

Some propagation methods for cloning holm oak (*Quercus ilex* L.) plants

Research Article

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Abstract: Holm oak (*Quercus ilex* L.), a typical evergreen tree of the Mediterranean area, is very important due to its ecological and economical values. Propagation of this species is extremely difficult and traditionally carried out only by seed germination. In this work, mature acorns were germinated *in vitro* and in peat substrate in aseptic and non-aseptic conditions. Explants from the seedlings obtained were propagated *in vitro* in WPM plus 4 μ M BA. Plant regeneration was achieved from hypocotyls and root segments cultured *in vitro* on modified Gamborg medium plus 20 μ M BA and 20 μ M NAA. 13.8% of the hypocotyls and approximately 30% of the root segments developed both shoots and roots after 30 days of culture. Rooting of stem segments was obtained both *in vitro* and *ex vitro* by basal dipping in IBA solutions. Within *ex vitro* rooting, mother plant age had major influence on the percentage of rooting of the cuttings as the younger plants showed higher ability to root. In this way, *Q. ilex* plants could be propagated and cloned. The procedure described here would be a very useful tool for breeding programs since vegetative propagation of selected individuals can be achieved.

Keywords: Holm oak • *In vitro* • Plant regeneration • *Quercus ilex* • Rooting • Seed germination

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Abbreviations

BA – benzyladenine;
IBA – indole-3-butyric acid;
MS – Murashige and Skoog medium;
NAA – α -naphthalene acetic acid;
PVC – polyvinyl chloride;
WPM – woody plant medium.

1. Introduction

The holm oak (*Quercus ilex* L.) is an abundant evergreen forest tree of the Mediterranean basin. In Spain alone, around 3 million hectares are covered with this species [1]. The holm oak tree has a very high ecological and economic value due to its adaptation to the common disturbances in the Mediterranean ecosystem (fires, clear-cutting, herbivory) with a high capacity for resprouting [2]. The species plays a vital role in soil and water

conservation [3,4], showing good adaptation to summer droughts [5]. Its acorns are important nourishment for many wild and domestic animals, which influences the hunting and agricultural industries. Truffles, which grow in an ectomycorrhizal symbiotic relationship with the roots of the holm oak tree, present another value of the species. All of these factors, as well as the use of its wood for fuel and construction, contribute significantly to rural development and to the stabilization of the population in these economically depressed areas [1].

In Spain and Portugal, *Q. ilex*, together with *Q. suber*, define the unique open woods (artificially created and maintained) considered as the paradigm of sustainable agrosilviculture practices and included as special areas of conservation in EU directive 92/43 (Council of Europe, UNEP & ECNC, 1996). However, these so-called “dehesas” are currently threatened by the lack of natural regeneration resulting in decaying, aged adult trees [6]. In addition, a high incidence of the oak decline disease has been observed over the last three decades, causing

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heavy losses in the oak forests of the southwestern Iberian peninsula and aggravating the stresses already present in these ecosystems [7,8]. Thus, *Q. ilex* “dehesas” would need intervention to ensure their regeneration due to their extremely low densities [9].

Traditionally, the holm oak tree has been propagated only by seed germination because the vegetative cuttings or *in vitro* explants are almost impossible to root [10,11]. Other oak species are less recalcitrant to vegetative propagation [11]. Propagation technologies for clonal production of the species have been impractical up to date due to this drawback. Somatic embryogenesis has been the most common approach used to attempt the multiplication of the holm oak tree [1], but it would be very useful to have simpler methodologies for mass vegetative propagation, both for plant selection and breeding activities. The aim of this work is to develop simple methods for holm oak plant vegetative propagation to produce clonal plants.

2. Experimental Procedures

Mature acorns of holm oak were collected from trees growing in the Sierra Norte natural park of Seville (Andalucía, Spain).

