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The Development of a Protocol for the Encapsulation-desiccation and *In Vitro* Culture of Embryonic Axes of *Quercus suber* L. and *Q. ilex* L.

By M. E. GONZÁLEZ-BENITO[1]), E. HERRADÓN[1]) and C. MARTIN[2])

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Summary

Quercus species have seeds recalcitrant against long-term storage. Cryopreservation of embryonic axes could be a feasible way of preserving their genetic diversity. Several cryopreservation protocols are based on desiccation, among them the so-called encapsulation-dehydration. However, it is previously necessary to establish an adequate *in vitro* culture development of desiccated axes. Embryonic axes of *Q. suber* and *Q. ilex* were aseptically excised, encapsulated in alginate beads, cultured in a sucrose-rich liquid medium, desiccated for different periods in a flow bench and cultured on basal WPM medium. Moisture content of encapsulated axes dropped from 74% to 71% (fresh weigh basis) to 24.5% to 21% after 6 h desiccation, respectively for the two species. Germination decreased to 20% in both species. Germination and shoot elongation of encapsulated embryos (non-desiccated or desiccated for 4 h) was studied for both species after culture on WPM medium supplemented with different concentrations of BAP and IBA. Medium with 0.1 mgl^{-1} BAP resulted in a high percentage of germination and development of shoots in both species.

Key words: aliginate bead, dehydration, *in vitro* culture, oak, *Quercus,* recalcitrant seed.

FDC: 165.442; 163; 176.1 *Quercus ilex;* 176.1 *Quercus suber.*

Introduction

In temperate developed areas, forests have suffered a reduction in their distribution due to the destruction of their habitat by human activities which led in many cases to habitat fragmentation (MCNEELY *et al.,* 1995). These events could provoke the genetic impoverishment of forest species. The genus *Quercus* is one of the most important ones of temperate regions of the northern hemisphere. Many species are autochthonous to the Iberian Peninsula and have a protective role in the ecosystems, besides their social and economic importance.

Seed or embryo cryopreservation is an adequate alternative for the storage of species with recalcitrant seeds (GROUT, 1986). However, the number of species where appropriate cryopreservation protocols have been developed is still limited (PENCE,

M. ELENA GONZÁLEZ-BENITO[1]), ESTHER HERRADÓN[2]) and CARMEN MARTÍN[2]), Departamento de Biología Vegetal, [1])Escuela Universitaria de Ingeniería Técnica Agrícola, [2])Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid, Ciudad Universitaria, 28040 Madrid, Spain. egonzalez@agricolas.upm.es.

1995). This low number is not surprising due to the difficulties of working with recalcitrant seeds. First of all, their short longevity makes it difficult to have available an amount of seeds of similar characteristics for a period of time long enough to carry out several assays. Secondly, some of the cryopreservation techniques are based on desiccation, to avoid the formation of ice crystals. Besides, the physiological and genetic variability of the starting plant material of different harvests adds further inconveniences (ENGELMANN, 1997).

Although attempts have been made to cryopreserve whole seeds (AHUJA, 1991), it is necessary, in many cases, to excise the axes and cultivate them *in vitro* in order to apply cryopreservation protocols to recalcitrant seeds. The cryopreservation of *Quercus* sp. embryonic axes and their subsequent *in vitro* culture often have resulted in callus growth, instead of organised growth (CHMIELARZ, 1997; PENCE, 1990, 1992). However, few published works have studied the effect of the culture medium on embryo or embryo axis development. POULSEN (1992) observed that embryo axes of *Quercus robur* developed better on WPM (LLOYD and MCCOWN, 1981) than on MS (MURASHIGE and SKOOG, 1962) medium.

We describe preliminary work carried out to determine the appropriate method of *in vitro* culture of *Q. suber* and *Q. ilex* embryo axes and the interaction with their encapsulation-desiccation as a first step to develop a cryopreservation protocol.

