Tree and Forestry Science and Biotechnology ©2011 Global Science Books

Application of Plant Regeneration of Selected Cork Oak Trees by Somatic Embryogenesis to Implement Multivarietal Forestry for Cork Production

Inmaculada Hernández[1] • Beatriz Cuenca[2] • Elena Carneros[1] • Nieves Alonso-Blázquez[1] • Mar Ruiz[1] • Cristina Celestino[1] • Luis Ocaña[3] • Jesús Alegre[1] • Mariano Toribio[1*]

[1] Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario (IMIDRA). Finca El Encín. Apdo. 127. 28800 Alcalá de Henares, Madrid, Spain
[2] TRAGSA. Departamento de Mejora Agroforestal. Crta. Maceda-Valdrey, km.2, 32708 Maceda. Ourense, Spain
[3] TRAGSA. Subdirección de I+D+i. c/ Julián Camarillo, 6b, 28037 Madrid, Spain

Corresponding author: * **mariano.toribio@madrid.org**

ABSTRACT

To implement multivarietal forestry, the availability of an efficient and economically viable methodology for vegetative propagation is required. In addition field tested varieties have to be developed. Somatic embryogenesis is increasingly being considered as the most appropriate way of cloning forest species. The cork oak (*Quercus suber* L.), one of the most important Mediterranean tree species, has been considered recalcitrant to vegetative propagation for long time. In this article we review the current status of plant regeneration by somatic embryogenesis in this species, and report on a joined effort between IMIDRA, a public research institution, and TRAGSA, a public company, to apply this tissue culture derived methodology to cork oak genetic improvement. At IMIDRA, a protocol to induce somatic embryogenesis in leaves of adult cork oak trees, applicable to any genotype, that allow to clone elite trees has been developed. In the frame of the project SEFEAL-2 led by TRAGSA, this protocol is being implemented with the goal of cloning trees, selected for their cork quality and productivity in Extremadura (Spain). Data on current frequencies of induction of embryogenic lines, and of plant conversion and survival at the operational level are provided.

Keywords: *Quercus suber* L., tree biotechnology, cloning, *in vitro* plant tissue culture
Abbreviations: ABA, abscisic acid; **AFLP**, amplified fragment length polymorphism; **BA**, benzyladenine; **2,4-D**, 2,4-diclorophenoxy-acetic acid; **MVF**, multivarietal forestry; **NAA**, 1-naphthaleneacetic acid; **PGR**, plant growth regulator; **RAPD**, random amplified polymorphic DNA; **SE**, somatic embryogenesis; **SSR**, simple sequence repeats (microsatellites)

CONTENTS

INTRODUCTION

In programs of conservation and improvement of genetic resources, besides studying the variability of natural populations and determining criteria for selection of plant material to conserve or to use for breeding, the technique of propagation is essential and conditions the results to be obtained. Vegetative propagation, by exploiting the non-additive genetic variation, transfers to the offspring all the genetic potential of selected individuals, while giving uniformity (Zobel and Talbert 1984). In species in which it was possible to use vegetative propagation techniques, cloning as a mean of genetic improvement has been the option. Examples are among woody crops (olive, vine, fruit trees), but also among forest species (poplar, eucalyptus, etc.). However, vegetative propagation has been almost impossible for many forest species, mainly when using adult trees as ortets.

Plant regeneration by somatic embryogenesis (SE) is currently one of the main tools of plant biotechnology, because it allows for the practical application of different biotechnologies, such as the obtaining of virus-free plants and genetic transformation. It also allows the deployment of vegetative propagules with operational purposes in species in which it was not possible before (Merkle and Nairn 2005; Nehra *et al.* 2005). Moreover, due to the capacity of the embryogenic material to undergo cryopreservation, it is possible to maintain the juvenile potential of propagation while clones are evaluated. Tested clones become varieties to be deployed in plantations, allowing the development of multivarietal forestry, MVF (Park 2004). This strategy has been started for some species, mainly among conifers (Kli-

Received: 3 June, 2009. Accepted: 11 September, 2010.

Invited Review

maszewska *et al.* 2007).

The development of vegetative propagation methods has been consider of great interest in the genetic improvement of *Quercus* species (Savill and Kanowski 1993), since the production of improved seeds based on traditional seed-orchards is largely hampered by problems of graft incompatibility and irregular acorn production. The capacity for vegetative propagation of the species of this genus by traditional methods is very low. A lot of studies on plant regeneration by somatic embryogenesis of *Quercus* species have been published in the last twenty years (Wilhelm 2000). The present review describes the work done with one of their species, the cork oak, and provides data on the beginning of the implementation of MVF with material from selected trees of this species. There are few studies reporting induction of somatic embryogenesis (SE) in tissues from mature trees (Khaw *et al.* 1998; Merkle and Battle 2000; Conde *et al.* 2004; Toribio *et al*. 2004), but the cork oak is one of the forest species where it is now possible to capture all the genetic potential of selected phenotypes, and to transfer it to the vegetative progeny.

THE CORK OAK

The cork oak (*Quercus suber* L.) is one of the most characteristic woody species in the Mediterranean ecosystem. It belongs to the Section *Suber* of the subgenus *Cerris*. It is a medium size, evergreen tree, which reaches up to 20-25 m tall and has a broad crown (**Fig. 1A**). The growing of cork oak trees is variable, depending on the growing area. Flowering occurs over a long period, usually from April to June. Fruiting begins when the tree is around 15 to 20 years old. The fruit (acorns) does not all ripen at the same time; they can appear from September to February, most of them in October or November. Cork oak, as Mediterranean species, grows in areas with dry and hot summers and wet and warm winter. It usually grows in soils with low calcium content. As it can not stand frost, it does not spread in cold areas or high mountains.