2.1 Germination

After the elimination of the lignified endocarp, the acorns were disinfected by a 20 min immersion in a solution of NaClO (7% of active chlorine) and 37% HCl (30:1, v/v) at room temperature, followed by three rinses (10 min each) with sterilized distilled water.

In the first experiment, 124 disinfected seeds were individually sowed in test tubes (15x2.5 cm) with 20 ml of 1/3 strength MS medium [12] without plant growth regulators, plus 2% sucrose and 0.5% agar. After adjusting the pH to 5.8, the media were autoclaved at 120°C, 1 atm for 20 min. The tubes were covered with a plastic cap (25 mm polypropylene closures, SIGMA C5791), sealed with Parafilm and placed in the culture chamber at 23±1°C, first in penumbra (2.25 $\mu\text{E m}^{-2} \text{s}^{-1}$) until germination and then at 30 $\mu\text{E m}^{-2} \text{s}^{-1}$ of illumination with a 16 h photoperiod.

In the second experiment, 12 disinfected seeds were individually sowed in tubular containers made *ad hoc* in our laboratory and formed by a basal cylindrical opaque PVC piece to keep the substrate (600 ml of perlite, wet with 20% Hoagland solution) covered by an inserted cylindrical tube of transparent metacrilate (Figure 1). The set (tubular container) was a closed cylinder 7 cm in diameter and 34 cm high. The tubular containers together with the substrate were sterilized in an autoclave (120°C, 1 atm for 20 min) before the seeds were sown.

In the third experiment, 28 non-disinfected seeds were sown in alveolar trays, filled with Florabella peat (Klasmann-Deilmann GmbH), with 1.5 kg m⁻³ of added fertiliser (NPK 14:16:18) as substrate. The trays were watered frequently to keep the substrate wet. Tubular containers and trays were placed in a different growth chamber at 25°C, 111 $\mu\text{E m}^{-2} \text{s}^{-1}$ of illumination for a 17 h photoperiod.

2.2 *In vitro* culture and plant regeneration

The seedlings raised in the test tubes were cut into several two-node explants, distinguishing among the basal (n=8), medium (n=19) or apical (n=9) location in the plantlet, in order to check the *in vitro* growth ability of each kind of explant. Each explant was transferred to test tubes with WP medium [13] plus 4 μM BA, 2% sucrose and 0.5% agar, sterilized as described above. After capping and sealing with Parafilm, the tubes were placed in the culture chamber at 23±1°C, 30 $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination and a 16 h photoperiod. For *in vitro* plant regeneration, other small (~1 cm) explants were made from hypocotyls (n=29) and roots, the latter also divided among basal (n=26), medium (n=15) and apical (n=8). These explants were placed in Petri dishes (6 cm of diameter) with macro and microelements of Gamborg medium [14] plus vitamins of MS and the addition of 20 μM BA, 20 μM NAA, 1 mg l⁻¹ thiamine • HCl, 2% sucrose and 0.5% agar, sterilized as before. After sealing with Parafilm, the Petri dishes were placed in the culture chamber at 23±1°C, 30 $\mu\text{E m}^{-2} \text{s}^{-1}$ illumination and a 16 h photoperiod.

2.3 Rooting

The seedlings obtained were used to test their rooting ability in *in vitro* and *ex vitro* conditions. For *in vitro* tests,



Figure 1. Tubular containers made at the lab for holm oak seed germination.

62 explants derived from the tubular containers ($n=30$) and alveolar tray seedlings ($n=32$) were cultured in test tubes (15x25 cm) with 10 ml of WPM medium plus 4 μM BA, 2% sucrose and 0.5% agar, sterilized as described above. The explants from the trays were previously disinfected for 10 min in 70% ethanol, followed by 20 min in NaClO:HCl (30:1, v/v) solution and three rinses (10 min each) with sterilized water. No distinction of the explant position was made. The tubes were capped and sealed as before and placed in the culture chamber at $23\pm1^\circ\text{C}$, $30 \mu\text{E m}^{-2}\text{s}^{-1}$ illumination and a 16 h photoperiod. After 30 days under these conditions, half of the explants were basal dipped for 1 min in 1 mg ml^{-1} IBA and the other half in 2 mg ml^{-1} IBA and re-implanted in test tubes with the same medium but without plant growth regulators and cultured at $23\pm1^\circ\text{C}$, $30 \mu\text{E m}^{-2}\text{s}^{-1}$ illumination for a 16 h photoperiod.

