PROTOCOL OF SOMATIC EMBRYOGENESIS: HOLM OAK (*QUERCUS ILEX* L.).

P.V. Mauri*, & J.A. Manzanera

IMIA, El Encín, Apartado 127, 28800 Alcalá de Henares, Spain
Telephone: 34-918879400
Fax: 34-918879494
e-mail: pedro.mauri@imia.madrid.org
*: to whom correspondence should be addressed.

1. INTRODUCTION

Holm oak (*Quercus ilex*) is a characteristic evergreen woody species present in most countries surrounding the Mediterranean. The most important features of this hardwood tree are hardiness, resistance to drought, high ecological value as climacic, soil improving tree, game nourishment and refuge, etc. (figure 1a). Formerly, the wood was appreciated for its density and resistance in ship and house building and other traditional manufactures. Genetic variability is considerable, with a range of ecotypes with different morphologies, growth patterns and fruit (acorn) taste. The use of holm oak trees for symbiotic culture of truffle has a high economic importance and would contribute to rural development and to the stabilisation of the population in depressed areas. Nevertheless, basic research on this species has been scarce. Some problems include the variability of acorn production, the difficulty of conserving the acorns and the lack of conventional vegetative propagation, especially for adult elite trees (Cornu *et al*., 1977; L'Helgoual'ch and Espagnac, 1987).

Micropropagation of oaks from apical and axillary bud cultures has been tried in *Q. ilex* juvenile cultures (Bellarosa, 1989), and shoot regeneration has been obtained in related species (Vieitez *et al*., 1993). Micropropagation from adult oak trees is in general more difficult, with moderate rates of plant regeneration (e.g., San José *et al*., 1985; Manzanera and Pardos, 1990; Manzanera *et al.,* 1996; Sánchez *et al*., 1996).

Somatic embryogenesis is preferred to bud micropropagation for potential in culture automation and artificial seed production. Much research effort

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has been devoted to oak somatic embryogenesis leading to plant production. Somatic embryos have been induced from culturing *Q. rubra* immature zygotic embryos (Gingas and Lineberger, 1989), *Q. robur* immature zygotic embryos (Chalupa, 1990), *Q. robur* stems and leaves (Cuenca et al., 1999), *Q. suber* immature embryos (Bueno *et al*., 1992), *Q. suber* leaves (Fernández-Guijarro *et al*., 1995) and *Q. suber* anthers (Bueno *et al*., 1997), *Q. acutissima* immature zygotic embryos (Kim *et al*., 1994), *Q. variabilis* immature zygotic embryos (Kim *et al*., 1995) and *Q. canariensis* immature zygotic embryos (Bueno *et al*., 1996). In the past, there was hardly any work done on somatic embryogenesis of *Q. ilex*, except the first work done by Feraud-Keller and Espagnac (1989) and now recently by Mauri and Manzanera (2003).

The bottle-neck of somatic embryogenesis in *Q. ilex* is the maturation phase. Maturation has been hampered, in many woody species, by precocious germination and spontaneous repetitive embryogenesis, which may reach a 70% frequency. To prevent these unwanted side effects, osmotic treatments and the addition of abscisic acid (ABA) in the early stages of embryo development have been used in related species (Garcia-Martin *et al*., 2001). The role of ABA in the induction of maturation and dormancy of zygotic embryos and seeds has been studied in many other species as well its inhibitory effect on germination. Nevertheless, there are no references on the effects of exogenous application of ABA on somatic embryos of holm oak.

2. INDUCTION OF EMBRYOGENIC TISSUE

Induction of somatic embryogenesis was obtained from immature zygotic embryos (Mauri and Manzanera, 2003), collected in August 10th only (4.3 % frequency), approximately four months post anthesis. No positive results were recorded in immature embryos collected in July or September, or in nodal segments or leaves from seedlings or adult trees. Féraud-Keller and Espagnac (1989) reported somatic embryogenesis induction from the leaves of seedlings.

