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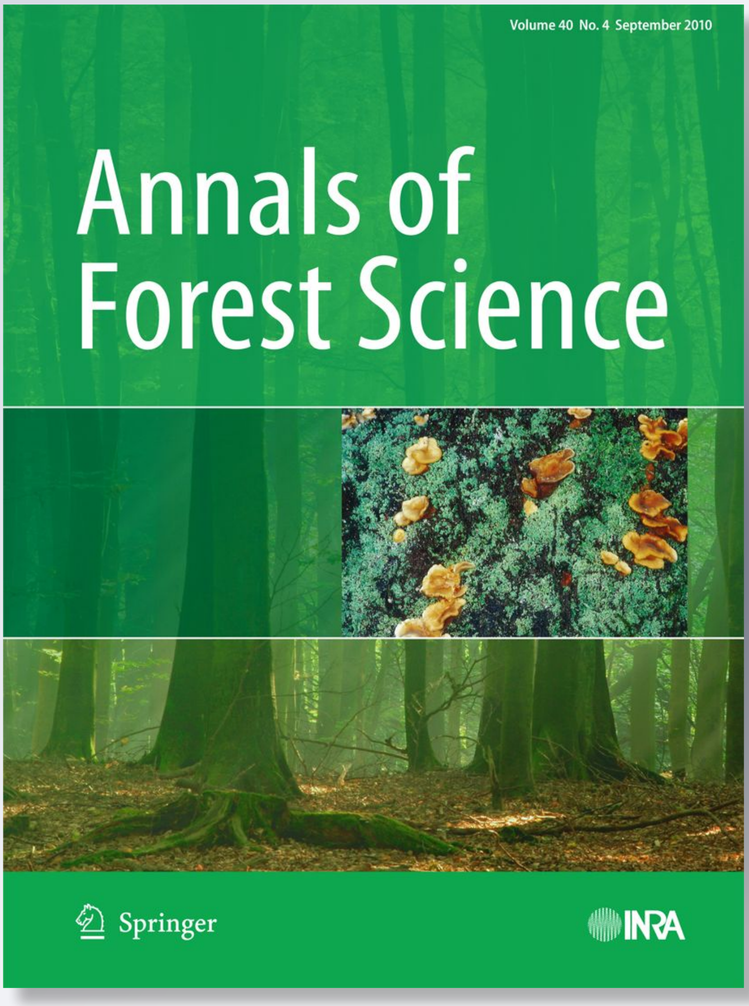
Annals of Forest Science
Official journal of the Institut National de la Recherche Agronomique (INRA)

ISSN 1286-4560
Volume 68
Number 3

Annals of Forest Science
(2011) 68:637-646
DOI 10.1007/s13595-011-0057-3



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ORIGINAL PAPER

Artificial regeneration with *Quercus ilex* L. and *Quercus suber* L. by direct seeding and planting in southern Spain

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Received: 6 March 2010 / Accepted: 14 October 2010 / Published online: 20 April 2011

Abstract

- ***Introduction*** The limited ability of *Quercus* species to regenerate naturally in Mediterranean forests has led to the development of various artificial regeneration methods; however, there is no general consensus as to what specific method is the best one for this purpose.
- ***Material and methods*** In this work, we assessed morphology, growth and survival of two *Quercus* species (*Quercus ilex* ssp. *ballota* and *Quercus suber*) using two different methods of artificial regeneration (viz. direct seeding and planting) and two seedling ages (1-year-old seedlings and 3-year-old seedlings) in southern Spain.
- ***Results and discussion*** The 1-year-old seedlings of both species were found to exhibit the highest survival percentages and direct-seeded plants intermediate survival values. For direct-seeded plants, seed mass was found to have a significantly positive effect on the establishment success in both species. No clear-cut trend in survival was detected in the 3-year-old seedlings. The survival of the 3-year-old *Q. suber* seedlings and the direct-seeded plants was similar, but not in *Q. ilex*, where the survival of the 3-year-old seedlings was the lowest. The latter result may have been a consequence of cultivation in smaller containers leading to root deformation and limiting plant access to water. Differences in survival could not be ascribed to morphological and growth variables or stomatal conductance.
- ***Conclusion*** Based on the results, all three artificial regeneration methods can be similarly effective provided appropriate nursery cultivation conditions are used and seeds are protected against predators, the best choice in each case being dictated by the particular restoration goals.

Keywords Artificial regeneration · Nursery · Oak · Seedling age · Sowing

1 Introduction

Holm oak [*Quercus ilex* L. ssp. *ballota* (Desf.)] and cork oak (*Quercus suber* L.) are two evergreen woody species widely represented in the wild and managed forests of the Iberian Peninsula. Also, they constitute two essential elements of the agro-sylvopastoral system known as "dehesa". Ensuring sustainable use of natural resources in savanna-like ecosystems (dehesas) is of great economic importance for rural areas.

Oak tree mortality has increased considerably over the past 20 years due to the effect of the combined action of pathogens, xylophagous insects and adverse climate conditions (Brasier 1996). Poor regeneration (Smit et al. 2009) and high adult mortality (Brasier 1996) in many areas have led to the implementation of artificial regeneration programmes, mainly in areas where the tree population has declined or disappeared, or the land has been converted to agriculture.

Artificial regeneration with *Quercus* trees is limited by many factors including acorn predation, slow growth, and

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Electronic supplementary material The online version of this article (doi:10.1007/s13595-011-0057-3) contains supplementary material, which is available to authorized users.

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low survival percentages especially during the first summer drought (Smit et al. 2009). In addition, *Quercus* seeds are recalcitrant, so they can only be stored for a few months.

No consensus exists as to which is the best available artificial regeneration method for *Quercus*. The greatest advantage of direct seeding is probably its low cost relative to planting as the latter requires nursery care and planting with higher associated costs (Stanturf et al. 1998). Moreover, direct-seeded plants can develop natural root systems on site and longer roots reaching deeper soil layers so that they have access to water more readily during the first summer drought, thus increasing survival (Lloret et al. 1999).