Materials and Methods

Plant material

Acorns of *Q. suber* L. and *Q. ilex* L. were collected in the autumn, sprayed with fungicide (Benlate 4 g l^{-1} and Previcur 3 ml l^{-1}) and stored in a chamber at 5 °C and 80% to 90% RH. Previously to their use in each experiment, acorns were placed in a beaker with water and floating ones were discarded. Acorns were washed with soap and water, pericarp was removed and cut, discarding the half without the embryo axis. The other halves were immersed in a 15% commercial bleach solution (0.75% NaOCl final concentration) for 20 min. After one rinse with sterile distilled water, embryo axes were removed and left in a Petri dish with 50 mgl^{-1} ascorbic acid sterile solution for at least 30 min, and covered with aluminium foil. Subsequently, that solution was removed and replaced by a 10% solution of commercial bleach. After 5 min, axes were rinsed three times with sterile distilled water.

Encapsulation-desiccation

Axes were encapsulated in alginate beads as follows: they were suspended in a medium-viscosity alginate solution (Sigma, 3% w/v, prepared in liquid WPM medium, with 0.35 M sucrose and no Ca^{+2}), drops of this solution, each containing one axis, were dispensed with a sterile pipette into WPM liquid medium supplemented with 100 mM $CaCl_2$, and kept there for at least 30 min. Subsequently, beads were incubated for 19 h in WPM liquid medium containing 0.75 M sucrose, shaking at 130 rpm. Beads were then removed from the medium and their surfaces dried with sterile filter paper. For desiccation, 20 beads were placed in an open Petri dish (9 cm diameter) and left in the airflow (0.46 m s^{-1}) of a laminar flow bench for different periods.

Moisture content determination

Encapsulated axes (axes surrounded by the alginate bead) were dried in an oven at 130 °C for 2 h and subsequently weighed after cooling in a chamber with silica gel; moisture content was expressed as percentage of fresh weigh. The actual moisture content of the axes was only determined in *Q. ilex* removing the alginate bead just after the 19 h culture in sucrose-rich liquid medium for non-desiccated ones or after desiccation, and following the same procedure as before.

In vitro culture

Each axis was cultured in a test tube containing 10 ml WPM medium, supplemented with 0.09 M sucrose; pH was adjusted

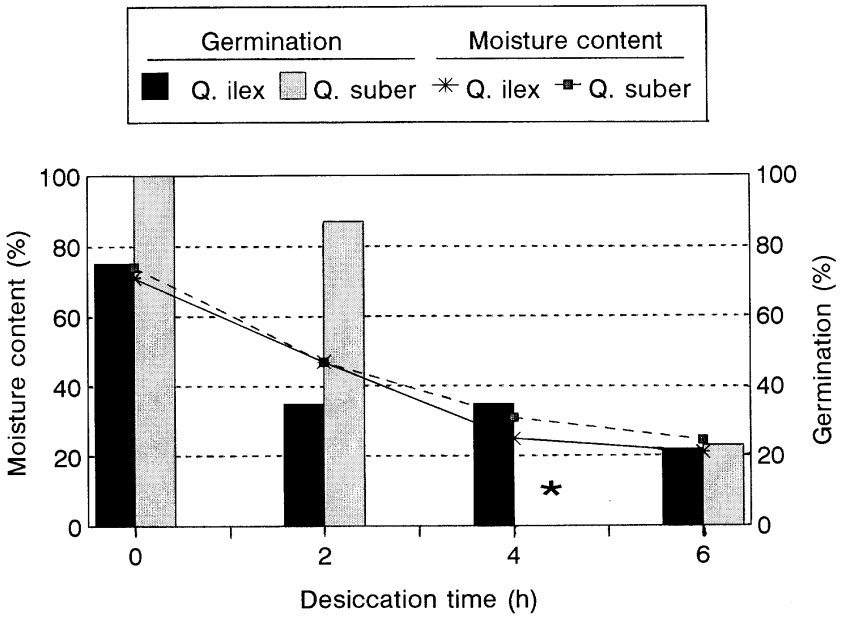


Figure 1. – Moisture content (fresh weigh basis) and germination percentage (after four weeks in culture) of encapsulated embryonic axes of *Quercus ilex* and *Q. suber* desiccated for different periods in a flow bench. (*: for *Q. suber* this treatment was not considered due to the high contamination percentage).