Acorns collected from the selected trees (figure 1b) are first rinsed in 70% (v/v) ethanol for 3 min, followed by immersion in 0.2% (w/v) Benlate® (Du Pont, 50% benomyl) with a few drops of Tween 20, then they are surface-sterilized for 20 min in a solution containing 0.5% (w/v) sodium hypochlorite prepared from commercial bleach to which 1-2 drops of Tween 20 are added. After rinsing three times in sterile distilled water,

they are left to imbibe for 10 min before dissection of the zygotic embryos. Remove the acorn coats with scalpel and forceps and transfer the embryos. Establish the excised zygotic embryos on G macronutrients (Gamborg *et al.*, 1968), with the micronutrients and cofactors of Murashige and Skoog (1962) (G*: Table 1), with 10 μM benzylaminopurine (BA) and 10 μM α-naphthaleneacetic acid (NAA) and 0.6 % (w/v) agar (Sigma, Type E) for one month. This plant growth regulator (PGR) combination was the best treatment for somatic embryo induction of all tested (Table 2). Adjust the pH of the medium to 5.8 with 1 N NaOH before autoclaving at 121 °C. We found that ten zygotic embryos per Petri dish are optimal for induction, which is realized at 25 °C in the dark. Wrap plates with a double layer of parafilm. Transfer the sterile embryos in petri plates containing G* medium with 0.5 μM BA and 0.5 μM NAA for 30 days. Transfer the embryos to basal medium free of PGR for 30 days. All through the induction period and even before embryogenic tissue can be observed, embryo cultures are transferred onto the fresh medium at every 4-week interval since this increases the frequency of induction as compared to cultures without subculture.

A white-translucent callus was induced on the zygotic embryo surface, which showed early embryo-like structures 3 to 8 weeks after the beginning of the induction treatment (figure 1c). Somatic embryos grow further and are eventually transferred to basal G* medium without PGR's when they reach 0.5-1.0 cm in diameter.

3. MAINTENANCE OF EMBRYOGENIC TISSUE

After 4-6 months, a proliferating, yellowish, friable callus is induced in the embryogenic cultures, from which somatic embryos develop after two weeks. On medium supplemented with 3 μM 2,4-dichlorophenoxyacetic acid (2,4-D) alone, non embryogenic callus is formed (Table 2). This non-embryogenic callus is normally characterised by a whitish appearance. Maintenance conditions are set at 25 °C with a 16-h photoperiod under light (50 μmol. $m^{-2}.s^{-1}$). Maintain embryogenic cultures by subculture every four weeks onto fresh basal medium without PGR and incubate at 25 ºC. Cultures are maintained on the same medium as for induction, i.e, G* medium without PGR.

Like other oak species, holm oak embryogenic tissue can be maintained either on solid (0.6 % agar) or in liquid medium (figure 1d-g) by serial subcultures to fresh medium every 28 or 14 days, respectively. Somatic

embryos have higher fresh weight (FW) increments in liquid medium (figure 2).

4. MATURATION

Holm oak somatic embryo development and maturation are carried out according to Mauri (1999). Maturation medium contains Schenk and Hildebrant's macronutrients (SH; 1972), with the micronutrients and cofactors of Murashige and Skoog (1962) supplemented with 0.1 or 1 μM ABA. Sucrose (90 mM, unless stated otherwise) was used as the carbon source. The medium was solidified with agar (Sigma, Type E) (6 g l^{-1}). Dispense the medium in Magenta™ glass flasks (59 x 66 mm, 30 ml medium) or in Magenta™ polycarbonate flasks (95 x 67 mm, 45 ml medium) for somatic embryo maturation. Adjust the pH of the medium to 5.8 with 0.1 M NaOH or 0.1 M HCl, and autoclave the medium at 1 atmosphere at 120 ºC for 20 min. Incubate the cultures in a climate-controlled chamber under a 16 h photoperiod, and a photon flux density of 50 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps.