There is no doubt that this species is of Mediterranean origin, although there are doubts about where diffusion centre was located, since there are authors that locate it in North Africa, others in the Atlantic region of the Iberian Peninsula or even in the area covered today by the Tyrrhenian Sea. There are also doubts about the spreads of cork oak and, in particular, how it managed to survive the Ice Age. It is thought that thanks to its polymorphic qualities the species continued to evolve and produce current biotypes (Anonymous 2000). The tree has spread mainly in the Mediterranean area under Atlantic influence, in the South of Europe and North Africa. The Iberian Peninsula has the biggest area (Portugal with 33% and Spain with 23%), followed by Argelia, Morocco, Italy, Tunisia and France (Anonymous 2000; IPROCOR 2006).

Besides having a great ecological value for the Mediterranean ecosystem, cork oak has also a great economic interest, since it is the only cork producing tree, its most distinctive characteristic. Cork is a natural and renewable product with diverse applications. It is produced by the phellem, a secondary meristem, in trunk and branches and is composed of death cells with trapped air, whose walls contain high amounts of suberin that confer cork very good gas and water insulating properties. This special bark has the natural mission to protect tree against fires, which often occur in Mediterranean forests. It regenerates after stripping. The first stripping of cork is made when the tree is 30-40 years old. This first cork, virgin cork, is thick and irregular. Cork is more uniform, and therefore more valuable, after every subsequent stripping. The commercial cork is stripped without severe damage at regular interval, usually every 8-14 years depending on the area, when it is 25 mm of thickness (**Fig. 1B**). It is stripped usually from June to August, period when the cork comes free from the trunk more easily (Caritat *et al.* 2000). There is a positive correlation between the thickness of cork and the rainfall, especially in fall and winter. Drought and/or temperature of dry season can also limit cork growth (Caritat *et al.* 2000). Average production per tree with normal development is 40-60 Kg of cork for each stripping. The quality of the cork begins to decline after 150 to 200 years, the age limit of the cork oak for commercial purposes; although in many cases the tree continues to grow to 250-350 years old (Anonymous 2000).

The extraordinary characteristic and properties of cork explains the interest in this natural product. Their properties and applications have been reviewed recently (Silva *et al.* 2005). The main use is in wine bottling, since wines that have to be aged in the bottle for many years require this high-quality closure. Currently it has also other uses in craftsmanship and industry: beehives, footwear, vehicle joints, building isolation, etc. Novel applications of cork include the use of by-products generated during processing of corkboards. Moiteiro *et al.* (2001) reported that some of them act as inhibitors of human lymphocyte proliferation and of the growth of a human cancer cell line. Recently, it has been described that phenolic compounds isolated from cork showed antioxidant activity, namely antiradical and reducing properties, and growth inhibitory effect on a human breast cancer cell line and two colon cancer cell lines (Fernandes *et al*. 2009).

Currently, cork production determines the management models of the cork oaks in Spain, although its interest is not exclusively in cork production. They produce other valuable products as the acorns, very appreciated for feeding Iberian pork, which involve also a high quality food industry; mushroom and several wild plants used as food and seasoning; its wood is also used to make tools and as excellent firewood and charcoal, etc.

Besides its economic interest, cork oaks have a great social and ecological value. They form part of open woodlands called “montados” in Portugal and “dehesas” in Spain. They are managed as silvopastoral systems that are very important for the rural development of the zone. Nowadays cork oaks are threatened because of forest fires, excess shepherding, tree decay, difficult natural regeneration and commercial competition of cork substitutes (Montero *et al*. 1998; Varela 1999; IFAPA 2006).

In the Iberian Peninsula 90% of cork oak woodlands belong to private owners, making difficult the development of improvement plans and strategies for conservation purposes (López de Heredia and Gil 2006). Although market demand of cork for wine bottling in Spain is increasing, the cork production has noticeably decreased in the last decades, with the consequence that, in the short term, low quality cork is been used. Therefore, it is necessary to undertake the cork oak reforestation in an effective and sustainable way, what needs of studies on the genetic characteristics of the species and of the implementation of genetic improvement plans.

INDUCTION OF SOMATIC EMBRYOGENESIS

Earlier embryogenic responses were observed in cotyledons of zygotic embryos (Toribio 1986; Toribio and Celestino 1989) and in nodal segments of seedlings (El Mâataoui and Espagnac 1987). However, as in most species, immature zygotic embryos proved to be highly responsive to the induction of somatic embryogenesis (El Mâataoui and Espagnac 1989). Complete plantlet regeneration from somatic embryos obtained from immature zygotic embryos was soon reported (Bueno *et al.* 1992; Manzanera *et al.* 1993). The induction response was obtained on medium containing different levels of 2,4-diclorophenoxyacetic acid (2,4-D), and the strong influence of the developmental stage of zygotic embryos on the frequency of somatic embryogenesis was evidenced. Moreover, induction could be achieved without plant growth regulators from zygotic embryos at the right stage of development (Fernández-Guijarro 1997).

The most important milestone in the regeneration of cork oak plants by somatic embryogenesis was the induction and plant recovery from leaves of young plants

Fig. 1 Cork oak, cork, and plant regeneration of selected cork oak trees by somatic embryogenesis. (**A**) *Elite* cork oak tree growing in a natural stand in La Almoraima (Cádiz, Spain). (**B**) Stripping cork from the trunk (Courtesy of the Institute CMC-IPROCOR. Extremadura, Spain). (**C**) Induction of somatic embryogenesis in an expanding leaf collected from an epicormic shoot. (**D**) Germinating somatic embryo. Bar = 1 cm. (**E**) Acclimatised somatic seedlings in 6 cm diameter forest containers. (**F**). Field trial of cork oak somatic seedlings from selected trees and their progenies established in Villaviciosa de Odón (Madrid, Spain).

(Fernández-Guijarro *et al.* 1994, 1995). SE induction was achieved with a three step induction process. All steps were carried out on the same basal medium composed by the macronutrients of SH (Schenk and Hildebrandt 1972) and

Table 1 Effect of genotype and time of collection on the induction of somatic embryogenesis in leaves of selected *Quercus suber* trees. N, total number of cultured leaves. SE, frequency of leaves forming somatic embryos.