However, direct-seeded plants remain in a more vulnerable condition over longer periods (Stanturf et al. 1998) and they also grow to a lesser extent (Zaczek et al. 1996). The one other disadvantage of acorns is vulnerability to predation by wild animals (Johnson et al. 2002), especially in small openings or forested areas over the first few weeks after sowing (Madsen and Löf 2005).

Nursery production of oak seedlings is believed to be a more reliable regeneration method than direct seeding, this being especially recommended when seed supply is limited (King and Keeland 1999). Thus, it considerably reduces initial predation, and results in increased growth and more efficient competition with grasses (Stanturf and Kennedy 1996). With respect to seedling age, Spanish legislation has set various quality conditions for nursery seedlings including a maximum seedling age of 1–2 years for *Q. ilex* and 1 year for *Q. suber*. However, those standards should be considered as reference values, and it has been shown in several studies (e.g. Navarro-Cerrillo et al. 2007; Jacobs et al. 2009). On the other hand, the growing use of plants to increase density and enrich existing *dehesas* has led nurseries to grow older *Q. ilex* and *Q. suber* seedlings (3–4 years) in larger containers (1,000–3,000 cm^3). This has allowed the initial growth period in the field to be shortened, thus obtaining more visible results within a shorter time after planting. However, planting larger seedlings is more expensive than planting small ones (Stanturf et al. 2004).

Which attributes explain seedling performance in the field? Various morphological traits including aerial biomass, height and the root/shoot biomass ratio dictate growth and survival (Navarro-Cerrillo et al. 2007) and have frequently been used to predict seedling performance in the field (Davis and Jacobs 2005; Jacobs et al. 2005). There is, however, no general agreement about the most suitable plant morphology for the Mediterranean environment. Some studies (Del Campo et al. 2010; Villar-Salvador et al. 2004) have found no relationship between plant performance and seedling morphology; also, the plant's early response is believed to be more markedly dependent on the particular local conditions (Dey et al. 2008; Palacios et al. 2009). One of the main causes of failure in reforestation actions is summer drought. Water stress affects planting stock and seedling establishment (Grossnickle 1988) and can be reflected in stomatal conductance changes (Stewart and Bernier 1995). In fact, stomatal conductance reflects the water use strategy of plants; thus, stomatal closure avoids excessive transpiration and xylem cavitation (Cochard et al. 1996), but can compromise photosynthesis and reduce the growth potential of plants as a result.

In short, the choice of a particular artificial regeneration method is dictated by a number of factors including site conditions, seedling quality and water status, the particular species to be regenerated and the specific objectives of the restoration project (Dey et al. 2008). Identifying the best possible artificial regeneration method in each case usually entails assessing them under different conditions and examining the morphological and physiological processes that underlie seedling outplanting performance. No previous simultaneous assessment of direct seeding and planting of 1- and 3-year-old seedling with *Q. ilex* and *Q. suber* oaks has, to the authors' knowledge, been done. The primary purposes of this study were, therefore, as follows: (a) to compare seedling performance of plants using three methods: direct seeding and planting of 1- and 3-year-old seedlings of the two most common oak species in the Iberian Peninsula; (b) to confirm whether morphological or physiological attributes can be used to explain seedling performance in the field; and (c) to identify the most suitable artificial regeneration method for these two species.

2 Material and methods

2.1 Site description

The study was carried out in an experimental plot at the University of Córdoba (Campus de Rabanales, Córdoba, Spain, 37° 51′ N, 4° 48′ W, 100 m a.s.l.) that was formerly used as agricultural land. The site has a small slope (<2%) and the soil is a fluvisol consisting of heterometric silical gravels containing occasional calcareous boulders. Physical and chemical soil characteristics are shown in Appendix S1. The climate is dry Mediterranean, with a mean annual temperature of 17.6°C, an average rainfall of 609 mm and dry summers. The average temperature and atmospheric humidity for the period from April to October 2008 were recorded with a data logger (HOBO Pro Series 8 Temp, RH) located in the area. The average temperature during the first 8 months (February–September 2008) was 23.9±5.1°C and the accumulated precipitation was 604 mm (Appendix S2).

2.2 Seed collection and seedling assessment

From October to December 2007, approximately 800 acorns of *Q. ilex* L. subsp. *ballota* (Desf.) Samp. and *Q. suber* L. with no apparent damage were collected from the Natural Park of Sierra de Cardeña and Montoro (Sierra Morena, Córdoba, Spain, 38° 21′ N, 3° 12′ W; for *Q. ilex*, the provenance region was Extremadurense, and for *Q. suber*, the provenance region was Sierra Morena Oriental; Jiménez et al. 1996; Martín-Albertos et al. 1998). Seeds were obtained from at least six trees in the same population. Acorns were stored at 2–5°C in a cold chamber until January 2008. The acorns to be used for direct seeding were selected by the flotation method. A sub-sample of each species was used to determine fresh weight and then oven-dried at 70°C for at least 48 h. Seed dry mass was determined after removing the pericarp from the cotyledons. Linear regression equations between acorn fresh mass (A_{FM}) and seed dry mass (S_{DM}) were established for each species in order to obtain a precise enough estimate of seed dry mass from acorn fresh mass. The equation for *Q. ilex* was $S_{DM} = -0.2878 + 0.5412 \times A_{FM}$ ($r^2 = 0.95, P < 0.001$), and for *Q. suber*, the equation was $S_{DM} = -0.1167 + 0.6019 \times A_{FM}(r^2 = 0.98, P < 0.001)$.