Culture immature somatic embryos on SH medium supplemented with 0.1 or 1 μM ABA for 11 monthly subcultures. It will be observed that recurrent embryogenesis is significantly reduced and maturation increased with continuous culture on medium with ABA when compared with a medium without it (figure 3). Spontaneous germination is lower than 5%. Higher concentrations of ABA (10 μM) are not recommended becuase growth is arrested, while somatic embryos cultured on a medium supplemented with 0.1 and 1 μM ABA reach the highest values of dry weight (figure 4).

The addition of ABA to the culture medium significantly reduces unwanted recurrent embryogenesis in mature somatic embryos without affecting the germination of embryos subjected to stratification at 4 ºC. ABA is important for cotyledonary embryo development. It inhibits the polyembryony and allows embryo separation and further embryo development. ABA plays a role in embryo development and meristem dormancy and prepares tissues to tolerate physiological stages previous to germination.

5. GERMINATION AND TRANSFER TO SOIL

Transfer the matured somatic embryos (1 cm-size white embryos) to flasks containing basal SH medium without ABA and store them in a refrigerator

for a stratification treatment at 4ºC. The optimal period of stratification is normally 2-months (figure 5). After the stratification period, transfer the mature embryos to SH* germination medium (table 1). Incubate the cultures in light (50 μmol. $m^{-2}.s^{-1}$) with a 16-hour photoperiod and a continuos temperature of 25 ºC both during the day and at night. After 6-8 weeks, somatic embryos germinate and develop somatic seedlings (figure 1h-i). Transplant somatic seedlings, germinated *in vitro,* to pots filled with soil substrate. Grow the somatic seedlings in pots for 6 months in the greenhouse at 25 ºC.

Somatic embryos previously cultured on 0.1 and 1 μM ABA for long periods of time and stratified for two months reached germination rates as high as that of the control without ABA, i.e. 73%, while the 10 μM ABA treatment significantly reduces germination rate (data not shown). Stratification can neutralise the effect of ABA as a germination inhibitor. But stratification also promotes germination of somatic embryos in different species, such as *Q. suber* (Bueno *et al*., 1992; Manzanera *et al*., 1993; González-Benito *et al*., 2002). In holm oak, the stratification of somatic embryos at 4ºC has been significantly positive on germination for treatments longer than one month, two months being the optimum. Another positive effect of stratification was the shortening of the mean germination time, which was reduced to 3 to 7 days for the two month treatment at 4 ºC.

Sucrose concentration in the medium significantly affected germination. The best rates were obtained between 90 and 450 mM sucrose, which had no statistical differences between them (figure 6). No significant difference was observed between embryos cultured in light and those cultured in darkness. When comparing temperature regimes, i.e., continuous temperature at 25 ºC or a thermoperiod of 25 ºC/15 ºC, no statistical differences were recorded in embryo germination.

The best germination rates were obtained with the addition of 90 to 450 mM sucrose in the culture medium, as in *Q. suber* (Garcia-Martin *et al*., 2001). Higher concentrations (630 mM) of sucrose significantly inhibited germination rate (figure 6). Temperature was not a critical factor. In *Q. robur* acorns, light negatively affected germination (Finch-Savage and Clay, 1994) but in our case, light and darkness treatments provided similar germination rates, in agreement with observations of somatic embryos from other species, such as *Q. suber* (Fernández-Guijarro et al., 1995) or *Q. robur* (Chalupa, 1990).

7. CONCLUDING REMARKS

Somatic embryogenesis has been induced in *Q. ilex* by culturing immature zygotic embryos (Mauri and Manzanera, 2003) and leaves (Feraud-Keller and Espagnac, 1989).

To overcome the difficulties encountered in plant production, efforts were devoted during the last 10 years to understand different physiological aspects of this species. This approach was beneficial as it helped to develop a complete protocol of somatic embryogenesis, that is from induction of embryogenic tissues to plants in the field passing through short-term preservation of embryos, *in vitro* acclimatization, etc. Furthermore, most of these conditions are directly applicable to other species.

