1, P_1 and $K_{1/50}$, or $P_{1/50}$ and K_1.

Only N fertilization had a significant effect ($p=0.003$) on root and leaf SS concentration, and both were higher for seedlings given $N_{1/20}$ (5.05 % and 3.16 % respectively) than N_1 (3.60 % and 2.08 % respectively). Only root St content was significantly higher ($p=0.006$) after addition of N_1 (1423.3 mg) than $N_{1/20}$ (1064.2 mg). The effects of P and K and all nutrients interactions were not significant ($p>0.120$) for any NSC parameter.

3.2 Frost tolerance and root growth capacity

There were significant negative correlations between Fv/Fm and VD on both dates (December, February) and at both times of measurement (4 h, 24 h). These correlations were better for measurements at 24 h ($r = -0.924$, $p<0.001$, $n=400$) than at 4 h ($r = -0.894$, $p<0.001$, $n=400$). The results at 24 h showed that for Fv/Fm≤0.64, VD was always ≥ 50%; for Fv/Fm≥0.77, VD was ≤ 50%; and for 0.64<Fv/Fm<0.77, the values of VD were unpredictable, but always equal to or lower than 60%.

There were significant differences in VD at all tested temperatures, except between −6°C and −7°C, with lower temperatures causing more damage. The VD was higher in December than in February (30.4% and 22.0% respectively). Assessment at 24 h showed higher VD than at 4 h (37.8% and 15.8% respectively). The interaction of temperature and measurement time was

Table 2 Morphological values of seedlings and leaf nutrient contents in December 2007 of seedlings cultivated with different nutrient doses of N (N_1 and $N_{1/20}$), P (P_1 and $P_{1/50}$), K (K_1 and $K_{1/50}$). For each parameter and nutrient treatment, different letters mean significant differences ($p<0.05$) between nutrient doses.

H: height, *D*: stem diameter, ShDW: shoot dry weight, RDW: root dry weight, SRDW: shoot-to-root dray weight ratio

	N		P		K	
	N_1	$N_{1/20}$	P_1	$P_{1/50}$	K_1	$K_{1/50}$
H (cm)	18.62 a	14.77 b	16.49 a	16.90 a	17.66 a	15.73 b
D (mm)	3.95 a	2.97 b	3.37 a	3.55 a	3.46 a	3.45 a
ShDW (g)	2.33 a	1.49 b	1.84 a	1.78 a	2.05 a	1.78 b
RDW (g)	6.27 a	5.04 b	5.66 a	5.64 a	5.85 a	5.46 a
SRDW	0.38 a	0.30 b	0.35 a	0.32 b	0.35a	0.32 b
N content (mg)	15.5 a	9.1 b	13.7 a	11.1 b	14.8 a	10.3 b
P content (mg)	2.3 a	1.1 b	1.8 a	1.6 a	2.0 a	1.5 b
K content (mg)	12.6 a	6.7 b	10.8 a	8.8 a	12.2 a	7.6 b

significant (p <0.001), with the increment in damage between measurements at 4 h and 24 h being more evident at −7°C and −8°C than at −6°C and −10°C. N was the most important nutritional factor affecting VD. N_1 seedlings had lower VD (20.0%) than did $N_{1/20}$ seedlings (32.2%) (Fig. 1). Significant differences in VD with P dose levels (p=0.003) occurred at only −8°C, at which temperature $P_{1/50}$ seedlings were more damaged than were P_1 seedlings.

Seedlings given N_1 had significantly more new roots after the RGC test than did seedlings given $N_{1/20}$ ($p<0.05$), with respect to all variables studied: 33.7 mg ThRW (N_1), 23.1 mg ThRW ($N_{1/20}$), 8.8 mg FRW (N_1), and 4.9 mg FRW ($N_{1/20}$). In December, significant differences were apparent for both variables studied, but, in February there was a significant difference only in FRW. Test date significantly affected only FRW (p=0.034), with a greater FRW value in December than in February.