Genotype	May 1999		January 2001		February 2002		May 2002		February 2003		June 2003		November 2003		Overall mean	
	N	SE (%)	N	SE (%)	N	SE (%)	N	SE (%)	N	SE (%)	N	SE (%)	N	SE (%)	N	SE (%)
Alm1	-	-	-	-	135	23	0	-	161	16	200	0	144	13	640	12
Alm2	-	-	-	-	184	5	108	0	208	5	242	0	116	4	858	3
Alm3	11	91	46	0	156	33	0	-	179	18	27	0	130	43	549	27
Alm4	293	8	48	2	209	12	24	38	179	22	172	0	106	43	1031	14
Alm5	337	38	28	28	201	33	37	5	165	43	169	0	124	5	1061	27
Alm6	260	20	21	19	260	23	6	0	189	12	97	1	103	11	936	16
Alm80	19	16	14	0	185	5	72	7	235	3	195	2	122	10	842	5
Overall mean	920	23	157	8	1330	19	247	7	1316	16	1102	1	845	18	5917	15

From Hernández 2007

the other components from MS (Murashige and Skoog 1962). The primary induction step included 10 μM of benzyladenine (BA) and 10 μM 1-naphthaleneacetic acid (NAA) and was performed in darkness for 30 days. Then the plant growth regulators (PGRs) were reduced to 0.5 μM of BA and 0.5 μM NAA for the secondary induction step, which was carried out in growth chamber with a 16 h photoperiod at 25 ± 1°C for 30 days. The manifestation phase was accomplished on the same medium but lacking PGRs, under the same growing conditions (Fernández-Guijarro *et al.* 1995). During this third month somatic embryos appeared almost directly on the leaf surface, or on a slight proliferation of callus, and began to multiply by secondary embryogenesis (**Fig. 1C**).

A further important advance was the observation that this induction protocol could be successfully applied to leaves from adult trees (Toribio *et al.* 2000). A key decision was to use as initial explants leaves from epicormic shoots obtained from pieces of branches that were forced to sprout under controlled conditions (Vieitez *et al.* 1994). From the first studies the strong influence of genotype on the frequency of induction was evidenced. Somatic embryos were obtained from 12 out of the 19 tested genotypes, with frequencies ranging from 0 to 28% of the cultured leaves (Hernández *et al.* 2001). This protocol is based on the inductive effect of the mixture of NAA and BAP, but the induction of SE in cork oak leaves has also been reported using a mixture of 2,4-D and zeatin (Pinto *et al.* 2002). Additional factors influencing the induction of SE were also identified. The degree of development of leaves has a strong effect, because when they were larger than 1.5 cm embryogenesis could not be obtained. In addition, higher NAA concentrations in the primary induction step led to higher frequencies of induction (Hernández *et al.* 2003a). Early data showed a significant effect of harvesting time of branches on the frequency of SE induction. Although these early data seemed to suggest a seasonal effect, with the highest frequency of induction in the spring than in winter (Hernández *et al.* 2003b), further evidence indicates that the time of collection is more important than the season. **Table 1** show frequencies of SE induction in leaves from seven cork oak trees, selected by their cork quality and productivity in a natural stand (La Almoraima, Cádiz, Spain), whose branches were collected at various times over a period of several years. All effects, genotype, time of collection and their interaction were significant. Except for the collection of January 2001, in which the budding of the branches and the frequencies of induction were very low, the autumn-winter collections are more stable. In general, the spring collections provided lower frequencies of induction. The May 1999 collection gave the highest frequencies of induction, perhaps due to the particular climate conditions of that year. All the selected trees could be cloned by SE (Hernández 2007).

PROLIFERATION AND MAINTENANCE OF EMBRYOGENIC LINES

From the pioneering studies it was observed that cork oak somatic embryos produced secondary embryos easily (El Mâataoui and Espagnac 1989). Moreover, this ability can be maintained for years on medium lacking plant growth regulators (Fernández-Guijarro *et al.* 1995). This feature of the embryogenic system of cork oak assures the continuous supply of embryos through the year, without seasonal limitations, and without apparent decline of the productivity of most embryogenic lines after many years of culture. Genotype influences the proliferation ability of embryogenic lines and the number of detachable embryos that can be obtained from them (Hernández *et al.* 2003b).

Although some abnormalities such as more than two cotyledons and fused embryos are often observed, most cork oak somatic embryos show the same histological structure of their zygotic counterparts (Puigderrajols *et al.* 1996). Secondary embryogenesis usually appears when the primary embryo has reached an advanced stage of differentiation. It is mainly of multicellular origin, and takes place from the surface of the proliferating root cap of the primary embryo (Puigderrajols *et al.* 1996). Embryo axis is competent to form secondary embryos and usually prevents this response in cotyledons, although these are competent when cultured on medium with plant growth regulators (Puigderrajols *et al.* 2000). While the multicellular origin is the main pathway of regeneration in semisolid cultures, the unicellular origin of secondary embryos has also been showed (Puigderrajols *et al.* 1996, 2001). Isolated embryos formed from isolated cells or group of cells detached from the friable mass with embryogenic ability can develop in liquid cultures up to the cotyledonary stage. This possible double origin of somatic embryos should be taken into account when performing techniques of transformation, and when developing protocols for mass propagation in biorreactors.

Cryopreservation of selected material is essential for managing the large number of embryogenic lines required to implement multivarietal forestry, and to conserve valuable material while field tests are in progress. As in most embryogenic cultures, either from hardwood or softwood species (Merkle and Nairn 2005; Klimaszewska *et al.* 2007), embryogenic lines of cork oak can be easily cryopreserved. Somatic embryos were recovered from three cryostored embryogenic lines that underwent a simple vitrification procedure, and germinated at rates similar to control (Valladares *et al.* 2004). Similar success was obtained using encapsulation of embryogenic clusters in an alginate beads and subsequent dehydration previous to freeze in liquid nitrogen. Cryopreserved somatic embryos were viable, and no significant changes were observed in either the ploidy level or patterns of DNA markers, simple sequence repeats (SSRs) and amplified fragment length polymorphisms (AFLPs) (Fernandes *et al.* 2008). Recently, these protocols have been succesfully used to cryopreserve 51 cork oak genotypes in a project involving conservation of field collections (Vidal *et al*. 2010).