For *ex vitro* condition, 18 plants were recovered from the alveolar tray seedlings and grown in the growth chamber for 2 years. The plants were irrigated (maintaining field capacity) with a 20% Hoagland solution. At the end of the first year of cultivation, 32 cuttings (10 cm length) with a few leaves were collected from 9 plants, washed with a fungicide solution composed of a mixture of S and Cu (0.3% and 0.2% respectively) and basal dipped for 1 min in 2 mg ml^{-1} IBA. Each cutting was then planted in 100 ml PVC pots with wet sterilized perlite and placed into transparent PVC boxes (72 x 40 x 20 cm). The covered boxes were placed in the growth chamber at 25°C , $111 \mu\text{E m}^{-2}\text{s}^{-1}$ of illumination for a 17 h photoperiod and opened every day to allow for ventilation and control of substrate moisture. Percentage of rooting was checked after 3 months. The same was performed with 31 cuttings obtained from the remaining 9 plants after 2 years of cultivation.

Student's *t* test and ANOVA were used for statistical analysis of the results using Statgraph 4.0, except for the data on plant regeneration. In this case, to cope with the limited sample size and with some low success frequencies, we carried out pairwise comparisons between treatments using the non-parametric Fisher's exact test implemented in the SISA package (<http://www.quantitativeskills.com/sisa/statistics/fisher.htm>). In order to control the Type I error inflation resulting from multiple testing we applied the False Discovery Rate procedure, as recommended by [15].

3. Results and Discussion

3.1 Germination

Since seed germination is the traditional tool for *Q. ilex* propagation, we tested different methods for this task.

Seeds cultured *in vitro* produced a heavy darkening of the medium due to phenolic exudation and oxidation. This effect was observed very early after sowing and, according to previous studies, it is a serious handicap compromising further development of the plantlets [16]. It became necessary to change the seeds to fresh media every 2 days until no more exudation was observed (3–4 times along the experiment) to avoid damaging the material. Despite this, 15 days after sowing, radicle emergence was observed in about a 65% of the seeds, although only 21% developed roots. At completion, 12.9% of the seeds became true plants with healthy growth (Table 1). After 45 days the seedlings had filled the test tube.

High germination percentages were observed in tubular containers and alveolar trays of 90% and 100% respectively. All seedlings developed root systems within 45 days, producing very healthy plants (Table 1). The only difference observed was that the growth of alveolar tray seedlings was slower than those raised in tubular containers.

These results indicate that the germination of holm oak seeds can be quick and easy, particularly when carried out in peat substrate. In this sense, tubular containers present the advantage of producing aseptic plants that can be used directly *in vitro* for cloning or propagation purposes.

3.2 *In vitro* culture and plant regeneration

The seedlings obtained were propagated *in vitro* in WP medium with the addition of 4 μM of BA. Basal explants presented significantly higher growth than explants from other origins (Table 2). When the explants came from

Method	Nº	Radicle emission (%)	Rooted (%)	True plants (%)
<i>In vitro</i>	124	65.3	20.9	12.9
Tubular containers	12	90	90	90
Trays	28	100	100	100

Table 1. Results of seed germination by three different methods, 45 days after sowing.

Explant origin	Nº	Growth (mm)	Shoots (nº)
Apical	9	8.03 a	1.67 a
Medium	19	6.38 a	1.63 a
Basal	9	35.55 b	3.50 a

Table 2. *In vitro* growth of two-node explants of *Q. ilex* in WPM medium after 30 days.