In January 2008, several sets of *Q. ilex* and *Q. suber* seedlings cultivated in a forest nursery were selected (San Jerónimo, Consejería de Medio Ambiente, Junta de Andalucía, Seville, Spain). For *Q. ilex*, the provenance region was Extremadurense, and for *Q. suber*, the provenance region was Sierra Morena Occidental (Jiménez et al. 1996; Martín-Albertos et al. 1998). The 1-year-old seedlings of *Q. ilex* (height, 13.8±2.2 cm; stem basal diameter, 0.35±0.07 cm) and *Q. suber* (height, 34.7±9.7 cm; stem basal diameter, 0.36±0.07 cm) were grown in 400 cm^3 Forestpot® containers. The 3-year-old seedlings of *Q. ilex* (height, 61±12.4 cm; stem basal diameter, 1.31±0.29 cm) were grown in Forestpot® 3,000 cm^3 containers and *Q. suber* seedlings of the same age (no measurements available) cultivated in 7,200 cm^3 individual pots. A randomly selected sample of 15–20 seedlings of each species and age was harvested (first harvest planting) before planting for the measurement of root, stem, and leaf dry biomass after oven-drying at 70°C for at least 2 days. All leaves were scanned (HP Scan-jet 6300c) for calculation of total leaf area with the image analysis software Image Pro-plus 4 (Media Cybernetics, Inc.). Specific leaf area (SLA) was calculated as the ratio of leaf area to leaf dry mass. Biomass allocation to leaves (LMF, leaf mass fraction), stems (stem mass fraction), and roots (root mass fraction) was calculated as the ratio of organ biomass to total biomass. Roots were manually classified as fine (less than 1-mm thick) or coarse (more than 1-mm thick).

2.3 Experimental design

The plot studied was tilled to a depth of 40 cm with an agricultural harrow and the soil removed with a single shank ripper. The experiment was arranged as a factorial design involving two species (*Q. ilex* and *Q. suber*) and three age levels (seeds, 1-year-old seedlings and 3-year-old seedlings) in a completely randomized three-block design. Hereafter, the three age levels will be designated as "artificial regeneration methods". Seeds were sown and seedlings planted in 2×2 m spacing in each block. For direct seeding, 150 acorns of each species were distributed in each block. Three seeds per point were sown, at a depth of 5 cm, to ensure emergence. Seeds were placed 10 cm apart. The fresh mass of each acorn was recorded before seeding. In addition, 50 1-year-old seedlings and 50 3-year-old seedlings of each species were planted in each block. Therefore, a total of 750 samples from each species were used. Both direct seeding and planting were performed in late January 2008. Weed control during the experiment was done by manual removal and motorized cultivator during the spring.

2.4 Survival assessment

Acorn emergence and seedling survival were assessed at 15-day intervals from February to September 2008, and once more in September 2009. Two survival percentages were calculated, one for plants surviving after the first summer (September 2008) and the other for those still alive after the second (September 2009). Two performance-related variables (survival and success) were determined for directly seeded samples. Survival was calculated as the proportion of plants remaining alive after summer relative to emerged plants, and success as the number of plants surviving the summer divided by the total number of acorns sown. Therefore, the success was a combination of emergence and survival. We also estimated plot success as the proportion of planting spots with at least one plant established relative to planting spots.

2.5 Morphological measurements

In April 2008, a sub-sample of 15 seedlings from directly sown seeds of each species was randomly selected from the three blocks for removal of their aerial fraction (first harvest seeding) and measurement of the dry mass of each part, leaf area and SLA as described above.

At the end of September of 2008, a sub-sample of 15 seedlings from each factorial treatment was randomly selected from the three blocks. Whole seedlings were removed from the soil, and their roots washed and cleaned (second harvest). The mean root depth was 28.4±4.9 cm. Total biomass was split into leaves, stems and roots, and morphological variables were calculated by following the above-described procedure. Absolute shoot and root mean increments were calculated as the mean differences between shoot or root dry mass of each method at the second and first harvest. Shoot and root mean increments for seedlings from directly sown seeds were taken to be the shoot or root dry mass values obtained at the second harvest as no shoots or roots existed at seeding time. The relative growth rate (RGR) for each method was calculated from the following expression (Hoffmann and Poorter 2002):

$$RGR = [ln(M_2) - ln(M_1)]/(t_2 - t_1)$$

where ln (M_2) and ln (M_1) denote the mean ln-transformed plant dry mass at time t_1 and t_2, respectively. M_2 and t_2 corresponded to the second harvest for all artificial regeneration methods, whereas M_1 and t_1 differed between methods. For 1- and 3-year-old cultivated seedlings, M_1 and t_1 corresponded to the first harvest before planting; for seedlings from directly sown seeds, M_1 and t_1 corresponded to the first seeding harvest (after emergence of seedlings). The standard deviation for RGR was calculated according to Cornelissen et al. (1996).

2.6 Physiological measurements

Two types of measurements were done: stomatal conductance and leaf water content. A porometer (delta T porometer AP4) was used to measure leaf stomatal conductance. Measurements were made every 2 weeks at midday (10–12 a.m. solar time) on four randomly selected replicates per species during the dry season (June to August 2008). Measurements were made on completely expanded young leaves receiving full sunlight.

From May to October 2008, ten seedlings per method were selected monthly for measurement of leaf water content. A young leaf directly hit by sunlight was collected from each replicate. Leaves were rapidly transferred to individual plastic pots that were previously weighed and covered with parafilm. Samples were brought back to the laboratory for weighing (within 1 h of collection) and then oven-dried at 70°C for at least 48 h to measure dry mass. Leaf water content was calculated as $WC = (FW - DW) \times 100/FW$, where FW and DW are fresh and dry weight, respectively.

2.7 Data analyses

Differences in seedling survival for each species between methods were assessed by using log-rank survival curves constructed by following the Kaplan–Meier procedure (Kaplan and Meier 1958). This analysis considers both seedling longevity and status (dead or alive) at the last survival assessment. In each species, differences in morphological and physiological attributes were analysed by one-way ANOVA with method as factor and a post hoc Tukey test. When necessary, data were converted into logarithmic form in order to fulfill normality and variance homogeneity requirements. A non-parametric Kruskal–Wallis test was applied in those cases where the transformed data failed to fulfill the ANOVA assumptions. The potential effect of blocks was excluded from the statistical analysis since they introduced no changes in survival trends. All statistical analyses were done with the software STATISTICA 8.0. (Statsoft, Inc.).