EMBRYO MATURATION, GERMINATION AND PLANT RECOVERY

Proper maturation of somatic embryos involves the cessation of secondary embryogenesis, the development of the isolated embryo to the cotyledonary state, and the accumulation of reserve substances. Generally the attainment of all these objectives is carried out through the application of simple maturation treatments to proliferating embryogenic masses. Then, frequencies of germination, conversion into plants and survival of somatic seedlings are considered as indicators of proper embryo maturation. Cork oak embryogenic lines proliferate in semisolid medium without PGRs as embryo clusters with embryos that reach the cotyledonary stage, but usually they remain fused and additional rounds of secondary embryogenesis appear. Sometimes, with frequencies dependant on genotype (Hernández *et al.* 2003b) some isolated embryos without signs of secondary embryogenesis, with large opaque-white cotyledons, can be easily picked out of the proliferating cultures. It is generally considered that these embryos have matured spontaneously. But usually small and translucent immature embryos, isolated from cultures of proliferation, are used as initial explants for maturation treatments. Regularly, maturation treatments in many species involve the application of abscisic acid (ABA) and culture media with high osmolarity.

In cork oak somatic embryos the endogenous ABA concentration increased from the immature to the mature stage (García-Martín *et al.* 2005). However, the effect of exogenous applications of this PGR on maturation is contradictory. While in some cases enhanced the frequency of embryos showing secondary embryogenesis (Fernández-Guijarro 1997), in other cases promoted maturation increasing fresh and dry weight without affecting the relative water content (García-Martín *et al.* 2005). These different effects are probably due to the importance of the state of development of each somatic embryo at the time of applying the ABA. In practice, most protocols for maturation in species of the genus *Quercus* do not incorporate the exogenous addition of ABA (Wilhelm 2000). However, as in other woody species, activated charcoal added to culture medium can be used to reduce secondary embryogenesis and to enhance growth of cork oak somatic embryos (Pintos *et al*. 2010).

Treatments with different osmotica, among which sucrose at high concentration is the most widely used, followed by chilling, have been used to mature and germinate cork oak somatic embryos (Bueno *et al.* 1992; Manzanera *el al.* 1993; Fernández-Guijarro *et al.* 1994, 1995). Data on germination, conversion, and acclimatization of somatic seedlings are variable, likely due to the strong influence of genotype on these responses (Hernández *et al.* 2003b). Conversion frequencies up to 75% when embryos were matured with very high sucrose concentration followed by two-month cold stratification have been reported (García-Martín *et al.* 2001). The application of low concentrations of BAP to germinating cork oak embryos improved shoot development and did not arrest root growth (González-Benito *et al.* 2002). On average, 10% of the somatic embryos induced on leaves of an adult tree germinated and 40% of these germinated embryos converted into plants (Pinto *et al.* 2002). Micorrhization has been used to improve *ex vitro* weaning of *in vitro* produced plants. The synthesis of ectomycorrhizas that promoted development of lateral roots of cork oak somatic embryos was reported (Díez *et al.* 2000).

Interestingly, the quality of the embryos seems to have little influence on the frequency of conversion, but does have an important effect on the frequencies of acclimatization. **Table 2** shows data of conversion frequencies of three types of somatic embryos taken from embryogenic lines from three elite cork oak trees. Type A are spontaneously matured embryos, directly picked out from proliferating cultures (opaque-white, 18 mm average length, 225 mg average fresh weight). Type B are embryos that were selected at an immature stage (translucent, 4 mm average length, 12 mg average fresh weight) and cultured on basal proliferating medium for three four-week subcultures. Type C embryos are equal to those of type B, but remained in the basal medium for the number of subcultures required to meet 25 mm average size and 1,300 mg average fresh weight. Differences in conversion frequencies with genotype were observed, but there was little difference with the type of embryo. However, the converted embryos (**Fig. 1D**) of different types survived at different rates after a six month acclimatization period (**Fig. 1E**), ranging from 5% of type A to 29% of type C (Hernández 2007).

Detailed protocols for cloning cork oak were published (Toribio *et al.* 2005). The encapsulation of cork oak somatic embryos in alginate beads to store them as seeds, and a method for determining the fresh weight of embryos depending on their size have been reported (Pintos *et al.* 2008).

GENETIC STABILITY

Although it was considered that somatic embryogenesis is less susceptible of genetic alterations than other *in vitro* based regeneration techniques because it requires the expression of many different genes (Vasil 1995), there are enough reports on somaclonal variation among somatic embryo derived propagules to justify the need of early monitoring. No variation among the random amplified polymorphic DNA (RAPD) profiles of cork oak somatic embryos within twelve different embryogenic lines was observed. RAPD patterns after several generations of recurrent embryogenesis were stable within genotypes and with time in culture (Gallego *et al.* 1997). When using AFLPs, which produced a significantly higher number of markers, again no change was detected in the pattern of these markers among somatic embryos within the same embryogenic lines (Hornero *et al.* 2001). The embryogenic lines of these studies were obtained from the induction in immature zygotic embryos. However, when embryogenic lines obtained from the induction in leaves from adult trees were studied, a low level of polymorphism (5.6-7.3%) in the AFLP pattern of two out of the three studied lines was detected (Hornero *et al.* 2001). Similarly, uniform microsatellite patterns were generally observed within and between somatic embryos and the respective donor genotypes, except one case that showed a mutation percentage of 2.5% (Lopes *et al.* 2006).

In addition, no changes were found in the ploidy level of somatic embryos and regenerated plants obtained from embryogenic lines induced in leaves of adult trees (Loureiro *et al.* 2005).

Table 2 Conversion frequencies (C) of somatic embryos obtained from three elite cork oak trees (*Quercus suber*), depending on the maturation procedure. A, B and C, maturation procedures. N, total number of cultured somatic embryos.