3.3 Field performance

For the first year after out-planting, seedlings planted in December 2007 had better vigor than did those planted in February 2008; in July 2008, seedling vigor was better than in December 2008. In addition, the interaction between measurement date and K dose was significant. In December 2008, seedlings previously treated in the nursery with K_1 showed better vigor than did those treated with $K_{1/50}$ (Fig. 2). Seedlings planted in December also had a significantly higher SDI on December 2008 than did those planted in February (Fig. 3). However, there were no significant differences for HI between seedlings planted at different dates.

At the beginning of the first dry season (July 2008), seedlings planted in December 2007 had significantly higher water potential than did those planted in February 2008 = (Ψ=−2.19±0.15 MPa and Ψ= −2.62±0.17 MPa respectively). However, there were no significant differences between planting dates in this parameter when measured in September 2008 (Ψ=−3.53±0.08 MPa overall).

4 Discussion

4.1 Morphological and nutritional status

Our results indicate that autumnal high-dose N fertilization (N_1) was more effective than was low-dose N ($N_{1/20}$) in increasing ShDW, RDW and SRDW of Holm oak at the end of a growing period in a nursery. The size and weight of the plants were within the range of the commercial seedlings usually produced in this region and according to the state regulations currently in force (Navarro et al. 2009; del Campo et al. 2010). The higher values of ShDW and SRDW were in agreement with the results of previous

Table 3 *P* values derived from the general lineal model of the main effects *N*, *P* and *K* fertilization on nutrient concentration and nutrient content, in leaves (L), roots (R) and stem (S), of *Quercus ilex* seedlings in December 2007

n.s.: not significant ($p>0.05$); *: $p\leq0.05$; **: $p\leq0.01$; ***: $p\leq0.001$

	N			P			K		
	L	R	S	L	R	S	L	R	S
%N	n.s.	n.s.	n.s.	*	n.s.	n.s.	**	n.s.	n.s.
%P	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
%K	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
N content	***	**	**	**	n.s.	n.s.	***	*	n.s.
P content	***	n.s.	*	n.s.	n.s.	n.s.	*	n.s.	n.s.
K content	**	n.s.	n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.

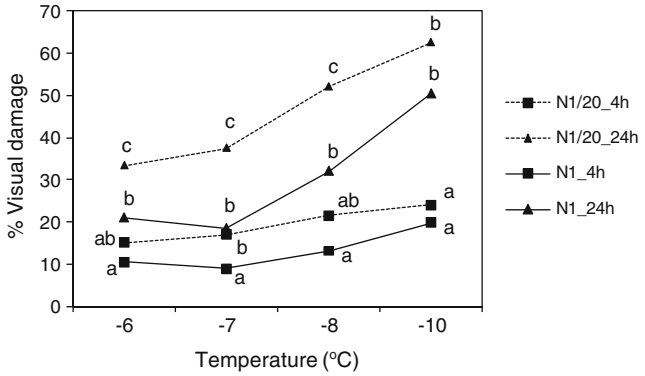

Fig. 1 Visual damage (VD) in relation to different doses of N applied, for each test temperature and measurement time. Different letters indicate significant differences ($p<0.05$) between different doses of N and measurement time for each test temperature

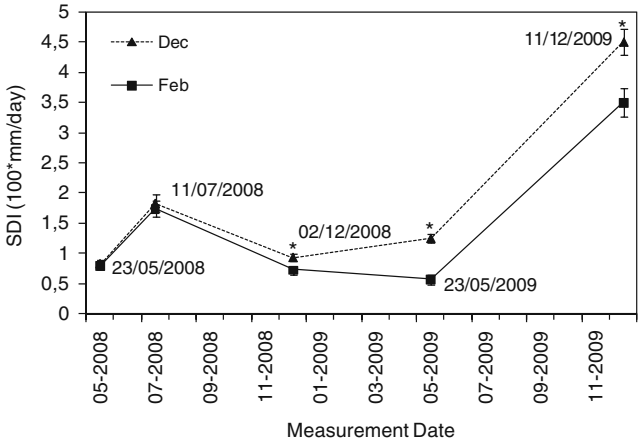

Fig. 3 Estimated stem diameter growth (SDI) (± SE) for each planting date during the studied period. * shows significant differences ($p<0.05$) between planting date to a given measurement date