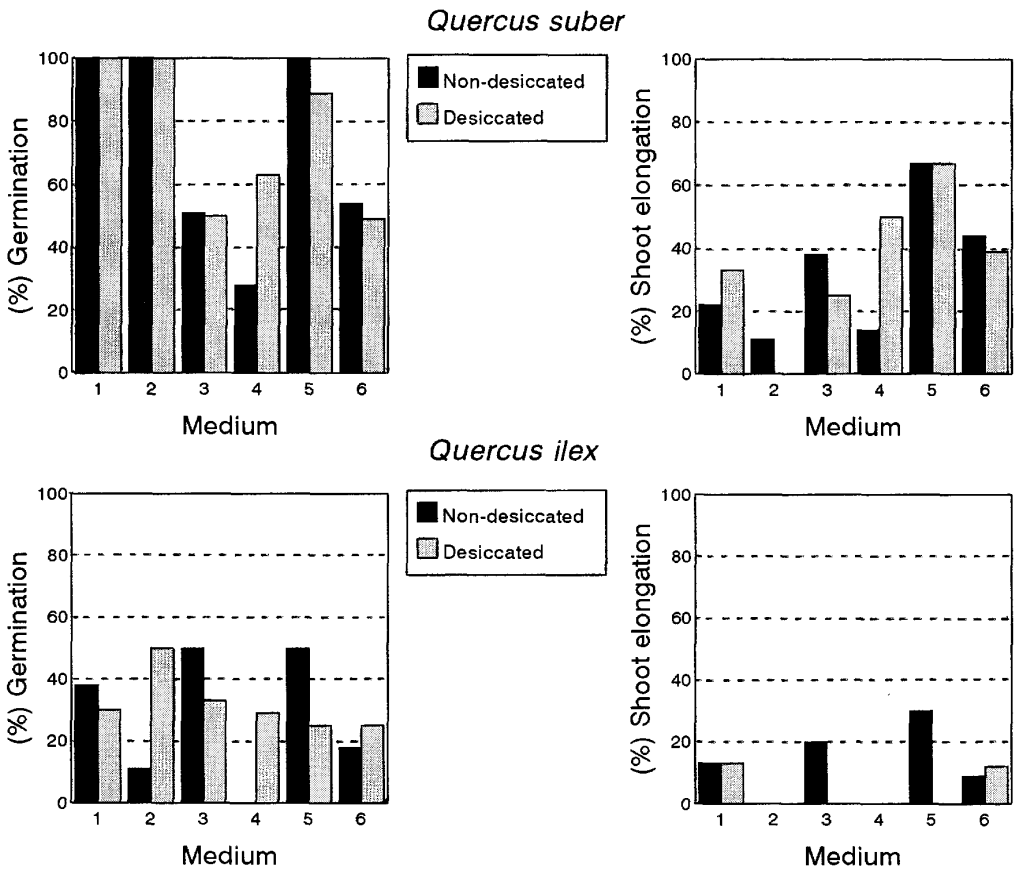
to 5.7 to 5.8. Agar (Difco, 6 gl^{-1}) was added prior autoclaving for 20 min (120 °C, 1 atm). Incubation took place at 25 ± 2 °C in darkness for the first week and afterwards with a 16 h photoperiod and an irradiance of 50 µmol m^2s^{-1}, provided by cool white fluorescent tubes. Twenty axes were cultured per treatment in each experiment.

In a first experiment encapsulated axes were desiccated for several periods and their germination on basal WPM medium and the moisture content were studied. The axes were obtained from acorns stored for 4 months. In a second experiment, encapsulated axes (desiccated for 4 h or not) were cultured on WPM medium containing different concentrations of BAP (6-benzylaminopurine) and/or IBA (indole-3-acetic acid). In this experiment axes were excised from acorns stored for 6 months. The effect of preculture of embryo axes, before encapsulation, for two days on sucrose-rich WPM semi-solid media (0.3 M, 0.5 M and 0.7 M sucrose) was studied in a third experiment with *Q. suber* (acorns had been stored for 7 months). After the culture of beads in WPM liquid medium + 0.75 M sucrose, half of them were desiccated for 4 h in the flow bench.

In all experiments, germination percentage (axes which showed either root or shoot elongation, or both, respect to non-contaminated axes) was recorded after four weeks in culture. In one experiment, percentage of shoot elongation (axes which showed shoot elongation respect to non-contaminated axes) was also considered.