3 Results

3.1 Survival

Eight months after planting, 1-year-old *Q. suber* seedlings exhibited the highest survival percentages (*ca.* 68%), and 3-year-old *Q. ilex* seedlings, the lowest (*ca.* 25%; Table 1). Twenty months after planting, all seedlings exhibited lower survival than after 8 months, but both survival percentages

Table 1 Survival, seedling success and plot success for the first and second year in the two *Quercus* species studied using the three artificial regeneration methods

		Survival (%)		Seedling success (%)		Plot success (%)	
		First year	Second year	First year	Second year	First year	Second year
Q. ilex	ds	51.5±14.7	34.96±9.2	17.2±9.9	11.7±7.2	42.66±18.03	24.66±11.01
	1ys	61.1±19.8	45.28±19.3				
	3ys	24.9±18.7	17.74±10.3				
Q. suber	ds	47.9±9.4	33.6±2.4	31.8±13.9	22.3±7.4	44.66±8.08	32.33±8.50
	1ys	67.7±14.6	60.5±11.1				
	3ys	45.1±13.6	37.6±25.1				

Seedling success (percentage of seedlings established from the acorns sown) and plot success (percentage of planting spots with at least one plant established). First year and second year were 8 and 20 months after being planted (after first and second dry season). Mean value and standard deviation of the three blocks' percentages are shown. The three artificial regeneration methods are: *ds* direct-seeded, *1ys* 1-year-old seedling, and *3ys* 3-year-old seedling

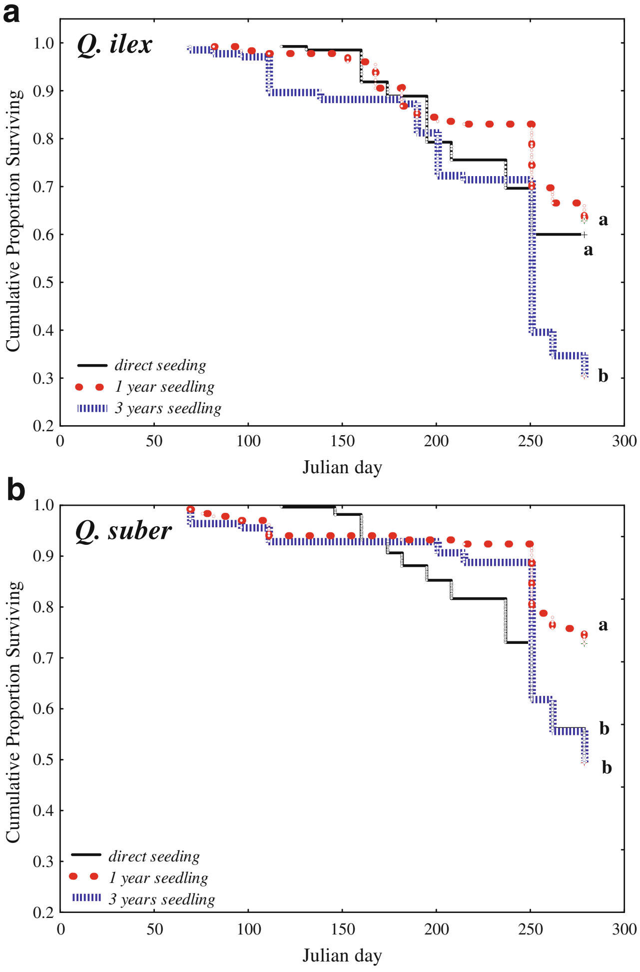

Fig. 1 Percent survival of *Q. ilex* (**a**) and *Q. suber* (**b**) seedlings during the first dry season. Different letters represent statistically different groups according to Kaplan–Meier survival analysis (log-rank tests; $P<0.05$)

showed a high correlation coefficient ($r=0.95$, $P=0.003$). Therefore, survival patterns were similar in both periods, thus meaning that methods with the highest survival after 8 months also had the highest survival after 20 months. At species level, log-rank tests revealed significantly lower survival of 3-year-old *Q. ilex* seedlings relative to the other methods (Fig. 1a). Also, 1-year-old *Q. suber* seedlings exhibited significantly higher survival percentages than 3-year-old seedlings of the same species and directly seeded plants (Fig. 1b).

Seedling success (a combination of emergence and survival) for the first year in directly seeded plants was about 17% for *Q. ilex* and 32% for *Q. suber*, and plot success (percentage of planting spots with at least one plant established) was about 43% for *Q. ilex* and 45% for *Q. suber* for the first year (Table 1). The second year seedling success decreased to 12% in *Q. ilex* and to 22% in *Q. suber* (Table 1; about a 30% decrease with respect to the first year). Plot success also decreased the second year for both species (Table 1).

Seedling success was significantly affected by seed mass ($F=14.3$, $P<0.001$). In both species, successful seedlings had larger acorns than the unsuccessful ones, the effect being significant for *Q. ilex* (Fig. 2). Because seedling success was a combination of emergence and survival, the seed mass may have influenced either variable. Interestingly, the favourable effect of seed mass was felt on survival in *Q. ilex* ($F=9.24$, $P=0.002$) but on emergence in *Q. suber* ($F=10.66$, $P=0.001$).

3.2 Seedling growth and morphology

Morphological attributes differed with seedling age, both on the planting date (Appendix S3) and 8 months later (Table 2). The change in root biomass was generally lower in the older seedlings, and so were the RGR, SLA and LMF for both species (Table 2). The root/shoot ratio of the older seedlings was higher except for the 3-year-old *Q. suber* seedlings, which exhibited the lowest ratios. The 1-year-old *Q. suber* seedlings had the highest fine roots proportion (16.7±4.3%) both at the first harvest (i.e. planting time; Appendix S3) and at the second harvest (Table 2).

3.3 Ecophysiological measurements

Figure 3 shows the stomatal conductance evolution for the dry period (June to August 2008). The 3-year-old seedlings of both species had lower mean conductance values than both 1-year-old seedlings and the plants from directly sown seeds throughout the dry season (Fig. 3). The mean maximum conductance at the start of the dry period ranged from 90±67 mmol m^{-2} s^{-1} for 3-year-old *Q. ilex* seedlings