studies (Broncano et al. 1998; Villar-Salvador et al. 2004a). However, some studies (Villar-Salvador et al. 2004a; Oliet et al. 2009a) reported no increase in root growth following N fertilization. The difference may be explained because we continued fertilization until mid-December, whereas fertilization was concluded in early autumn in the cited studies, and so it could not help root and cambial activity during autumn. We also found that autumnal P and K fertilization enhanced SRDW; and in particular, K fertilization significantly increased shoot growth. Oliet et al. (2009b) also found an increase in shoot growth for N- and P-fertilized plants. Such elevated shoot growth could be attributable to the greater leaf nutrient contents of K and P fertilizer plants, and particularly to the higher leaf N concentration and content of seedlings fertilized with higher doses of P and K.

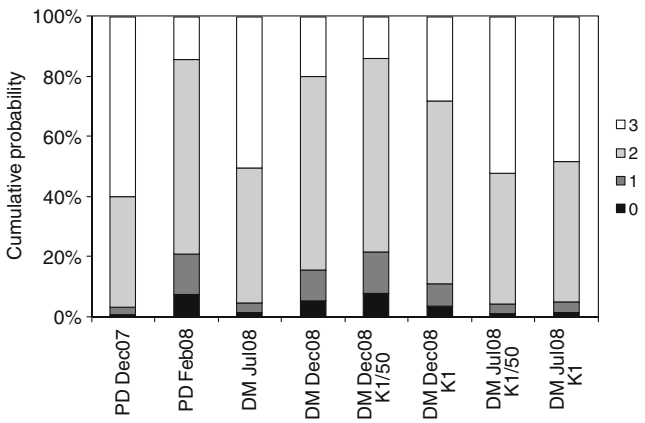

Fig. 2 Estimated cumulative probability (%) of belonging to one of the categories considered for evaluated vigour status: zero seedlings dead, one seedling defoliation higher than 50%, two seedlings defoliation lower than 50%, and three seedlings completely healthy. It was only represented factors or interactions with statistically significant differences. The model has proved to be significant for planting date (DP), measurement date (DM) and the interaction between DM and K dose

Nutrient concentrations at the end of our nursery period were similar to those reported in previous studies of Holm oak seedlings (del Campo et al. 2010; Navarro et al. 2009; Oliet et al. 2009a). N fertilization was the main factor affecting plant nutrient content: greater autumn N supply significantly increased such content, mainly because of the higher biomass of seedlings, and elevated leaf P concentration. Oliet et al. (2009a) reported higher N, P, and K concentrations in fertilized seedlings than in non-fertilized seedlings, but found no effect of different N fertilization levels on these concentrations, even though the differences between their N doses were greater than those of the present study, and they applied different fertilization treatments during all the nursery growing period. In the case of roots, we found no significant differences in N, P, or K concentration, in contrast to other studies on Holm oak (Villar-Salvador et al. 2004a; Oliet et al. 2009a) which found higher N, P, and K levels in fertilized seedlings. This difference is probably attributable to the "dilution" of nutrients in the new seedling root growth and the accumulation of these over the 40-week growing period, not only the hardening phase. The positive effect of P and K on leaf N concentration may be because of several reasons (Marschner 1995): (i) a requirement of these nutrients for synthesizing of nucleic acids, for supporting root respiration, which is a precondition of N uptake, or for the photosynthetic metabolism, (ii) a direct relationship of P level with cellular energy metabolism, in particular as a source of phosphate esters and energy-rich phosphates (e.g., ATP) that is necessary for reduction of NO_3^- to NH_4^+, or (iii) the K-mediated activation of numerous enzymes and ATPases required for protein synthesis.