In conclusion, somatic embryogenesis can be induced in holm oak immature zygotic embryos during a narrow developmental window in August. A possible explanation is that at the end of the maturation period in September, the tissues of late zygotic embryos become more specialised and lose embryogenic capacity. The exogenous application of 0.1 to 1 μM ABA to immature somatic embryos induces maturation and lowers secondary embryogenesis, improving synchronisation of the cultures. The main factor for breaking dormancy and promoting germination in holm oak somatic embryos is a stratification treatment at 4ºC for two months.

Further research work should be devoted to the optimisation of the maturation rates and to the improvement of plant acclimation to greenhouse and nursery conditions. This would secure the large-scale production of holm oak somatic seedlings.

REFERENCES

Bellarosa R (1989) Oak (*Quercus* spp.). In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol. 5 Trees II. (387-401) Springer-Verlag, Berlin, Heidelberg.

Bueno MA, Astorga R & Manzanera JA (1992) Plant regeneration through somatic embryogenesis in *Quercus suber*. Physiol Plant 85: 30-34.

Bueno MA, Gómez A, Vicente O & Manzanera JA (1996) Stability in ploidy level during somatic embryogenesis in *Quercus canariensis*. In: MR Ahuja, W Boerjan & DB Neale (eds). Somatic Cell Genetics and Molecular Genetics of Trees. (23-28). Kluwer Academic Publishers ISBN 0-7923-4179-1.

Bueno MA, Gómez A, Boscaiu M, Manzanera JA & Vicente O (1997) Stress induced haploid plant production from anther cultures of *Quercus suber*. Physiol Plant 99: 335-341.

Chalupa V (1990) Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). Plant Cell Reports 9: 398-401.

Cornu D, Delran S, Garbaye J & Le Tacon F (1977) Research of the best rooting conditions for green shoots of oak (*Q. petraea* (M) Liebl.) and beech (*Fagus sylvatica* L.) (in French). Ann Sci For 34: 1-16.

Cuenca B, San José MC, Martínez MT, Ballester A & Vieitez AM (1999) Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. Plant Cell Reports 18: 538-543.

Féraud-Keller C & Espagnac H (1989) Conditions for the appearance of somatic embryogenesis on callus from leaf tissue cultures of holm oak (*Quercus ilex*) (in French). Can. J. Bot. 67: 1066-1070.

Fernández-Guijarro B, Celestino C and Toribio M (1995) Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber* L. Plant Cell Tiss. Org. Cult. 41: 99-106.

Finch-Savage, W.E.; Clay, H.A. (1994) Evidence that ethylene, light and abscisic acid interact to inhibit germination in the recalcitrant seeds of *Quercus robur* L. Journal of Experimental Botany. 45: 1295-1299.

Gamborg OL, Miller RA & Ojima K (1968) Nutrient requirement of suspensions cultures of soybean root cells. Exp. Cell. Res. 50:151.

Garcia-Martin G, Gonzalez-Benito ME & Manzanera JA (2001) *Quercus suber* L. somatic embryo germination and plant conversion: pretreatments and germination conditions. In Vitro Cellular and Developmental Biology-Plant 37: 190-198.

Gingas VM & Lineberger RD (1989) Asexual embryogenesis and plant regeneration in *Quercus*. Plant Cell Tiss. Org. Cult. 17: 191-203.

González-Benito, M.E.; García-Martín. G.; Manzanera, J.A. (2002) Shoot development in *Quercus suber* L. somatic embryos. In Vitro Cellular and Developmental Biology-Plant 38: 477-480.

Kim YW, Lee BC, Lee SK & Jang SS (1994) Somatic embryogenesis and plant regeneration in *Quercus acutissima*. Plant Cell Reports 13: 315-318.

Kim YW, Youn Y & Noh ER. (1995) Somatic embryogenesis and germination from immature embryos of *Quercus variabilis*. Research Report of the Forest Genetics Research Institute Kyonggido 31: 147-152.