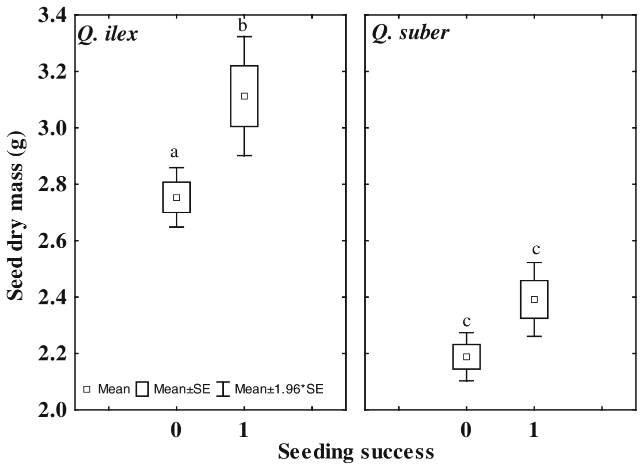

Fig. 2 Differences in initial seed dry mass between emerged and live seedlings (*1*) and non-emerged or dead seedlings after 8 months. Different letters represent statistically different groups according to the post hoc Tukey test ($P<0.05$)

Table 2 Summary of the biomass allocation and growth variables (mean±SD) in *Q. ilex* and *Q. suber* at the end of the first dry season

	Q. ilex			*Q. suber*		
	ds	1ys	3ys	ds	1ys	3ys
Root biomass (g)	0.72±0.45	3.97±1.50	39.42±10.20	0.83±0.43	6.73±3.86	47.13±11.39
Stem biomass (g)	0.61±0.28	2.30±1.05	25.15±15.82	0.51±0.32	4.03±2.17	60.48±14.88
Leaf biomass (g)	0.60±0.33	2.07±0.59	9.26±6.58	0.40±0.21	1.79±0.95	16.15±5.40
Total biomass (g)	1.80±0.87	8.15±2.46	76.04±31.38	1.82±0.72	12.64±5.51	124.26±25.16
Δ root biomass* (g)	0.72±0.45	−0.43±1.49	−1.64±10.20	0.83±0.43	0.53±3.86	n.a.
Δ shoot biomass* (g)	1.21±0.57	1.84±1.44	8.56±19.46	0.91±0.46	1.64±2.52	n.a.
RGR (mg g^{-1} day^{-1})	4.1±1.35	2.2±0.88	1.2±6.02	4.9 ± 2.45	1.3±3.52	n.a.
SLA (m^2 kg^{-1})	9.70±5.22 a	7.53±3.92 a	4.53±0.30 b	12.73±6.97 A	12.82±7.70 A	10.79±6.18 A
LMF (kg kg^{-1})	0.30±0.10 a	0.25±0.04 a	0.11±0.05 b	0.21±0.05 A	0.15±0.04 B	0.13±0.04 B
SMF (kg kg^{-1})	0.33±0.07 a	0.26±0.05 a	0.34±0.10 a	0.32±0.15 A	0.32±0.20 A	0.49±0.06 A
RMF (kg kg^{-1})	0.38±0.12 b	0.48±0.08 a,b	0.55±0.11 a	0.47±0.16 A	0.54±0.18 A	0.38±0.06 A
Fine roots (%)	9.02±12.47 a	9.18±6.07 a	15.44±5.24 a	2.77±4.42 B	17.12±15.78 A	4.96±3.41 B
Root/shoot	0.66±0.32 b	0.96±0.28 a,b	1.38±0.70 a	0.90±0.50 A,B	1.40±0.71 A	0.63±0.18 B

Values of root, stem and leaf biomass, specific leaf area (SLA), leaf mass fraction (LMF), stem mass fraction (SMF), root mass fraction (RMF), root/shoot and percentage of fine roots are from plants in second harvest (after 8 months of being planted). Shoot and root mean increments were calculated as the mean differences between shoot or root dry mass of each afforesation method at the second and first harvest. At each species, different letters in rows (lower case letters for *Q. ilex* and capital letters for *Q. suber*) represent statistically different groups according to post hoc Tukey test ($P<0.05$) or to Kruskal–Wallis test when data did not fulfill ANOVA assumptions (*Q. ilex* SLA and *Q. suber* SMF). Fine roots percentage data were square root transformed in order to fulfill ANOVA requirements

ds direct-seeded, *1ys* 1-year-old seedling, *3ys* 3-year-old seedling, *n.a.* not available

*Shoot and root mean increments were calculated as the mean differences between shoot or root dry mass of each afforesation method at the second and first harvest

to 226±95 mmol m^{-2} s^{-1} for direct-seeded *Q. suber* plants (Fig. 3). Conductance decreased considerably throughout the dry season in all plant types. At the end of the dry period, 3-year-old *Q. suber* seedlings exhibited the lowest conductance values (6±0.5 mmol m^{-2} s^{-1}) and 1-year-old *Q. suber* seedlings, the highest (90±29 mmol m^{-2} s^{-1}), the differences being significant—the conductance of 3-year-old *Q. suber* seedlings was significantly lower than those for the other treatments ($F=10.97$, $P=0.003$). There were also substantial differences between individual specimens of the same species (Fig. 3).

Leaf water content varied little with time (results not shown). The mean water content was highest for direct-seeded *Q. suber* plants (56.6±9.5) and lowest for 3-year-old *Q. ilex* seedlings (47.6±5.7); the differences, however, were not statistically significant.

4 Discussion

In this study, we simultaneously evaluated direct seeding and planting of 1- and 3-year-old seedlings of the two most common oak species in the Mediterranean region (*Q. ilex* and *Q. suber*). We found that 1-year-old seedlings of both species perform much better than the other methods. In fact, this age has been recommended as being optimal for planting in Mediterranean conditions (Villar-Salvador 2003). However, the other methods (direct seeding and planting 3-year-old seedlings) may offer other advantages.

Seedlings obtained by direct sowing had medium survival percentages (about 50% the first year and 33% the second). Mendoza et al. (2009) had found that direct seedling survival depended on the specific habitat and was lower (from 30% to 50%) in open sites that are comparable to our study area. However, Navarro et al. (2006) found high survival percentages (80%) in directly sown seedlings 2 years after seeding, and proposed the seeding method as a viable choice for forest restoration.