Different letters in columns indicate statistical significance at $P\leq 0.05$ (ANOVA-test)

seedlings germinated in trays, they showed a higher incidence of contamination after transfer to *in vitro* culture (15%).

Hypocotyls and root segments obtained from seedlings germinated *in vitro* displayed plant regeneration when cultured in modified Gamborg medium (Table 3; Figure 2A, B). All the explants produced roots. All the segments produced new shoots with the exception of apical root segments. 75% of the apical root segments emitted roots, much higher than the other explants, but they failed to regenerate aerial components. By contrast, hypocotyls and medium and basal root segments developed shoots in percentages ranging from 20% (medium root) to 38.4% (basal root) (Table 3).

Some of these explants developed whole plants with both shoots and roots. Root segments were the most effective with percentages of regeneration around 30% while nearly 14% of the hypocotyls produced whole plants. To date, regeneration in *Q. ilex* has only been achieved through somatic embryogenesis [1,17,18]. To our knowledge, this is the first observation of regeneration from hypocotyls and root explants in this species. Similar explants prepared from seedlings germinated in tubular containers showed no regeneration when cultured in the same conditions. This behaviour could be linked to a less juvenile character of that material.

3.3 Rooting

Rooting of the explants was effectively achieved by basal dipping in IBA solutions (Table 4). *In vitro* rooting reached percentages that ranged from 28-40%. The best results were obtained for an IBA concentration of 2 mg ml⁻¹ although no significant differences were found between the two concentrations tested or the provenance of the explants (tubular containers or trays)

(Table 4). As before, no previous observations of *in vitro* rooting of holm oak explants is known to exist.

Cuttings from plants germinated in trays also responded to basal dipping in IBA (Table 5). In this case, rooting efficiency depended on the age of the mother plant. The younger plants displayed a significantly higher percentage of rooting (65.6%) than 2-year-old plants (25.8%), indicating a loss of rooting ability as plant maturation proceeds. These results improve those reported by [10] who obtained a maximum of 20% of rooting for 1- or 2-year-old plants.

According to [19,20] and nursery uses, the holm oak tree is propagated mainly by seed germination.

Explant type	Nº	Plants (%)	Only shoots (%)	Only roots (%)
Hypocotyl	29	13.8 a	34.5 ab	20.7 a
Apical root	8	0.0 a	0.0 a	75.0 b
Medium root	15	33.3 a	20.0 ab	13.3 a
Basal root	26	30.7 a	38.4 b	7.7 a

Table 3. *In vitro* behaviour of hypocotyls and root explants in modified Gamborg medium after 30 days.

Different letters in columns indicate statistical significance at $P \leq 0.05$ (Fisher's exact test)

Explant origin	Nº	IBA 1 mg ml ⁻¹	IBA 2 mg ml ⁻¹
Tubular container	30	30	40
Trays	32	28	34.4

Table 4. *In vitro* rooting (%) in WPM medium without growth regulators of IBA basal dipped explants from holm oak plants germinated in tubular containers and trays.

No significant differences were found (Student's *t* test)



Figure 2. Plant regeneration from root (A) and hypocotyl (B) segments after 30 days of culture in modified Gamborg medium plus 20 μ M BA and 20 μ M NAA.

Mother plant age	Nº	Rooting %
1 year	32	65.6b
2 years	31	25.8a

Table 5. Percentage of rooting after 3 months of cuttings collected from 1- or 2-year old holm oak plantlets germinated in trays and placed in 100 ml pots with wet perlite. Cuttings were basal dipped in 2 mg ml⁻¹ IBA.

Different letters in columns differ at $P \leq 0.05$ (Student's *t* test)

The natural resprouting of this species [2,21] is the way forest restoration typically occurs [22,23]. There is no prior literature related to vegetative propagation of this oak species except for the induction, maturation and germination of holm oak somatic embryos

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