L'Helgoual´ch M & Espagnac H (1987) First observations on the adventitious rhizogenic capacity of holm oak (*Quercus ilex* L.) (in French). Annal Sci For 44: 325-334.

Manzanera, J.A.; Astorga, R.; Bueno, M.A. (1993) Somatic embryo induction and germination in *Quercus suber* L. Silvae Genetica. 42: 90-93.

Manzanera JA & Pardos JA (1990) Micropropagation of juvenile and adult *Quercus suber* L. Plant Cell Tissue Organ Cult 21: 1-8.

Manzanera JA, Bueno MA and Pardos JA (1996) *Quercus robur* L. (Pedunculate Oak). In: Bajaj YPS (ed). Biotechnology in agriculture and forestry 35. Trees IV.

(321-341) Springer Verlag, Berlin
Mauri PV (1999) Inducción de embryogenesis somática en encina y estudios sobre los procesos de desarrollo y maduración. Tesis doctoral: 1-235. UPM. Madrid.
Mauri PV & Manzanera JA (2003) Induction, development and maturation of holm oak (*Quercus ilex* L.) somatic embryos. Plant Cell Tissue Organ Cult 74: 229-235.
Murashige T & Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
San José MC, Vieitez AM & Vieitez E (1985) Establishment and in vitro multiplication of shoots from genus *Quercus* (in Spanish). Fiton 45: 31-40.
Sánchez MC, San Jose MC, Ballester A & Vieitez AM (1996) Requirements for in vitro rooting of *Quercus robur* and *Quercus rubra* shoots derived from mature trees. Tree Physiology 16: 673-680.
Schenk RU & Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50: 199-204.
Vieitez AM, Pintos F, San Jose MC & Ballester A (1993) In vitro shoot proliferation determined by explant orientation of juvenile and mature *Quercus rubra* L. Tree Physiology 12: 107-117.