Oak seedlings obtained by direct seeding must rely heavily on available acorn reserves. We found plants from large acorns to be more successful than those from small acorns as a result of the combined effect of emergence and survival. A relationship between seed mass and both seedling and root mass in *Quercus* has been established (Quero et al. 2007), based on which seeding large acorns can be expected to provide more vigorous seedlings with an increased survival likelihood (Lloret et al. 1999), especially under adverse environmental conditions, such as drought

With respect to the 3-year-old seedlings, several studies have found that large seedlings have higher survival and

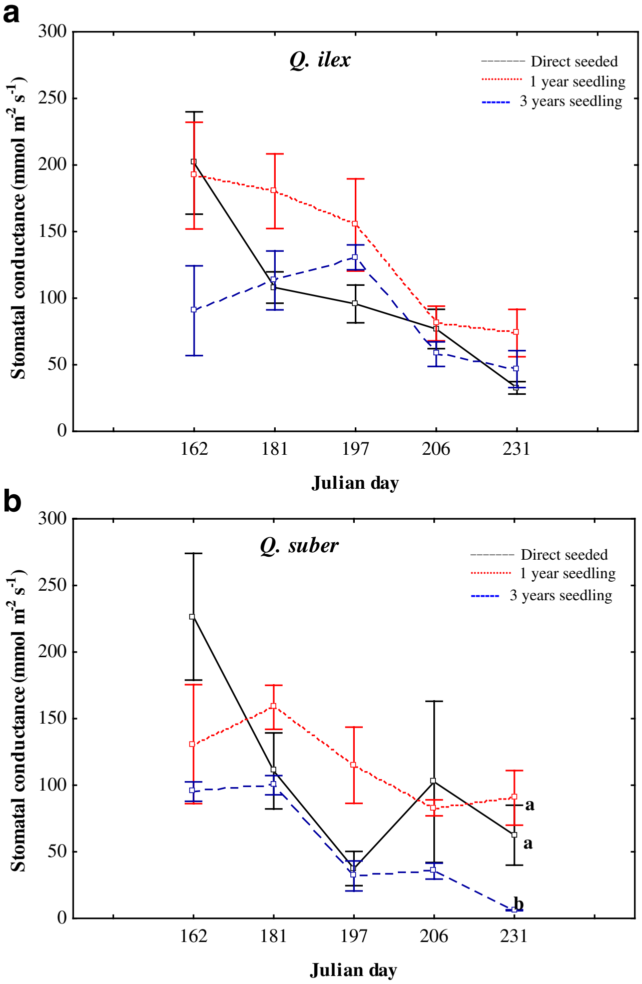

Fig. 3 Stomatal conductance of *Q. ilex* (**a**) and *Q. suber* (**b**) at midday during the dry season. *Bars* represent standard errors and *different letters* represent statistically different groups according to the post hoc Tukey test ($P<0.05$) on a given date

growth relative to small seedlings in the Mediterranean environments (Puértolas et al. 2003; Villar-Salvador et al. 2008; Luis et al. 2009; Oliet et al. 2009). Large seedlings may have higher net carbon gain because its greater photosynthetic surface area than small seedlings (Villar-Salvador 2003), and it may stimulate root growth during the wet season and thus allow seedlings to extract water in the dry season, ensuring plant survival (Padilla and Pugnaire 2007).

However, no clear conclusion can be drawn as regards survival in 3-year-old seedlings in our study. Thus, *Q. suber* exhibited medium percentages (about 50% in the first year) that were comparable to those for direct-seeded plants, and *Q. ilex* had very low values (25%) that departed significantly from those for the other methods. Initial seedling growth ("vigor") is a critical factor related to morphological and physiological attributes during the establishment period (Ashton et al. 1999). Older plants may exhibit abnormal root growth from the effect of their cultivation in small containers (Pemán et al. 2006) and develop inadequately after planting as a result (Dey et al. 2008), thereby being at an increased risk of dying during the first summer drought. In this study, containers that were used to grow 3-year-old *Q. ilex* seedlings were considerably smaller than those for 3-year-old *Q. suber* seedlings. Seedlings grown in deep containers have similar root systems to those of naturally grown plants and exhibit a greater root development capacity and hydraulic conductance than those growing in smaller containers (Pemán et al. 2006; Chirino et al. 2008). Moreover, removal of seedling roots from the soil after 8 months of growth in the field revealed that the nursery seedlings had developed few new roots outside the nursery container root volume (see values around zero in Table 2); by contrast, the roots of direct-seeded plants had grown deeper into the soil. The depth of the root system allows seedlings to access water during the dry season (Pemán et al. 2006). In this sense, Padilla and Pugnaire (2007) found a strong positive relationship between survival and maximum rooting depth in the five Mediterranean woody species. The relatively small container of 3-year-old *Q. ilex* may have been critical, determining less vigorous plants with less deep roots than the others that may partly account for their poor survival due to the effect of their strengthening container constraints on root growth.

Although seedlings with an increased root/shoot ratio can be expected to survive quite well in water deficit situations (Jacobs et al. 2009; Lloret et al. 1999), we found no lower root/shoots ratios in 3-year-old *Q. ilex* seedlings from the effect of their previous cultivation in small nursery containers or their lower survival. Similarly, previous studies in Mediterranean found no relationship between root/shoot ratio and seedling performance (Del Campo et al. 2010; Padilla and Pugnaire 2007; Villar-Salvador et al. 2004) or container size (Chirino et al. 2008). Our results are suggestive of slightly greater root mass allocation in the larger plants, but this requires confirmation by a careful study of the morphology and structure of the roots. Possibly, there was a markedly thickened main root, so the actual surface area available for water and nutrient uptake was probably small. Jacobs et al. (2009) also found that larger root volume does not confer seedlings with drought avoidance, and the most effective drought avoidance mechanisms were root growth, stomatal regulation, reduced leaf area and higher growth allocation to roots relative to shoots. On the other hand, one-year-old *Q. suber* seedlings showed a high proportion of fine roots at planting time and must therefore have had an increased physiological capacity for water and nutrient uptake (Eissenstat 1992).

Apart from fine roots proportion, performance differences can seemingly not be explained in terms of the morphological variables studied. Previous studies, which aimed to identify morphological attributes thus allowing seedling field performance to be predicted, found either no definite relationships or relationships that changed with the meteorological conditions of the planting year (Del Campo et al. 2010; Palacios et al. 2009). Also, species may differ in the response depending on the phenotypic plasticity. For example, Cuesta et al. (2010) found that holm oak seedlings under different nursery conditions did not show differences in mortality after transplanting while Aleppo pine did, suggesting that the lower phenotypic plasticity of late successional species (oaks) may explain these results.