Our results indicate that SS concentrations in roots and leaves were slightly lower in seedlings given N_1 than in those given $N_{1/20}$. This is probably because of a higher carbon consumption by N_1 plants that grew more vigorously than $N_{1/20}$ ones, as SS concentrations may be determined by the balance between source activity and the demand by growth and maintenance according to growth phenology (Fernández et al. 2008; Palacio et al. 2008; Sanz-Pérez et al. 2007). The higher root St content of seedlings treated with N_1, by comparison with $N_{1/20}$, indicates that no "dilution" effect on root St concentration was evident, even though the N_1 plants had higher root biomass.

4.2 Frost tolerance and root growth capacity

Previous studies have examined the effect of cold stress on the photosynthetic efficiency of Holm oak, based on chlorophyll fluorescence measurements (Oliveira and Peñuelas 2004). Fv/Fm assessment is a simple, rapid, and non-destructive method evaluating frost-induced plant damage. A high correlation between Fv/Fm values and frost damage after a freezing test has previously been found in other species (Fernández et al. 2007, 2008). Therefore, Fv/Fm measurements could be useful to assess frost damage by a quantitative method instead of a more "subjective" visual method (VD). The lower VD of our seedlings in February relative to December could be caused by accumulation of more chilling hours, which enhanced cold hardiness (Fernández et al. 2008). Previous studies have reported different effects (increase, no effect or decrease) of N fertilization on such hardiness (see cited literature in Vilagrosa et al. 2006 and Fernández 2008). In the present study, N fertilization increased cold hardiness, although we achieved 50% VD at about −10°C, whereas in other studies at the same nursery, using seeds of the same provenance, and similar containers and growing media, we obtained 50% VD at −13°C. Temperature variation during the nursery period may explain this difference.

An association between SS concentration and cold hardiness in various oak species has been reported (Mollá et al. 2006; Morin et al. 2007; Fernández et al. 2008). In contrast, we found that seedlings of greater cold hardiness (N_1 treatment) had a lower SS concentration than did seedlings treated with $N_{1/20}$. However, the variation in SS content of plants given different N treatments was small, and SS concentrations were relatively low (3.60–5.05%) in respect to the other cited studies that reported SS levels of 5.0–9.5%., Because cell membranes are the primary sites of injury during freezing, most of the cell alterations during the hardening period are aimed at preserving the integrity of membranes (Zwiazek et al. 2001). Therefore, although accumulation of SS can improve cold hardiness by their cryogenic and osmotic properties, elevation of the levels of other solutes, including lipids, amino acids, proteins (apoplastic proteins having antifreeze activity, cryoprotective proteins, dehydrins, etc), organic acids, glycosides, and organic salts can also improve hardiness (Larcher 2000; Zwiazek et al. 2001). This may explain the positive effect of N fertilization on cold hardiness found in the present work. Nevertheless, the relationship between cold hardiness and the composition of these organic constituents needs to be defined for this and other Mediterranean species. Besides, a more frequent measurement of freezing tolerance and SS analysis than in the present study, from late summer onwards, through fall and into winter (Colombo et al. 2003), would be the only way to really understand how nutrition affects the development of plant cold hardiness.

In addition, we also found that N fertilization increased the leaf K content. K plays a key role in osmoregulation (Hsiao and Läuchli 1986) and frost damage is inversely related to leaf K content (Marschner 1995). We found a positive effect of P fertilization on cold hardiness only for plants exposed to −8°C; this effect was minor compared with that induced by N fertilization, and was probably related to a P-mediated increase in leaf N concentration.