Genotype	Maturation procedure							
	A		B		C		Overall mean	
	N	C (% ± se)	N	C (% ± se)	N	C (% ± se)	N	C (% ± se)
Alm3	448	55 ± 4	279	63 ± 4	352	62 ± 3	1079	60 ± 2
Alm4	438	34 ± 2	353	28 ± 4	434	42 ± 4	1225	35 ± 2
Alm6	398	32 ± 4	272	41 ± 6	349	45 ± 6	1019	39 ± 3
Overall mean	1284	41 ± 3	904	44 ± 4	1135	49 ± 3	3323	45 ± 2

From Hernández 2007

FIELD TESTING

Although somatic embryos resemble true seeds without coats, the performance of somatic seedlings compared to sexual seedlings has to be tested. Studies comparing emblings and seedling have been published for several species, mainly among conifers such as *Pseudotsuga menziesii* (Benowicz *et al.* 2002) and *Pinus taeda* (Pullman *et al.* 2003), but also for hardwoods such as *Hevea brasiliensis* (Carron *et al.* 2000). Evaluation of large field trials have also been reported (Carron *et al.* 2009). Most of these studies indicate that somatic and zygotic seedlings have similar structural, phenological, and physiological attributes. However, in some cases lower performance of somatic embryo derived plants have been recorded, which may be attributable to different reasons such as aging of callus lines, somaclonal variation or acclimatization problems with root distortions (Carron *et al.* 2009).

Cork oak plants converted from somatic embryos looks like plants raised from acorns (Hernández *et al.* 2003b; Lopes *et al*. 2006), although initial growth is less vigorous and the first leaves are smaller. One of the main concerns when vegetative propagules are obtained from adult donors is that they may retain mature features due to the ageing process. In fact, many clonal propagation programs based on cuttings from hedged mother trees were affected by undesired long-term growth reductions due to precocious ageing (Smith 1999). Therefore, as a protocol for cloning adult cork oak trees by somatic embryogenesis is available, the complete rejuvenation of plants obtained from somatic embryos induced in leaves of such trees has to be proved. A field trial involving clonal material from five selected trees and their half-sib progenies, both seedlings from acorns and somatic seedlings obtained from embryos induced in leaves of young seedlings, has been established (**Fig. 1F**) (Celestino *et al.* 2007). The design allow for the comparisons somatic *vs.* zygotic origin and somatic from mature trees *vs.* somatic from juvenile seedlings, testing also the effect of genotype. After the winter following the implantation in autum all plants from acorns survived, whereas only did half of the somatic seedlings. There were no differences between emblings of adult and juvenile origin. Dead plants were replaced, and the survival rate after the summer was 70%. After the first growing season seedlings doubled in heigth to emblings, whereas no differences were noticed between those of adult and juvenile origin (Celestino *et al.* 2007). After two additional growth periods, somatic seedlings of mature origin grew sligthly better than somatic seedlings of juvenile one, and plants from somatic embryos tended to minimize the initial advantage of plants from acorns (Celestino *et al.* 2009). This initial advantage is likely due to the large amount of reserves that the zygotic embryo accumulates in their cotyledons, many more than the somatic embryo.

SCALING-UP

All the studies mentioned before were performed on semisolid medium and with a limited number of genotypes. The scaling-up of the regeneration process can be viewed from two points of view: (1) the application of the previously defined protocols to many selected genotypes for developing selected varieties of cork oak, and (2) the development of methods of culture in liquid medium and bioreactors, to assure that mass propagation at a reasonable cost of the selected varieties can be carried out when the clonal tests have concluded.

Studies on the second aspect are in progress, dealing with effects of type of vessel and shaking on oxygen exchange, shear forces and their influence on the evolution of embryogenic clusters (Jiménez *et al.* 2009).

The application of current protocols developed by public research institutions to many genotypes has been initiated by TRAGSA, a Spanish public company, so that multivarietal forestry can be implemented for cork oak. Seventy *elite* cork oak trees were selected in four Spanish provenances of Extremadura (Southwest Spain) mainly on the basis of their cork quality and productivity (Bueno *et al.* 2002). To date samples have been collected from 59 genotypes, 51 of them gave epicormic shoots, and embryogenic lines were obtained in 44 of them. Therefore, SE was achieved in 86% of the tested genotypes at the first attempt (Hernández *et al.* 2009). The average induction frequency was 17%, which is quite in line with the previous data (**Table 1**). The induction frequency varied with genotype between 0 and 56%. Embryogenic lines were amplified by recurrent embryogenesis on semisolid medium without PGRs as described previously. Embryos that matured spontaneously, and isolated immature embryos that underwent a maturation treatment, were cold stratified and germinated. Conversion frequencies varied with genotype between 9 and 87% in the case of spontaneously matured embryos (average 59%), and between 37 and 75% in the case of embryos that were subjected to maturation treatment (average 55%).

Average survival of the plants regenerated from embryos that matured spontaneously was 17%, varying according to genotype between 0 and 33%, whereas those from embryos subjected to maturation treatment was 26%, ranging between 12 and 52% according to genotype. These results were obtained working with 1,667 spontaneously matured embryos and 2,559 embryos subjected to maturation treatment (Hernández *et al.* 2009). There was no difference between the frequencies of conversion, but there were significant differences between the frequencies of survival for the two types of maturation processes, which is in agreement with previous results. Therefore it seems that the degree of maturation of cork oak somatic embryos has little influence on frequencies of conversion, but strong influence on the survival of regenerated plants.

CONCLUDING REMARKS

The advance in the cloning of cork oak by somatic embryogenesis has been quite considerable in the last years. Using this way of regeneration, the *Agrobacterium*-mediated genetic transformation of the species has been accomplished (Álvarez *et al.* 2004; Sánchez *et al.* 2005; Álvarez *et al.* 2007), which opens the way to studies on functional genomics. In addition, the availability of protocols to clone adult trees will allow for the modification of specific traits of selected phenotypes, as demonstrate the recent introduction of the *bar* gene into the cork oak genome to confer herbicide tolerance (Álvarez *et al.* 2009).