All regeneration methods exhibited similar trends in stomatal conductance with time. Physiological parameters related to water use may change with plant age and developmental stage (Cavender-Bares and Bazzaz 2000); also, differences between species may not be apparent at the seedling stage (Mediavilla and Escudero 2004). Thus, although *Q. ilex* is known to develop an extremely conservative strategy for water use, it exhibits a low stomatal sensitivity and high conductance at the seedling stage (Mediavilla and Escudero 2004). The decrease in conductance in the dry season was a response to prevent xylem cavitation through stomatal control (Vilagrosa et al. 2003). Although all plant types exhibited a similar strategy, directly seeded and 1-year-old seedlings seemingly had a better water status—reflected in increased stomatal conductance—during the dry period. The 3-year-old seedlings (especially those of *Q. suber*) exhibited a higher stomatal sensitivity, a strategy which may reduce its growth rate. Also, older seedlings had lower leaf water contents (*Q. ilex*) and SLA values, both traits being closely related to RGR (Cornelissen et al. 1996; Ruiz-Robleto and Villar 2005). In fact, SLA and RGR decreased with age, which was to be expected since younger seedlings need to develop more leaves and have higher photosynthesis rates in order to reach the growth potential they require for successful establishment. Increased SLA values are suggestive of rapid production of biomass, but are related to low values of efficient conservation of nutrients (Poorter and Garnier 1999) and water (Poorter et al. 2009). On the other hand, the more conservative strategy of the older seedlings could be successful under extremely dry or stressing conditions. Thus, under the conditions of a high evaporation demand and low water availability, large seedlings may undergo more marked reductions in growth and conductance than small seedlings (Stewart and Bernier 1995), as we indeed found here. Moreover, older oak seedlings had lower LMF values at both harvests. Finally, leaf dieback has been noted as a response to high water stress by reducing passive water losses (Vilagrosa et al. 2003) and may have been the specific strategy adopted by our 3-year-old oak seedlings.

It should be noted that the collection of seeds for seeding and for seedlings were from different populations, so it could introduce confounding effects. However, for *Q. ilex*, the region of provenance of seeds and seedlings was the same (Extremadurense region). For *Q. suber*, the provenance of seeds was different (Sierra Morena Oriental) than of seedlings (Sierra Morena Occidental), although the two regions are very proximate and had a similar climate. Moreover, Hamrick (2004) suggests that woody species have more than 90% of their total genetic diversity within populations rather than among them. In summary, we do not think it may be an important source of error.

In short, *Q. ilex* and *Q. suber* establishment should be considered either by seeding or planting showing each method's advantages and disadvantages (Appendix S4). For both alternatives, seeding offers the most promise in terms of cost-effectiveness (about 845 euros ha^{-1}, see Appendix S5), but this method presents some disadvantages. For example, the seeding method can fail due to the effect of acorn predation by wild and domestic animals (Smit et al. 2009) or may be difficult to execute when seed is in short supply (*Quercus* may have infrequent mast years, and they show recalcitrant seeds that cannot be stored for long periods). Planting 1-year-old seedlings showed the highest survival percentages, but this is more expensive than direct seeding (about 1,200 euros ha^{-1}, see Appendix S5). Both methods would need to control the weeds with an approximate cost of 150 euros per year. Planting 3-year-old seedlings showed medium to low survival percentages and is the most expensive method (about 2,000 euros ha^{-1}, see Appendix S5). However, this method offers the advantage of shortening the initial growth period, which appears to be an important threshold affecting the economic attractiveness of plantations for *Q. ilex* and *Q. suber* planting in southern Spain (or in Mediterranean areas). Shortening the period of establishment is a useful silvicultural strategy in areas where intense pressure from browsing animals exerts a strong influence on vegetation. In areas where wild or domestic animals exclosures are needed to permit the development of regeneration, the duration of effective exclusion seldom exceeds 3–5 years. Therefore, the use of 2–3-year-old seedlings of *Quercus* spp. could be a positive alternative. Moreover, although larger seedlings are more expensive to plant than smaller ones, the former can be planted at lower densities in order to reduce costs (Dey et al. 2008). Previous studies (Rey Benayas and Camacho-Cruz 2004) have indicated a positive growth response of this stock type where cultural treatments are possible. However, a complex number of physiological mechanisms may underlie differences in seedling response related to size and root development (Tsakaldimi et al. 2009; Pemán et al. 2006). Specifically, leaf water conditions and soil–root interaction probably differed in that large seedling plants resulted in more water stress and lower fine root functionality, which leads to the loss of initial response of growth and survival (Wilson et al. 2007) during the first several months of the growing season. By contrast, small seedlings from nursery and from seeds have been associated with a better morphological condition and more efficient physiological status (Tsakaldimi et al. 2009).

5 Conclusions

Two different artificial regeneration methods (viz. direct seeding and planting) and two seedling ages (1-year-old seedlings and 3-year-old seedlings) were found to result in differential plant survival percentages. Each method, however, has some advantages. Thus, seeding was the least expensive method, with the roots growing in depth, but having medium survival percentages. Planting 1-year-old seedlings showed the highest survival percentages but was more expensive than seeding. Planting 3-year-old seedlings showed medium to low survival percentages depending on pot size and is the most expensive method, but it shortens the vegetative period in the field. Success percentages for direct-seeded plants can be raised by using large acorns. Regarding plant size, although 3-year-old seedlings of *Q. ilex* exhibited decreased survival percentages, this method has the potential advantage of shortening the plant juvenile period. However, it requires using large containers, which increases costs. Large seedlings grown in small containers undergo root deformation and are exposed to increased water stress, which diminish stomatal conductance and RGR rates.

Acknowledgements This study was supported by grant FPI-MEC, awarded to V.G-R (BES-2006-13059), and by the coordinated Spanish MEC Project DINAMED (CGL2005-05830-C03-02/BOS), INTERBOS (CGL2008-04503-CO3-02) and FEDER funds. We thank “Viveros San Jerónimo” (Consejeria de Medio Ambiente de Andalucía, Spain) for providing the studied plants. Thanks to Dr. A. del Campo, one anonymous referees and Dr. Jacobs, who made useful comments on a previous version of the manuscript. We also wish to thank José Manuel Quero, Pedro Lara, Fernando Puig and Bartolomé Arévalo for facilitating the seed collection in the Parque Natural Sierra de Cardeña and Montoro (Córdoba, Spain). Our research group is a member of the GLOBIMED network on forest ecology.