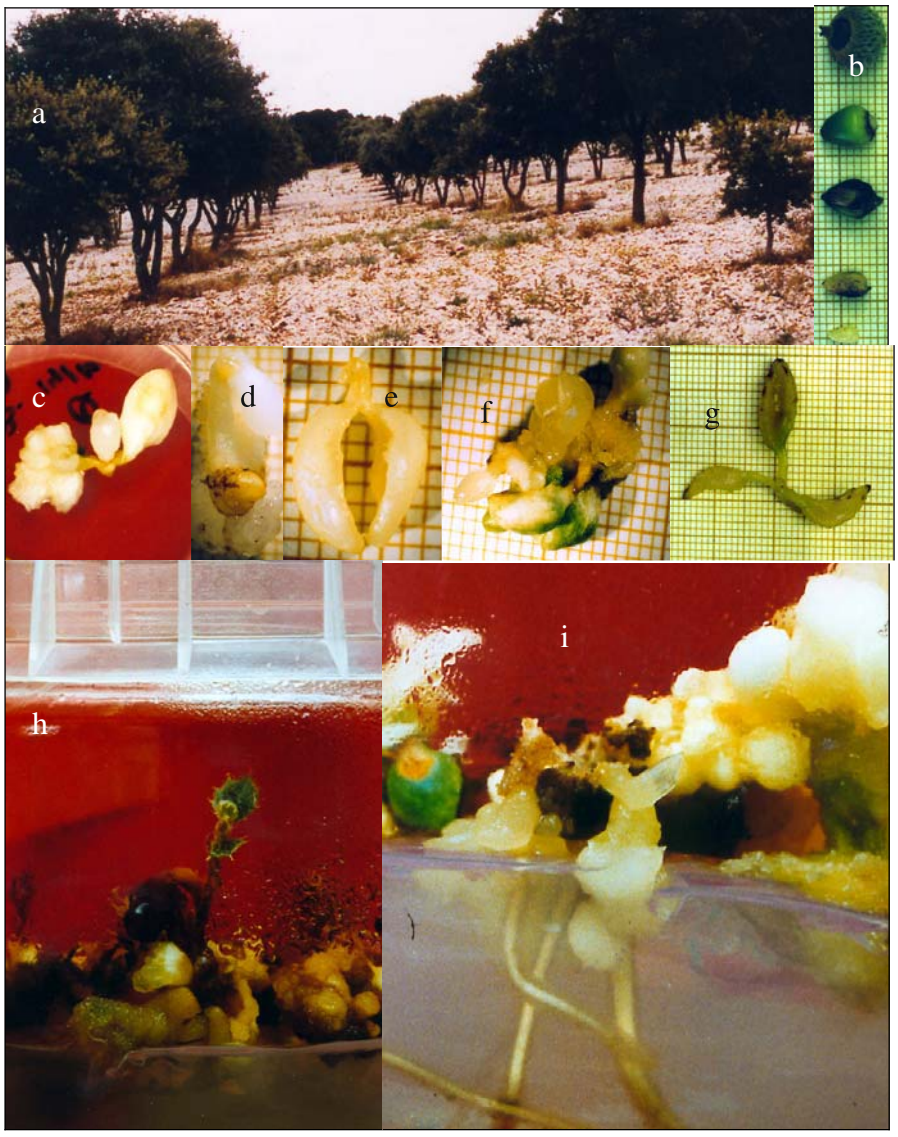

Figure 1. Somatic embryogenesis in Holm oak: (a) Holm oak trees (b) Holm oak acorns (c) Somatic embryos on induction medium. (d) Zygotic embryo in solid medium. (e; f) Somatic embryos matured according to solid maturation medium and (g) liquid medium. (h) Plantlet from a somatic embryo on germination medium and (i) Somatic embryos on germination medium.

Table 1. Composition of the different media used for the different stages of somatic embryogenesis in Holm oak.

	Induction medium (G*) (mg/L)	Maturation medium (SH*) (mg/L)	Germination medium (SH*) (mg/L)
KNO_3	2500	2500	2500
$MgSO_4.7H_2O$	250	400	400
$CaCl_2.2H_2O$	150	200	200
$(NH_4)_2 SO_4$	134	-	-
$NaH_2 PO_4.H_2O$	150	300	300
KI	0.83	0.83	0.83
H_3BO_3	6.2	6.2	6.2
$MnSO_4.H_2O$	16.9	16.9	16.9
$ZnSO_4.7H_2O$	8.6	8.6	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025
$CoCl.6H_2O$	0.025	0.025	0.025
$FeSO_4.7H_2O$	278.5	278.5	278.5
Na_2-EDTA	372.5	372.5	372.5
Nicotinic Acid	0.5	0.5	0.5
Pyridoxine-HCL	0.5	0.5	0.5
Thiamine-HCl	0.1	0.1	0.1
Myo-inositol	100	100	100
Glycine	2.0	2.0	2.0
Sucrose	3 %	3 %	3 %
BA	0.5-10* μM	-	-
NAA	0.5-10* μM	-	-
ABA	-	0.1-1μM	-
Sigma, Type E-agar	0.6	0.6	0.6
pH	5.8	5.8	5.8

* 10.0 μM = for the first month.

* 0.5 μM = for the second month.

Table 2. Effect of plant growth regulator composition on the percentage of somatic embryo induction. in Holm oak. BA: benzyl-adenine; NAA: naphthalene acetic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid. Percentages followed by the same letter were not significantly different at the 0.05 level.

Plant growth regulators	**Induction percentage (%)**
10 μM BA + 10 μM NAA	40 a
10 μM BA + 3 μM 2,4-D	30 a
5 μM 2,4-D	20 ab
3 μM 2,4-D	0 b
Control	33 a

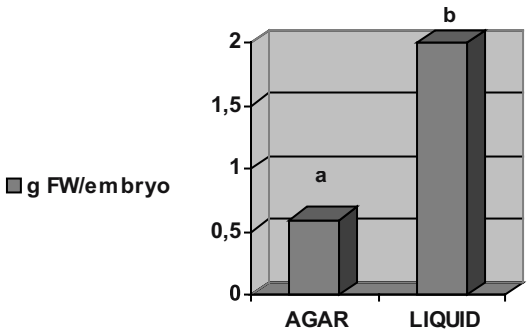

Figure 2. Holm oak somatic embryo mean fresh weight (FW, g) after 30 days in culture, either in agar-solidified (AGAR) or in liquid medium (LIQUID). Treatments with the same letter are not significantly different at the 0.05 level.