At present, the protocols developed for this species are able to produce the few hundreds of plants required for genetic tests, and for *in situ* or *ex situ* conservation purposes. An endangered tree with a very singular genotype growing in Minorca (Balearic Islands) has been recently cloned (Hernández *et al.* 2008; Lorenzo *et al*. 2009). Currently, the genetic tests to validate the selected trees as varieties are been established by TRAGSA in several field trials.

ACKNOWLEDGEMENTS

We thank funding from Spanish national projects AGL2007-66345-CO2-01 and PROFIT CIT-010000-2007-5, and from the TRAGSA's funded project SEFEAL-2.

REFERENCES

Álvarez R, Alonso P, Cortizo M, Celestino C, Hernández I, Toribio M, Ordás RJ (2004) Genetic transformation of selected mature cork oak (*Quercus suber* L.) trees. *Plant Cell Reports* **23**, 218-223

Álvarez R, Ordás RJ (2007) Improved genetic transformation protocol for cork oak (*Quercus suber* L.). *Plant Cell, Tissue and Organ Culture* **91**, 45-52

Álvarez R, Álvarez JM, Humara JM, Revilla A, Ordás RJ (2009) Genetic transformation of cork oak (*Quercus suber* L.) for herbicide resistance. *Biotechnology Letters* **31**, 1477-1483

Anonymous (2000) *O Sobreiro e a Cortiça – The Cork Oak and Cork*, Direcçao-Geral de Desenvolvimento Rural, Lisboa, Portugal, 93 pp

Benowicz A, Grossnickle SC, El-Kassaby YA (2002) Field assessment of

Douglas-fir somatic and zygotic seedlings with respect to gas exchange, water relation and frost hardiness. *Canadian Journal of Forest Research* **32**, 1822-1828

Bueno M, Astorga R, Manzanera J (1992) Plant regeneration through somatic embryogenesis in *Quercus suber*. *Physiologia Plantarum* **85**, 30-34

Bueno MA, Manzanera JA, González-Adrados JR, Pintos B, Cuenca B, Ocaña L, Santos I (2002) Cork-oak phenotypic selection based on cork production and quality: biotechnological propagation and commercial application. In: *International Congress Sustainable Forestry Wood Products and Biotechnology*, 11-14 November, Vitoria-Gazteiz, Spain, pp 473-478

Caritat A, Gutiérrez E, Molinas M (2000) Influence of weather on cork-ring width. *Tree Physiology* **20**, 893-900

Carron MP, Le Roux Y, Tison J, Dea BG, Caussanel V, Clair J, Keli J (2000) Compared root system architectures in seedlings and *in vitro* plantlets of *Hevea brasiliensis*, in the initial years of growth in the field. *Plant and Soil* **223**, 73-85

Carron MP, Lardet L, Leconte A, Dea BG, Keli J, Granet F, Julien J, Teerawatanasuk K, Montoro P (2009) Field trials network emphasizes the improvement of growth and yield through micropropagation in rubber tree (*Hevea brasiliensis*, Muëll.-Arg.). *Acta Horticulturae* **812**, 485-492

Celestino C, Hernández I, López-Vela D, Carneros E, Alegre J, Fernández-Guijarro B, Cardo L, Toribio M (2007) First data from a field trial of *Quercus suber* plants regenerated from mature selected trees and from their half-sib progenies by somatic embryogenesis. *Acta Horticulturae* **748**, 215-218

Celestino C, Fernández-Guijarro B, Hernández I, López-Vela D, Carneros E, Jiménez J, Cardo L, Alegre J, Toribio M (2009) Growth data from a field trial of *Quercus suber* plants regenerated from mature selected trees and from their half-sib progenies by somatic embryogenesis. *Acta Horticulturae* **812**, 493-498

Conde P, Loureiro J, Santos C (2004) Somatic embryogenesis and plant regeneration from leaves of *Ulmus minor* Mill. *Plant Cell Reports* **22**, 632-639

Díez J, Manjón JL, Kóvacs GM, Celestino C, Toribio M (2000) Mycorrhization of vitroplants raised from somatic embryos of cork oak (*Quercus suber* L.). *Applied Soil Ecology* **15**, 119-123

El Mâataoui M, Espagnac H (1987) Néoformation de structures de type embryons somatiques sur des cultures de tissus de chêne liège (*Quercus suber* L.). *Comptes rendus de l'Académie des Sciences Paris* **304 (III)**, 83-88

El Mâataoui M, Espagnac H (1989) Comportement *in vitro* d'embryons zygotiques de chêne liège (*Quercus suber* L.) excisés à divers stades de leur développement. *Annals of Science Forestière* **46 (Suppl)**, 145s-148s

Fernandes A, Fernandes I, Cruz L, Mateus N, Cabral M, Freitas V (2009) Antioxidant and biological properties of bioactive phenolic compounds from *Quercus suber* L. *Journal of Agricultural and Food Chemistry* **57**, 11154-11160

Fernandes P, Rodriguez E, Pinto G, Roldán-Ruiz I, De Loose M, Santos C (2008) Cryopreservation of *Quercus suber* somatic embryos by encapsulation-dehydration and evaluation of genetic stability. *Tree Physiology* **28**, 1841-1850

Fernández-Guijarro B (1997) Embriogénesis somática en alcornoque (*Quercus suber* L.) PhD thesis, Polytechnic University of Madrid, 195 pp

Fernández-Guijarro B, Celestino C, Toribio M (1994) Somatic embryogenesis in *Quercus suber* L. In: Pardos JA, Ahuja MR, Elena-Rossello R (Eds) *Biotechnology of Trees*, Investigación Agraria - Sistemas y Recursos Forestales, Fuera de Serie nº 4, INIA-MAPA, Madrid, Spain, pp 105-110