RGC is considered a measure of seedling vigor, and provides an evaluation of photosynthetic efficiency or the amount of stored carbohydrates (Ritchie 1985). We found that autumn N fertilization (N_1) increased RGC, shoot growth, and starch content, possibly because the greater size of shoots affected assimilation capacity (Villar-Salvador et al. 2004a). RGC has been used as a predictor of transplantation performance of forest species, and is frequently correlated with cold hardiness (Simpson and Ritchie 1996). In our study, N fertilization (N_1) increased RGC and cold hardiness, as also reported by Mollá et al. (2006), but had no effect on field performance. It may be attributable to the very favorable environmental conditions in the plot during the study period; the vast majority of our seedlings survived, with only 2% mortality for December plantings and 8% mortality for February plantings; and although predawn leaf water potential dropped to −3.5 MPa the first summer, it did not drop below −4.0 MPa, typical of Holm oak seedling under severe water stress (Villar-Salvador et al. 2004b).

4.3 Field performance

During our study period, seedlings planted in December had better vigor than did those planted in February. Palacios et al. (2009) also found that field performance of Holm oak was strongly affected by planting date. As our results confirm, summer (the dry season) was the most stressful season, and seedlings displayed more vigor before than after summer. In summer, roots need to access water that is deeper in the soil (Palacios et al. 2009). Thus, in our study

plot, seedlings planted in December presumably developed more extensive root systems during the mild winter, during which time the shoots remained dormant. In fact, our water potential data confirm that, in the middle of summer, seedlings planted in December had better water status than did those planted in February. In addition, SDI was higher after the first dry season in seedlings planted in December, presumably because of a positive relationship between growth and water status. The variations in SDI between seedlings of different planting dates were enhanced after the second year in the field.

As we have said before, previous studies found varying effects of nursery fertilization on the field performance of Holm oak and other Mediterranean species. In the present study, only K fertilization affected seedling vigor, possibly because of the role of K in controlling stomatal aperture and osmotic adjustment (Hsiao and Läuchli 1986). A positive effect of K on field response (survival) was also found by del Campo et al. (2010), especially in a dryer year. However, although we found no variation in K concentration in plants given different K treatments, there was variation between treatments in leaf K content. In addition, we found that N fertilization improved cold hardiness, growth, and nutritional status at the end of the nursery period, but did not affect field performance. This may be because of the low plasticity of Holm oak seedlings with respect to nutrient availability, the application of N for only a brief period (over the final 12 weeks in the nursery), or to the generally favorable growing conditions at our field plot during the study period, so that all of our seedlings had sufficient N reserves for optimal field performance. Different results of the effect of N fertilization on field performance may be expected under more stressful field conditions, but it has been unpredictable for this study.

The results of our study indicate that autumn fertilization, especially with N, improved the quality of Holm oaks grown in a nursery in the Mediterranean Basin of Spain. The application of 100 mg N plant^{-1} (70 mg during the growing phase and 30 mg during the hardening phase) led to seedling complying with morpho-physiological standards. Taking into account the fact that an acorn can contain about 30 mg N (Oliet et al. 2009a), at the end of December the seedlings only kept between 34 % [$N_{1/20}$] and 37 % [N_1] of the N available in the acorn plus the N applied to the growing medium. In particular, nitrogen autumn fertilization (30 mg N vs 1.5 mg N) increased seedling growth, root growth capacity, cold hardiness, and the nutritional status of seedlings, but does not necessarily improve field performance. Field performance will depend strongly on edapho-climatic conditions in addition to plant quality. Besides, the application of 43.6 mg P and 83.1 mg K (13.1 mg P and 24.9 mg K during the hardening phase, respectively) did not decrease plant quality, but improved or had no effect on morpho-physiological parameters compared with 30.8 mg P and 58.6 mg K (0.3 mg P and 0.5 mg K during the hardening phase respectively). However, further studies are needed to determine the optimal fertilization schedule for improvement of field performance.

Our results indicate that early planting of Holm oak in Mediterranean areas with mild winters might allow for root growth, so that plants can better survive the dry summer season. As our results led to a low frost tolerance of the more tolerant treatment (about −10°C) compared with other studies (Fernández et al. 2008; Mollá et al. 2006), the post-transplantation response in harsher winter sites would be unpredictable for this seedling from a nursery subjected to a mild climate.

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