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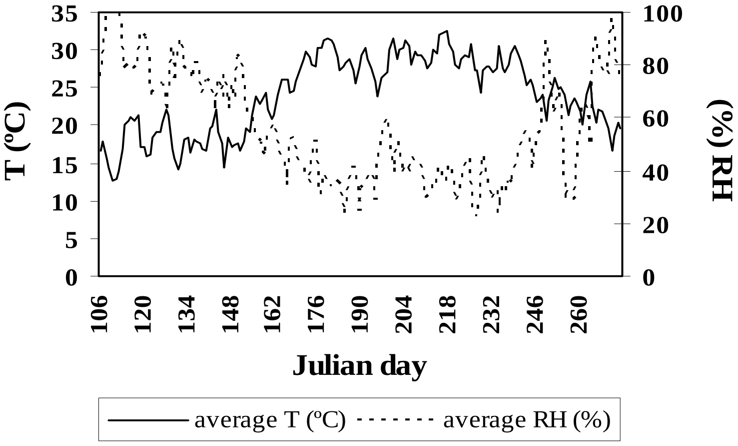
Appendix S1. Selected physicochemical soil properties of the study area. (Mean ± SD, n=15).

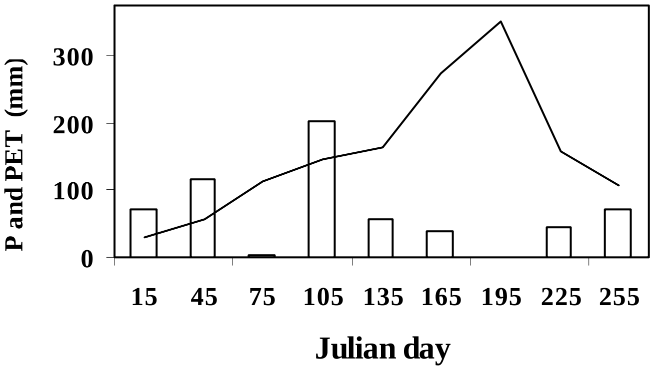
Clay (%)	10.43 ± 1.51
Sand (%)	53.37 ± 4.55
Mud (%)	36.20 ± 5.22
Penetration resistance (Mpa)	1.64 ± 0,3
Profundity of maximum penetration resistance value (cm)	34.18 ± 10.57
Maximum profundity of measurement (cm)	49.40 ± 2.20
Soil organic matter (%)	1.66 ± 0.30
pH	6.79 ± 0.31
Organic N (%)	0.09 ± 0.01
Assimilable P (ppm)	26.31 ± 7.87
Asimilable K (ppm)	344.33 ± 109.80
Ca (meq/100g)	5.00 ± 0.69
Mg (meq/100g)	1.80 ± 0.53
Na (meq/100g)	0.41 ± 0.03
K (meq/100g)	0.79 ± 0.26
Cation exchange capacity (meq/100g)	9.88 ± 0.73

Appendix S2. Mean temperature, relative humidity (*a*), precipitation (P, white bars) and potential evapotranspiration (PET, lines) (*b*) during the experiment.

a



b



Appendix S3. Summary of biomass allocation and miscellaneous morphological variables for *Q. ilex* and *Q. suber*.

Species		**Root biomass (g)**	**Stem biomass (g)**	**Leaf biomass (g)**	**Shoot biomass (g)**	**SLA (m^2 kg^{-1})**	**LMF (kg kg^{-1})**	**SMF (kg kg^{-1})**	**RMF (kg kg^{-1})**	**Root/shoot ratio**	**Fine roots (%)**
Q. ilex	**ds**		0.12 ± 0.05	0.56 ± 0.28	0.68 ± 0.31	7.94 ± 1.33					
	1ys	4.40 ± 2,09	1.23 ± 0.61	1.80 ± 0.74	2.53 ± 0.99	5.91 ± 2.26 **a**	0.25 ± 0.04 **a**	0.15 ± 0.04 **a**	0.60 ± 0.07	1.97 ± 1.55 **a**	10.21 ± 2.15 **a**
	3ys	41.06 ± 9.56	11.90 ± 7.93	7.30 ± 4.05	25.84 ± 4.97	4.77 ± 0.39 **b**	0.14 ± 0.03 **b**	0.24 ± 0.05 **b**	0.61 ± 0.05	1.61 ± 0.34 **a,b**	10.73 ± 4.67 **a**
Q. suber	**ds**		0.09 ± 0.03	0.36 ± 0.18	0.46 ± 0.20	11.14 ± 1.46					
	1ys	6.20 ± 2.56	1.97 ± 0.80	2.19 ± 0.70	4.17 ± 1.39	6.95 ± 0.71 **c**	0.22 ± 0.03 **a**	0.20 ± 0.07 **a.b**	0.58 ± 0.09	1.50 ± 0.47 **b**	16.67 ± 4.27 **b**
	3ys	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Data were obtained from seedlings before planting and on direct-seeded plants three months after sowing. Different letters in each column represent statistically different groups according to the post-hoc Tukey test ($P < 0.05$).ds direct-seeded plants; 1ys 1-year-old seedlings; 3ys 3-year-old seedlings. n.a., not available.

Appendix S4. Advantages and disadvantages of each artificial regeneration method.

	Direct seeding	**1-year seedling**	**3-year seedling**
Cost	low	medium	high
Root development	good	limited by container	very limited by container
Predation	high	null	null
Growth	low	medium	high

Appendix S5. Estimated costs (euros per ha) of the different artificial regeneration methods for 1 ha at a seedling density of 300 individuals ha^{-1}.

	Seeding preparation	**Seeds or plants**	**Establishment**	**Weed control**	**Tree shelters**	**Total**
Seeding	300	45	100	150	250	845
Small seedling	300	300	200	150	250	1200
Large seedling	300	900	400	150	250	2000