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

Gallego FJ, Martínez I, Celestino C, Toribio M (1997) Testing somaclonal variation using RAPDs in *Quercus suber* L. somatic embryos. *International Journal of Plant Sciences* **158**, 563-567

García-Martín G, González-Benito ME, Manzanera JA (2001) *Quercus suber* L. somatic embryo germination and plant conversion: pre-treatments and germination conditions. *In Vitro Cellular and Developmental Biology – Plant* **37**, 190-198

García-Martín G, Manzanera JA, González-Benito ME (2005) Effect of exogenous ABA on embryo maturation and quantification of endogenous levels of ABA and IAA in *Quercus suber* somatic embryos. *Plant Cell, Tissue and Organ Culture* **80**, 171-177

González-Benito ME, García-Martín G, Manzanera JA (2002) Shoot development in *Quercus suber* L. somatic embryos. *In Vitro Cellular and Developmental Biology – Plant* **38**, 477-480

Hernández I (2007) Regeneración clonal de alcornoques adultos (*Quercus suber* L.) mediante embriogénesis somática. PhD thesis, University of Alcalá, 215 pp

Hernández I, Celestino C, Martínez I, Manjón JL, Díez J, Fernández-Guijarro B, Toribio M (2001) Cloning mature cork oak (*Quercus suber* L.) trees by somatic embryogenesis. *Melhoramento* **37**, 50-57

Hernández I, Celestino C, Toribio M (2003a) Vegetative propagation of *Quercus suber* L. by somatic embryogenesis: I. Factors affecting the induction in leaves from mature cork oak trees. *Plant Cell Reports* **21**, 759-764

Hernández I, Celestino C, Alegre J, Toribio M (2003 b) Vegetative propagation of *Quercus suber* L. by somatic embryogenesis: II. Plant regeneration from selected cork oak trees. *Plant Cell Reports* **21**, 765-770

Hernández I, Celestino C, López-Vela D, Carneros E, Jiménez J, Alegre J, Gil L, Toribio M (2008) Plant regeneration from an endangered valuable cork oak tree by somatic embryogenesis. In: Vázquez J, Pereira H, González-Pérez A (Eds) *Suberwood: New Challenges for Integration of Cork Oak Forest and Products*, University of Huelva Publisher, Huelva, Spain, pp 205-212

Hernández I, Cuenca B, Carneros E, Alonso-Blázquez N, Ruiz M, Celestino C, Ocaña L, Alegre J, Toribio M (2009) Regeneración clonal de alcornoques selectos mediante embriogénesis somática. In: *5th Congreso Forestal Español*, 21-25 September, 2009, Ávila, Spain. ISBN 978-84-936854-6-1 (digital version)

Hornero J, Martínez I, Celestino C, Gallego FJ, Torres V, Toribio M (2001) Early checking of genetic stability of cork oak somatic embryos by AFLP analysis. *International Journal of Plant Sciences* **162**, 827-833

IFAPA (Instituto de Investigación y Formación Agraria y Pesquera) (2006) I Seminario y I Jornada de la Seca de la encina y del alcornoque. "La seca", un reto a la investigación. Junta de Andalucía (Eds). ISBN 84-8474-182-6

IPROCOR - Instituto CMC (Instituto del Corcho, la Madera y el Carbón Vegetal) (2006) El alcornoque. Folletos Divulgativos. Junta de Extremadura. www.iprocor.org

Jiménez J, Alonso-Blázquez N, Hernández I, Carneros E, Cuenca B, Ocaña L, Ruiz M, Celestino C, Toribio M, Alegre J (2009) Efecto de las características del biorreactor y de su manejo sobre el desarrollo de cultivos embriogénicos de alcornoque. In: *5th Congreso Forestal Español*, 21-25 September, 2009, Ávila, Spain, ISBN 978-84-936854-6-1 (digital version)

Khaw CH, Ng SK, Drew RA (1998) Performance of commercial scale clonal oil palm (*Elaeis guineensis* Jacq.) plantings in Malaysia. *Acta Horticulturae* **461**, 251-258

Klimaszewska K, Trontin JF, Becwar MR, Devillard C, Park Y-S, Lelu-Walter M-A (2007) Recent progress in somatic embryogenesis of four *Pinus* spp. *Tree and Forestry Science and Biotechnology* **1**, 11-25

Lopes T, Pinto P, Loureiro J, Costa A, Santos C (2006) Determination of genetic stability in long-term somatic embryogenic cultures and derived plantlets of cork oak using microsatellite markers. *Tree Physiology* **26**, 1145-1152

López de Heredia U, Gil L (2006) La diversidad en las especies forestales: un cambio de escala. El ejemplo del alcornoque. *Ecosistemas* **2**, 1-9

Lorenzo Z, Burgarella C, López de Heredia U, Lumaret R, Petit RJ, Soto A, Gil L (2009) Relevance of genetics for conservation policies: the case of Minorcan cork oaks. *Annals of Botany* **104**, 1069-1076

Loureiro J, Pinto G, Lopes T, Doležel J, Santos C (2005) Assessment of ploidy stability of the somatic embryogenesis process in *Quercus suber* L. using flow cytometry. *Planta* **221**, 815-822

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

Merkle SA, Battle PJ (2000) Enhancement of embryogenic culture initiation from tissues of mature sweetgum trees. *Plant Cell Reports* **19**, 268-273

Merkle SA, Nairn CJ (2005) Hardwood tree biotechnology. *In Vitro Cellular and Developmental Biology – Plant* **41**, 602-619

Moiteiro C, Justino F, Tavares R, Marcelo-Curto MJ, Florencio MH, Nascimento MSJ, Pedro M, Cerqueira F, Pinto MMM (2001) Synthetic secofriedelane and friedelane derivatives as inhibitors of human lymphocyte proliferation and growth of human cancer cell lines *in vitro*. *Journal of Natural Products* **64**, 1273-1277

Montero G, San Miguel A, Cañellas I (1998) Systems of Mediterranean silvicultura "La Dehesa". In: Jiménez R, Lamo de Espinosa J (Eds) *Agricultura Sostenible. Agrofuturo*, Life and Mundi-Prensa, Madrid, Spain, pp 519-554