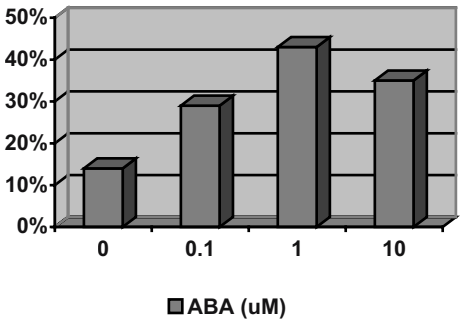

Figure 3. Maturation percentage (%) of Holm oak immature somatic embryos cultured in SH medium with the addition of ABA for 11 subcultures. Treatments with the same letter are not significantly different at the 0.05 level.

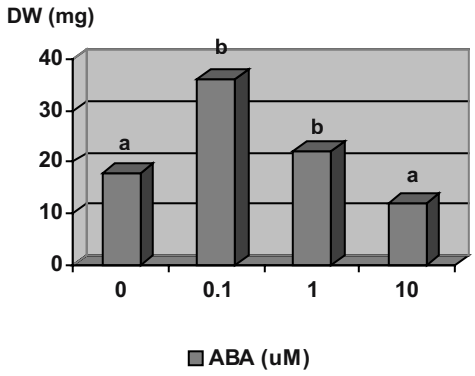

Figure 4. Dry weight (DW, mg) of Holm oak somatic embryos matured in SH medium with the addition of ABA for 11 subcultures. Treatments with the same letter are not significantly different at the 0.05 level.

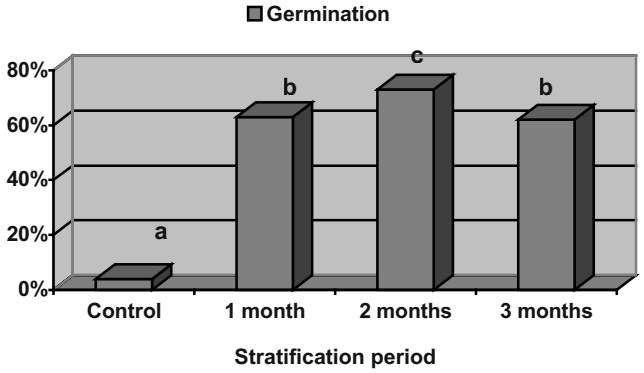

Figure 5. Percentage of germination of Holm oak mature somatic embryos previously cultured on SH medium with the addition of 0.1 μM ABA for 11 subcultures, and then stratified at 4 ºC for different periods. Treatments with the same letter are not significantly different at the 0.05 level.

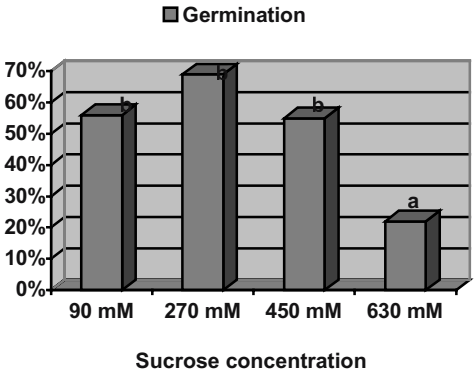

Figure 6. Germination percentage of Holm oak mature somatic embryos subjected to different sucrose concentrations in the germination medium, after two months stratification at 4 ºC. Treatments with the same letter are not significantly different at the 0.05 level.