Results and Discussion

Moisture content of encapsulated axes (axis + alginate bead) dropped from 71% and 74% to 21% and 24.5% after 6 h desiccation, respectively for *Q. ilex* and *Q. suber*. After that dehydration period germination decreased to approximately 20% in both species *(Fig. 1)*. Germination was in general high for *Q. suber* in mild desiccated or control axes, and germination dropped rapidly in *Q. ilex* embryos after 2 h dehydration. It was observed (in *Q. ilex*) that the actual axes moisture content was lower than that of the whole structure (alginate bead + axis) in non-desiccated ones (60.5% *vs* 71%), but it got closer with desiccation, until being very similar after the 6 h treatment (48% for 2 h, 22% for 4 h and 20% for 6 h).



Statistical analysis : Kruskal-Wallis					
Variation Source	Factor	*Q. suber*		*Q. ilex*	
		$Pr > \chi^2$	Significance level	$Pr > \chi^2$	Significance level
Medium	Germination	0,0002	***	0,2301	NS
	Shoots	0,0008	***	0,5445	NS
Desiccation	Germination	0,05	*	0,992	NS
	Shoots	0,6494	NS	0,1556	NS

NS= no significant

Figure 2. – Effect of growth regulator concentration on germination and shoot elongation percentages (after four weeks in culture) of embryo axes of *Q. suber* and *Q. ilex* encapsulated in alginate beads and desiccated for 4 h in the flow bench or not. Growth regulators combinations (mgl^{-1}): 1 = basal medium, 2 = 0.1 IBA, 3 = 0.01 BAP, 4 = 0.01 BAP + 0.1 IBA, 5 = 0.1 BAP, 6 = 0.1 BAP + 0.1 IBA.

Table 1. – Effect of a 2-day preculture of embryo axes of *Q. suber* before encapsulation and desiccation for 4 h, after four weeks in culture.

Sucrose concentration in preculture medium	Germination (%)	
	Non-desiccated	Desiccated
0,3 M	30	0
0,5 M	20	11
0,7 M	50	44

The response of desiccated and non-desiccated encapsulated axes was studied after culture in six combinations of BAP and IBA *(Fig. 2).* In *Q. suber,* medium without growth regulators or supplemented with 0.1 mgl^{-1} IBA or with 0.1 mgl^{-1} BAP resulted in a high percentage of axes germination. However, the last medium induced the highest percentage of shoot development for desiccated and non-desiccated axes. The germination response in *Q. ilex* was lower in all media compared with *Q. suber.* Shoot elongation was low, especially in desiccated axes, the maximum being 30% on medium supplemented with 0.1 mgl^{-1} BAP. The media with 0.1 mgl^{-1} BAP resulted in many cases in multiple shoots development in both species.

The use of 0.7 M sucrose in the preculture medium resulted in a higher germination percentage, especially after desiccation *(Table 1).* This effect of the sucrose, the protection against dehydration, has been reported previously in vegetative explants (González-Arnao *et al.,* 1996).

To use cryopreservation techniques based on encapsulation-dehydration it is necessary to obtain the appropriate development of axes after desiccation. Culture medium should be optimised to detect possible damage due to desiccation and freezing (Pence, 1995). In the two species tested in this work, shoot elongation was favoured by the use of 0.1 mgl^{-1} BAP, without the root development being affected. This fact could be important for the appropriate establishment of the plantlets in *ex vitro* conditions. In nature, *Quercus* sp. embryos produce a long taproot before shoot elongation. A development similar to that observed in nature has been already described with the use of BAP in the culture medium for *Q. suber* embryo axes (Bellarosa, 1981). The BAP concentration which resulted in the best results in the present work (0.1 mgl^{-1}) was the one which produced the highest shoot development and number of lateral shoots in the *in vitro* culture of apical shoots of *Q. suber* (Manzanera and Pardos, 1990).

A first step in the development of a cryopreservation protocol has been established by the appropriate growth of embryo axes of *Q. suber* and *Q. ilex* in culture after encapsulation and desiccation to approximately 20% moisture content.

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