Nehra NS, Becwar MR, Rottmann WH, Pearson L, Chowdhury K, Chang SJ, Wilde HD, Kodrzycki RJ, Zhang CS, Gause KC, Parks DW, Hinchee MA (2005) Forest biotechnology: Innovative methods, emerging opportunities. *In Vitro Cellular and Developmental Biology – Plant* **41**, 701-717

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497

Park YS (2004) Commercial implementation of multi-varietal forestry using conifer somatic embryogenesis. In: *Proccedings IUFRO joint Conference of Division 2, Forest Genetics and Tree Breeding in the Age of Genomics: Progress and Future*, 1-5 November, 2004, Charleston, SC, USA, p 139 (Abstract)

Pinto G, Valentim H, Costa A, Castro S, Santos C (2002) Somatic embryogenesis in leaf callus from a mature *Quercus suber* L. tree. *In Vitro Cellular and Developmental Biology – Plant* **38**, 569-572

Pintos B, Bueno MA, Cuenca B, Manzanera JA (2008) Synthetic seed production from encapsulated somatic embryos of cork-oak (*Quercus suber* L.) and automated growth monitoring. *Plant Cell, Tissue and Organ Culture* **95**, 217-225

Pintos B, Manzanera JA, Bueno MA (2010) Oak somatic and gametic embryos maturation is affected by charcoal and specific amino acids mixture. *Annals of Forest Science* **67**, 205p1-205p9

Puigderrajols P, Fernández-Guijarro B, Toribio M, Molinas M (1996) Origin and early development of secondary embryos in *Quercus suber* L. *International Journal of Plant Sciences* **157**, 674-684

Puigderrajols P, Celestino C, Suils M, Toribio M, Molinas M (2000) Histology of organogenic and embryogenic responses in cotyledons of somatic embryos of *Quercus suber* L. *International Journal of Plant Sciences* **161**,

353-362

Puigderrajols P, Mir G, Molinas M (2001) Ultrastructure of early secondary embryogenesis by multicellular and unicellular pathways in cork oak (*Quercus suber* L.). *Annals of Botany* **87**, 179-189

Pullman GS, Johnson S, Peter G, Cairney J, Xu N (2003) Improving loblolly pine somatic embryo maturation: comparison of somatic and zygotic embryo morphology, germination, and gene expression. *Plant Cell Reports* **21**, 747-758

Sánchez N, Manzanera JA, Pintos B, Bueno MA (2005) *Agrobacterium*–mediated transformation of cork-oak (*Quercus suber* L.) somatic embryos. *New Forests* **29**, 169-176

Savill PS, Kanowski PJ (1993) Tree improvement programs for European oaks: goals and strategies. *Annales des Sciences Forestières* **50**, 368s-383s

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany* **50**, 199-204

Silva SP, Sabino MA, Fernandes EM, Correlo VM, Boesel LF, Reis RL (2005) Cork: properties, capabilities and applications. *International Materials Reviews* **50**, 345-365

Smith DR (1999) Successful rejuvenation of radiata pine. In: *Proceedings 25th Biennial Southern Forest Tree Improvement Conference*, 11-14 July, New Orleans, Louisiana, USA, pp 158-167

Toribio M (1986) Callus initiation and primary morphogenic responses from *Quercus suber* L. cotyledons cultured *in vitro*. In: *Proceedings 18th IUFRO World Congress. Division 2, vol. II*, 7-21 September, Ljubljana, Yugoslavia, p 863 (Abstract)

Toribio M, Celestino C (1989) Cultivo *in vitro* del alcornoque. *Scientia Gerundensis* **15**, 11-21

Toribio M, Celestino C, Gallego J, Martínez I (2000) Induction of somatic embryogenesis in tissues from mature oak trees. In: Ríordáin FÓ (Ed) *COST Action 822, Development of integrated systems for large-scale propagation of elite plants using in vitro techniques*. (EUR 19237, Report of activities 1998), Office for Official Publications of the European Communities, Luxembourg, pp 236-237

Toribio M, Fernández C, Celestino C, Martínez MT, San-José MC, Vieitez AM (2004) Somatic embryogenesis in mature *Quercus robur* trees. *Plant Cell, Tissue and Organ Culture* **76**, 283-287

Toribio M, Celestino C, Molinas M (2005) Cork oak, *Quercus suber* L. In: Jain SM, Gupta PK (Eds) *Protocol for Somatic Embryogenesis in Woody Plants*. Forestry Sciences (Vol 77, Chapter 35), Springer, The Netherlands, pp 445-457

Valladares S, Toribio M, Celestino C, Vieitez AM (2004) Cryopreservation of embryogenic cultures from mature *Quercus suber* trees using vitrification. *CryoLetters* **25**, 177-186

Varela MC (1999) Cork and the cork oak system. Special issue: Mediterranean forests. *Unasylva* (English Edn) **50 (2), issue 197**, 42-44

Vasil IK (1995) Cellular and molecular genetic improvement of cereals. In: Terzi M, Cella R, Falavigna A (Eds) *Current Issues in Plant Molecular and Cellular Biology*, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 5-18

Vidal N, Vieitez AM, Fernández MR, Cuenca B, Ballester A (2010) Establishment of cryopreserved gene banks of European chestnut and cork oak. *European Journal of Forest Research* **129**, 635-643

Vieitez AM, Sánchez MC, Amo-Marco JB, Ballester A (1994) Forced flushing of branch segments as a method for obtaining reactive explants of mature *Quercus robur* trees for micropropagation. *Plant Cell, Tissue and Organ Culture* **37**, 287-295

Wilhelm E (2000) Somatic embryogenesis in oak (*Quercus* spp.). *In Vitro Cellular and Developmental Biology – Plant* **36**, 349-357

Zobel B, Talbert J (1984) *Applied Forest Tree Improvement*, John Willey and Sons, New York, USA, 505 pp